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US 5466593 A
KATAOKA, H. et al., Cancer Research. 2000, vol. 60, pages 6148-6159
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1996, vol. 271, pages 3615-3618
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(54) Title: MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR

Clone #	Sib#	H1				H2										H3												Affinity
		30	31	32	33	49	50	51	52	52A	53	54	55	56	57	58	95	96	97	98	99	100	100A	100B	100C	100D	100E	100F	101	102	
33	2	T	S	S	A	G	I	I	N	P	N	G	G	Y	T	N	S	S	R	L	A	G	A					M	D	Y	12 nM
35	1	T	G	S	A	G	I	I	N	P	N	S	G	Y	T	D	S	A	R	I	R	G						F	D	Y	12 nM
37	1	N	S	N	G	G	W	I	Y	P	A	G	G	A	T	D	W	G	W	G								F	D	Y	56 nM
39	1	N	G	T	Y	G	G	I	Y	P	A	G	G	A	T	Y	W	W	A	W	P	A						F	D	Y	6 nM
42	1	N	G	T	W	G	G	I	Y	P	A	G	G	A	T	D	W	R	A	V	P	S						F	D	Y	30 nM
49	1	T	G	T	Y	G	W	I	S	P	Y	N	G	D	T	Y	D	W	F	G	F	G	E					F	D	Y	2 nM
58	1	T	G	S	A	A	I	I	N	P	N	G	G	Y	T	Y	S	A	R	F	S							F	D	Y	1 nM
61	1	S	G	N	W	A	E	I	N	P	Y	N	G	S	T	N	F	Y	R	W	S	V	N	S	V			M	D	Y	20 nM
74	1	T	N	Y	W	G	G	I	Y	P	A	G	G	A	T	D	Y	S	I	P	A							F	D	Y	60 nM
75	1	S	N	S	G	G	W	I	Y	P	T	G	G	A	T	D	F	W	W	R	S							F	D	Y	6 nM
86	1	S	D	S	S	A	R	I	Y	P	T	S	G	N	T	N	G	L	K	V	P	F	Y	A	N	A	A	M	D	Y	19 nM
90	1	S	G	S	A	A	I	I	N	P	T	G	G	Y	T	N	S	R	G	H	Y	A						M	D	Y	40 nM
91	2	T	G	N	G	A	W	I	S	P	Y	G	G	S	T	N	G	H	R	V								F	D	Y	100 nM
95	4	N	N	T	G	G	W	I	Y	P	A	G	G	A	T	D	F	F	P	V	A							F	D	Y	4 nM

(57) Abstract: The invention provides methods and compositions for modulating hepatocyte growth factor activator function.

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MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR

RELATED APPLICATIONS

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application number 60/615,657 filed October 4, 2004, the contents of which are incorporated herein in their entirety by reference.

TECHNICAL FIELD

The present invention relates generally to the fields of molecular biology and growth factor regulation. More specifically, the invention concerns modulators of hepatocyte growth factor activator function, and uses of said modulators.

BACKGROUND

Hepatocyte growth factor (HGF) promotes cell proliferation, migration, angiogenesis, survival and morphogenesis by activating the receptor tyrosine kinase Met (reviewed in 8, 9). In addition to its importance in normal physiology, the HGF/Met pathway has been implicated in invasive tumor growth and tumor metastasis (8). HGF has high similarity to the serine protease plasminogen and is composed of a α-chain containing an N-domain and four Kringle domains and a β-chain with homology to chymotrypsin-like proteases. It is secreted into the extracellular matrix as an inactive single chain precursor (pro-HGF) and requires activation cleavage at Arg494 - Val495 to form the biologically competent, disulfide-linked α/β heterodimer (10-13). This step is mediated by pro-HGF converting serine proteases, such as hepatocyte growth factor activator (HGFA) (14). HGFA is inhibited by cell surface-expressed Kunitz-type inhibitors, such as the two hepatocyte growth factor activator inhibitor splice variants HAI-1 (16-17) and HAI-1B (15) and by HAI-2 (18). HAI-2 (also known as placental bikunin) (19) also potently inhibits factor XIa and plasma kallikrein (20), whereas HAI-1B has little or no inhibitory activity (15). Therefore, the biological availability of the pro-HGF pool in the extracellular matrix is regulated by the activities of pro-HGF convertases such as HGFA and their inhibitors.

Since activation of pro-HGF requires cleavage by a convertase such as HGFA, modulation of HGFA function and/or its interaction with its substrate could prove to be an efficacious therapeutic approach. In this regard, there is a clear need to identify clinically relevant agents capable of modulating activity of and/or specificifically interacting with HGFA. The invention fulfills this need and provides other benefits.

All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Australia or any other country.

DISCLOSURE OF THE INVENTION

A first aspect provides an isolated antibody that binds human hepatocyte growth factor activator (HGFA) and blocks HGFA proteolytic activity, wherein the antibody blocks HGFA proteolysis of the small molecule substrate methanesulphonyl-D-cyclohexylalanyl-butyl-arginine-paranitroanilide (FVIIa).

A second aspect provides a method of treating a disease associated with dysregulation of HGF/c-met signaling in a subject, comprising administering to the subject an effective amount of an antibody of the first aspect.

A third aspect provides use of the antibody of the first aspect in the manufacture of a medicament for treating a disease associated with dysregulation of HGF/c-Met signaling.

The invention provides methods, compositions, kits and articles of manufacture for modulating hepatocyte growth factor activator (HGFA) function, thereby modulating physiological effects of HGFA activity. Modulation of HGFA function can be effected by the use of antibodies as described herein.

The invention provides modulator molecules capable of use for modulating HGFA function. In one embodiment, HGFA function is modulated through inhibition of HGFA activity (e.g., proteolytic activity). Generally, the modulator molecules comprise an antibody as described herein. The modulator molecules are capable of effecting modulation either directly (e.g., by binding to HGFA and interefering with HGFA proteolytic activity) or indirectly (e.g., by targeting/directing an active agent to HGFA in a tissue or cell, wherein the active agent is capable of interfering with HGFA proteolytic activity). In one embodiment, the invention provides an antagonist molecule comprising an antibody that binds to HGFA. In one embodiment, binding of the antagonist to HGFA interferes with HGFA proteolytic activity. In one embodiment, binding of the antagonist to HGFA interferes with activation of HGF by HGFA. In one embodiment, the antibody binds to the active site of HGFA. In one embodiment, the antibody binds to HGFA at a position other than the HGFA active site (e.g., an exosite). In one embodiment, binding of the antibody to HGFA at a position other than the HGFA active site inhibits interaction of HGFA with its substrate molecule. In one embodiment, binding of the antibody to HGFA at a position other than the HGFA active site inhibits HGFA proteolytic activity.

Disclosed herein are antagonists that disrupt the HGF/c-met signaling pathway. For example, disclosed herein is a molecule that inhibits HGFA cleavage of proHGF (e.g., cleavage at the R494-V495 position). The molecule can exert its inhibitory function in any number of ways, including but not limited to binding to HGFA at its active site and/or at a site other than the active

site (e.g., an exosite) such that HGFA cleavage of proHGF is inhibited. The molecule can bind to HGFA in complexed or uncomplexed form. The molecule can also exert its inhibitory function by interfering with one or more aspects of the HGF activation process. For example, in one embodiment, an antagonist molecule of the invention binds to HGFA-proHGF complex such that cleavage of proHGF is inhibited. In one embodiment, binding of the molecule to proHGF or HGFA (singly or in complex) inhibits release of HGF subsequent to cleavage by HGFA. In one embodiment, an antagonist molecule of the disclosure does not inhibit HGF binding to c-met. For example, in one embodiment, an antagonist molecule is not an antibody or fragment thereof having similar inhibitory and/or binding ability as the antibody produced by hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-11894 (hybridoma 1A3.3.13) or HB-11895 (hybridoma 5D5.11.6). In one embodiment, an antagonist molecule inhibits biological activities associated with HGF/c-met activation.

Also disclosed is an antibody comprising a CDR-H1 region comprising the sequence of SEQ ID NO:3,6,9,12,15,18,21,24,27,30,33,36,39 or 42. Also disclosed is an antibody comprising a CDR-H2 region comprising the sequence of SEQ ID NO:4,7,10,13,16,19,22,25,28,31,34,37,40 or 43. Also disclosed is an antibody comprising a CDR-H3 region comprising the sequence of SEQ ID NO:5,8,11,14,17,20,23,26,29,32,35,38,41 or 44. In one embodiment, the antibody comprises a CDR-H1 region comprising the sequence of SEQ ID NO:3,6,9,12,15,18,21,24,27,30,33,36,39 or 42, and a CDR-H2 region comprising the sequence of SEQ ID NO:4,7,10,13,16,19,22,25,28,31,34,37,40 or 43. In one embodiment, the antibody comprises a CDR-H1 region comprising the sequence of SEQ ID NO:3,6,9,12,15,18,21,24,27,30,33,36,39 or 42, and a CDR-H3 region comprising the sequence of SEQ ID NO:5,8,11,14,17,20,23,26,29,32,35,38,41 or 44. In one embodiment, the antibody comprises a CDR-H2 region comprising the sequence of SEQ ID NO:4,7,10,13,16,19,22,25,28,31,34,37,40 or 43, and a CDR-H3 region comprising the sequence of SEQ ID NO:5,8,11,14,17,20,23,26,29,32,35,38,41 or 44. In one embodiment, the antibody comprises a CDR-H1 region comprising the sequence of SEQ ID NO:3,6,9,12,15,18,21,24,27,30,33,36,39 or 42, a CDR-H2 region comprising the sequence of SEQ ID NO:4,7,10,13,16,19,22,25,28,31,34,37,40 or 43, and a CDR-H3 region comprising the sequence of SEQ ID NO:5,8,11,14,17,20,23,26,29,32,35,38,41 or 44.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:3;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:4;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:5.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:6;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:7;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:8.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:9;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:10;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:11.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:12;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:13;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:14.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:15;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:16;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:17.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:18;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:19;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:20.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:21;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:22;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:23.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:24;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:25;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:26.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:27;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:28;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:29.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:30;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:31;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:32.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:33;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:34;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:35.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:36;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:37;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:38.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:39;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:40;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:41.

Also disclosed is an antibody comprising at least one, at least two, or all three of the following:

(i) a CDR-H1 sequence comprising the sequence of SEQ ID NO:42;

(ii) a CDR-H2 sequence comprising the sequence of SEQ ID NO:43;

(iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO:44.

The amino acid sequences of SEQ ID NOs:3-44 are numbered with respect to individual CDR (i.e., H1, H2 or H3) as indicated in Figure 1, the numbering being consistent with the Kabat numbering system as described below.

In one embodiment, an antibody of the disclosure comprises a heavy chain variable domain CDR sequence(s) comprising the sequence of at least one, at least two, or all three of the H1 (SEQ

ID NO: 71-84), H2 (SEQ ID NO: 85-98) and/or H3 (SEQ ID NO: 99-112) sequences for each clone depicted in Figures 1B, 1C and 1D.

Also disclosed are antibodies comprising heavy chain CDR sequences as depicted in Figure 1A, B, C and D. In some embodiment, these antibodies further comprise a light chain variable domain of humanized 4D5 antibody (huMAb4D5-8) (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (also referred to in U.S. Pat. No. 6,407,213 and Lee et al., J. Mol. Biol. (2004), 340(5):1073-93) as depicted in SEQ ID NO:45 below.

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val
Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn Thr Ala Val Ala Trp Tyr Gln Gln
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Phe Leu Glu Ser Gly
Val Pro Ser Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser

Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr (SEQ ID NO:45)

In one embodiment, the huMAb4D5-8 light chain variable domain sequence is modified at one or more of positions 30, 66 and 91 (Asn, Arg and His as indicated in bold/italics above, respectively). In one embodiment, the modified huMAb4D5-8 sequence comprises Ser in position 30, Gly in position 66 and/or Ser in position 91. Accordingly, in one embodiment, an antibody of the invention comprises a light chain variable domain comprising the sequence depicted in SEQ ID NO: 54 below:

1 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val ***Ser*** Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly Ser ***Gly*** Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln ***Ser*** Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys 107 (SEQ ID NO: 54) (CDR residues are underlined)

Substituted residues with respect to huMAb4D5-8 are indicated in bold/italics above.

Antibodies of the invention can further comprise any suitable framework and/or light chain variable domain sequences, provided HGFA binding activity is substantially retained. For example, in some embodiments, these antibodies further comprise a human subgroup III heavy chain framework consensus sequence. In one embodiment of these antibodies, the framework consensus sequence comprises substitution at position 71, 73 and/or 78. In some embodiments of these antibodies, position 71 is A, 73 is T and/or 78 is A. In one embodiment, these antibodies comprise heavy chain variable domain framework sequences of humanized 4D5 antibody (huMAb 4D5-8) (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (also referred to in U.S. Pat. No. 6,407,213 and Lee et al., J. Mol. Biol. (2004), 340(5):1073-93). In one embodiment, the humanized 4D5-8 antibody is as described in U.S. Pat. No. 6,407,213. In one embodiment, these antibodies further comprise a human κI light chain framework consensus sequence. In one embodiment, these antibodies comprise light chain variable domain sequences of humanized 4D5 antibody (huMAb 4D5-8) (SEQ ID NO:45) (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (also referred to in U.S. Pat. No. 6,407,213 and Lee et al., J. Mol. Biol. (2004), 340(5):1073-93), or the modified variant thereof as depicted in SEQ ID NO: 54.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequences of SEQ ID NO: 46, 47, 48 and 49 (FR1, 2, 3, and 4, respectively), and CDR H1, H2 and H3 sequences as depicted in Figure 1A, B, C, and/or D. In one embodiment, an antibody of the invention comprises a light chain variable domain,

wherein the framework sequence comprises the sequence of SEQ ID NO: 50, 51, 52 and 53 (FR1, 2, 3, and 4, respectively), and CDR L1, L2 and L3 sequences as depicted in SEQ ID NO: 54.

In one embodiment, an antibody comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NO: 59, 60, 61 and 62 (FR1, 2, 3 and 4, respectively) (FIG. 1E), and CDR H1, H2 and H3 sequences as depicted in Figure 1. In one embodiment, an antibody comprises a light chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NO: 55, 56, 57, and 58 (FR 1, 2, 3 and 4, respectively) (FIG. 1E), and CDR L1, L2 and L3 sequences as depicted in SEQ ID NO: 54.

In one embodiment, an antibody comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NO: 67, 68, 69 and 70 (FR 1, 2, 3 and 4, respectively) (FIG. 1F), and CDR H1, H2 and H3 sequences as depicted in FIG. 1A, B, C and/or D. In one embodiment, an antibody comprises a light chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NO: 63, 64, 65, and 66 (FR 1, 2, 3 and 4, respectively) (FIG. 1F), and CDR L1, L2 and L3 sequences as depicted in SEQ ID NO: 54.

Also disclosed is an antibody that competes with any of the above-mentioned antibodies for binding to HGFA. Also disclosed is an antibody that binds to the same epitope on HGFA as any of the above-mentioned antibodies. In one embodiment, an antibody is affinity matured, humanized, chimeric, or human. In one embodiment, an antibody is an antibody fragment (as described herein), or a substantially full length antibody. In one embodiment, an antibody comprises a wild type Fc region, or a variant thereof. In one embodiment, an antibody is an IgG (e.g., IgG1, IgG2, IgG3, IgG4), IgM, IgE or IgD.

Also disclosed is an antagonist molecule of the disclosure linked to a toxin such as a cytotoxic agent. These molecules/substances can be formulated or administered in combination with an additive/enhancing agent, such as a radiation and/or chemotherapeutic agent.

Also disclosed are methods and compositions useful for modulating disease states associated with dysregulation of the HGF/c-met signaling axis. Thus, also disclosed is a method of modulating c-met activation in a subject, said method comprising administering to the subject a modulator molecule of the invention that inhibits HGFA cleavage of proHGF, whereby c-met activation is modulated. Also disclosed is a method of treating a pathological condition associated with activation of c-met in a subject, said method comprising administering to the subject a modulator molecule of the invention that inhibits HGFA cleavage of proHGF, whereby c-met activation is inhibited. In one embodiment, the modulator molecule is an antibody that binds to HGFA.

The HGF/c-met signaling pathway is involved in multiple biological and physiological functions, including, e.g., cell growth stimulation (e.g. cell proliferation, cell survival, cell migration, cell morphogenesis) and angiogenesis. Thus, also disclosed is a method of inhibiting c-met activated cell growth (e.g. proliferation and/or survival), said method comprising contacting a

cell or tissue with an antagonist of thedisclosure, whereby cell proliferation associated with c-met activation is inhibited. Also disclosed is a method of inhibiting angiogenesis, said method comprising administering to a cell, tissue, and/or subject with a condition associated with abnormal angiogenesis an antagonist of thedisclosure, whereby angiogenesis is inhibited.

Also disclosed is use of a modulator molecule of the disclosure in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disease, such as a cancer, a tumor, a cell proliferative disorder, an immune (such as autoimmune) disorder and/or an angiogenesis-related disorder.

Also disclosed is use of a nucleic acid of the disclosure in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disease, such as a cancer, a tumor, a cell proliferative disorder, an immune (such as autoimmune) disorder and/or an angiogenesis-related disorder.

Also disclosed is use of an expression vector of the disclosure in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disease, such as a cancer, a tumor, a cell proliferative disorder, an immune (such as autoimmune) disorder and/or an angiogenesis-related disorder.

Also disclosed is use of a host cell of the disclosure in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disease, such as a cancer, a tumor, a cell proliferative disorder, an immune (such as autoimmune) disorder and/or an angiogenesis-related disorder.

Also disclosed is use of an article of manufacture of the disclosure in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disease, such as a cancer, a tumor, a cell proliferative disorder, an immune (such as autoimmune) disorder and/or an angiogenesis-related disorder.

Also disclosed is use of a kit of the disclosure in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disease, such as a cancer, a tumor, a cell proliferative disorder, an immune (such as autoimmune) disorder and/or an angiogenesis-related disorder

Also disclosed is a method of inhibiting c-met activated cell proliferation, said method comprising contacting a cell or tissue with an effective amount of a modulator molecule of the disclosure, whereby cell proliferation associated with c-met activation is inhibited.

Also disclosed is a method of treating a pathological condition associated with dysregulation of c-met activation in a subject, said method comprising administering to the subject an effective amount of a modulator molecule of the disclosure, whereby said condition is treated.

Also disclosed is a method of inhibiting the growth of a cell that expresses c-met or hepatocyte growth factor, or both, said method comprising contacting said cell with a modulator

molecule of the disclosure thereby causing an inhibition of growth of said cell. In one embodiment, the cell is contacted by HGF expressed by a different cell (e.g., through a paracrine effect).

Also disclosed is a method of therapeutically treating a mammal having a cancerous tumor comprising a cell that expresses c-met or hepatocyte growth factor, or both, said method comprising administering to said mammal an effective amount of an a modulator molecule of the disclosure, thereby effectively treating said mammal. In one embodiment, the cell is contacted by HGF expressed by a different cell (e.g., through a paracrine effect).

Also disclosed is a method for treating or preventing a cell proliferative disorder associated with increased expression or activity of HGFA, said method comprising administering to a subject in need of such treatment an effective amount of an a modulator molecule of the disclosure, thereby effectively treating or preventing said cell proliferative disorder. In one embodiment, said proliferative disorder is cancer.

Also disclosed is a method for treating or preventing a cell proliferative disorder associated with increased expression or activity of c-met or hepatocyte growth factor, or both, said method comprising administering to a subject in need of such treatment an effective amount of a modulator molecule of the disclosure, thereby effectively treating or preventing said cell proliferative disorder. In one embodiment, said proliferative disorder is cancer.

Also disclosed is a method for inhibiting the growth of a cell, wherein growth of said cell is at least in part dependent upon a growth potentiating effect of HGFA, said method comprising contacting said cell with an effective amount of a modulator molecule of the disclosure, thereby inhibiting the growth of said cell. In one embodiment, the cell is contacted by HGF expressed by a different cell (e.g., through a paracrine effect).

Also disclosed is a method for inhibiting the growth of a cell, wherein growth of said cell is at least in part dependent upon a growth potentiating effect of c-met or hepatocyte growth factor, or both, said method comprising contacting said cell with an effective amount of a modulator molecule of the disclosure, thereby inhibiting the growth of said cell. In one embodiment, the cell is contacted by HGF expressed by a different cell (e.g., through a paracrine effect).

Also disclosed is a method of therapeutically treating a tumor in a mammal, wherein the growth of said tumor is at least in part dependent upon a growth potentiating effect of HGFA, said method comprising contacting said cell with an effective amount of a modulator molecule of the disclosure, thereby effectively treating said tumor. In one embodiment, the cell is contacted by HGF expressed by a different cell (e.g., through a paracrine effect).

Also disclosed is a method of therapeutically treating a tumor in a mammal, wherein the growth of said tumor is at least in part dependent upon a growth potentiating effect of c-met or hepatocyte growth factor, or both, said method comprising contacting said cell with an effective amount of a modulator molecule of the disclosure, thereby effectively treating said tumor. In one

embodiment, the cell is contacted by HGF expressed by a different cell (e.g., through a paracrine effect).

Methods of the disclosure can be used to affect any suitable pathological state, for example, cells and/or tissues associated with dysregulation of the HGF/c-met signaling pathway, e.g. through increased HGF activity associated with HGFA activation of HGF. In one embodiment, a cell that is targeted in a method of the disclosure is a cancer cell. For example, a cancer cell can be one selected from the group consisting of a breast cancer cell, a colorectal cancer cell, a lung cancer cell, a papillary carcinoma cell (e.g., of the thyroid gland), a colon cancer cell, a pancreatic cancer cell, an ovarian cancer cell, a cervical cancer cell, a central nervous system cancer cell, an osteogenic sarcoma cell, a renal carcinoma cell, a hepatocellular carcinoma cell, a bladder cancer cell, a prostate cancer cell, a gastric carcinoma cell, a head and neck squamous carcinoma cell, a melanoma cell and a leukemia cell. In one embodiment, a cell that is targeted in a method of the disclosure is a hyperproliferative and/or hyperplastic cell. In one embodiment, a cell that is targeted in a method of the disclosure is a dysplastic cell. In yet another embodiment, a cell that is targeted in a method of the disclosure is a metastatic cell.

Methods of the disclosure can further comprise additional treatment steps. For example, in one embodiment, a method further comprises a step wherein a targeted cell and/or tissue (e.g., a cancer cell) is exposed to radiation treatment or a chemotherapeutic agent.

As described herein, HGF/c-met activation is an important biological process the dysregulation of which leads to numerous pathological conditions. Accordingly, in one embodiment of methods of the disclosure, a cell that is targeted (e.g., a cancer cell) is one in which activation of HGF/c-met is enhanced as compared to a normal cell of the same tissue origin. In one embodiment, a method of the disclosure causes the death of a targeted cell. For example, contact with a modulator molecule of the disclosure may result in a cell's inability to signal through the c-met pathway, which results in cell death.

Dysregulation of c-met activation (and thus signaling) can result from a number of cellular changes, including, for example, overexpression of HGF (c-met's cognate ligand) and/or HGFA, and/or increased activation of HGF by HGFA. Accordingly, in some embodiments, a method of the disclosure comprises targeting a tissue wherein one or more of HGFA, c-met and hepatoctye growth factor, is more abundantly expressed and/or present (e.g., a cancer) as compared to a normal tissue of the same origin. An HGF or c-met-expressing cell can be regulated by HGFA from a variety of sources, i.e. in an autocrine or paracrine manner. For example, in one embodiment of methods of the disclosure, a targeted cell is contacted/bound by hepatocyte growth factor activated by HGFA expressed in a different cell (e.g., via a paracrine effect). Said different cell can be of the same or of a different tissue origin. In one embodiment, a targeted cell is contacted/bound by HGF activated by HGFA expressed by the targeted cell itself (e.g., via an autocrine effect/loop).

Also disclosed are compositions comprising one or more modulator molecules of the disclosure and a carrier. In one embodiment, the carrier is pharmaceutically acceptable.

Also disclosed are nucleic acids encoding a modulator molecule of the invention. In one embodiment, a nucleic acid of the disclosure encodes a modulator molecule which is or comprises an antibody or fragment thereof.

Also disclosed are vectors comprising a nucleic acid of the disclosure.

Also disclosed are host cells comprising a nucleic acid or a vector of the disclosure. A vector can be of any type, for example a recombinant vector such as an expression vector. Any of a variety of host cells can be used. In one embodiment, a host cell is a prokaryotic cell, for example, *E. coli*. In one embodiment, a host cell is a eukaryotic cell, for example a mammalian cell such as Chinese Hamster Ovary (CHO) cell.

Also disclosed are methods for making a modulator molecule of the disclosure. For example, also disclosed is a method of making a modulator molecule which is or comprises an antibody (or fragment thereof), said method comprising expressing in a suitable host cell a recombinant vector of the disclosure encoding said antibody (or fragment thereof), and recovering said antibody.

Also disclosed is an article of manufacture comprising a container; and a composition contained within the container, wherein the composition comprises one or more modulator molecules of the disclosure. In one embodiment, the composition comprises a nucleic acid of the disclosure. In one embodiment, a composition comprising a modulator molecule further comprises a carrier, which in some embodiments is pharmaceutically acceptable. In one embodiment, an article of manufacture further comprises instructions for administering the composition (for e.g., the modulator molecule) to a subject.

Also disclosed is a kit comprising a first container comprising a composition comprising one or more modulator molecules of the disclosure; and a second container comprising a buffer. In one embodiment, the buffer is pharmaceutically acceptable. In one embodiment, a composition comprising a modulator molecule further comprises a carrier, which in some embodiments is pharmaceutically acceptable. In one embodiment, a kit further comprises instructions for administering the composition (for e.g., the modulator molecule) to a subject.

Also disclosed is a method of diagnosing a disease comprising determining the level of HGFA in a test sample of tissue cells by contacting the sample with an antibody of the disclosure, whereby HGFA bound by the antibody indicates presence and/or amount of HGFA in the sample. Also disclosed is a method of determining whether an individual is at risk for a disease comprising determining the level of HGFA in a test sample of tissue cell by contacting the test sample with an antibody of the disclosure and thereby determining the amount of HGFA present in the sample, wherein a higher level of HGFA in the test sample, as compared to a control sample comprising

normal tissue of the same cell origin as the test sample, is an indication that the individual is at risk for the disease. In one embodiment of methods of the disclosure, the level of HGFA is determined based on amount of HGFA polypeptide indicated by amount of HGFA bound by the antibody in the test sample. An antibody employed in the method may optionally be detectably labeled, attached to a solid support, or the like.

Also disclosed is a method of binding an antibody of the disclosure to HGFA present in a bodily fluid, for example blood.

Also disclosed is a method of binding an antibody of the disclosure to a cell that expresses and/or is responsive to HGFA, wherein the method comprises contacting said cell with said antibody under conditions which are suitable for binding of the antibody to HGFA and allowing binding therebetween. In one embodiment, binding of said antibody to HGFA on the cell inhibits an HGFA biological function. In one embodiment, said antibody does not inhibit interaction of HGFA with its substrate molecule. In one embodiment, said antibody binds to an HGFA molecule on the cell and inhibits binding of another molecule (such as pro-HGF) to the HGFA molecule.

Also disclosed is a method of targeting a therapeutic agent to an HGFA-associated tissue in a host, the method comprising administering to the host said therapeutic agent in a form that is linked to an antibody of the disclosure, whereby the agent is targeted to the HGFA-associated tissue in the host. In one embodiment, the antibody that binds HGFA is capable of specifically binding to HGFA located on a cell (either *in vitro* or *in vivo*), for example where HGFA is present on the surface of a cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 (A) Heavy chain CDR loop sequences of anti-HGFA antibodies. The figure shows the heavy chain CDR sequences, H1, H2, and H3. The light chain sequence is humanized 4D5 sequence (see Lee et al., *supra*). Sequence numbering is as follows: clone 33 (CDRH1 is SEQ ID NO:3; CDRH2 is SEQ ID NO:4; CDRH3 is SEQ ID NO:5); clone35 (CDRH1 is SEQ ID NO:6; CDRH2 is SEQ ID NO:7; CDRH3 is SEQ ID NO:8); clone 37 (CDRH1 is SEQ ID NO:9; CDRH2 is SEQ ID NO:10; CDRH3 is SEQ ID NO:11); clone 39 (CDRH1 is SEQ ID NO:12; CDRH2 is SEQ ID NO:13; CDRH3 is SEQ ID NO:14); clone 42 (CDRH1 is SEQ ID NO:15; CDRH2 is SEQ ID NO:16; CDRH3 is SEQ ID NO:17); clone 49 (CDRH1 is SEQ ID NO:18; CDRH2 is SEQ ID NO:19; CDRH3 is SEQ ID NO:20); clone 58 (CDRH1 is SEQ ID NO:21; CDRH2 is SEQ ID NO:22; CDRH3 is SEQ ID NO:23); clone 61 (CDRH1 is SEQ ID NO:24; CDRH2 is SEQ ID NO:25; CDRH3 is SEQ ID NO:26); clone 74 (CDRH1 is SEQ ID NO:27; CDRH2 is SEQ ID NO:28; CDRH3 is SEQ ID NO:29); clone 75 (CDRH1 is SEQ ID NO:30; CDRH2 is SEQ ID NO:31; CDRH3 is SEQ ID NO:32); clone 86 (CDRH1 is SEQ ID NO:33; CDRH2 is SEQ ID NO:34; CDRH3 is SEQ ID NO:35); clone 90 (CDRH1 is SEQ ID NO:36; CDRH2 is SEQ ID NO:37; CDRH3 is SEQ ID NO:38); clone 91 (CDRH1 is SEQ ID NO:39; CDRH2 is SEQ ID NO:40; CDRH3 is SEQ ID NO:41); clone 95 (CDRH1 is SEQ ID NO:42; CDRH2 is SEQ ID NO:43; CDRH3 is SEQ ID NO:44). Amino acid positions are numbered according to the Kabat numbering system as described below. IC50 values are also indicated in the last (right hand) column.

(B), (C) and (D) Heavy chain CDR loop sequences of anti-HGFA antibodies.

(E) and (F) Exemplary framework region sequences. (E) HuMAb4D5-8 framework region sequences. (F) HuMAb4D5-8 framework region sequences comprising modifications.

Fig. 2 Inhibition of HGFA-mediated proHGF activation by anti-HGFA antibodies. HGFA was incubated with ^{125}I-labelled proHGF and anti-HGFA antibodies for 4 hr at 37°C. Reactant concentrations were 50 μg/ml proHGF, 2 nM HGFA and 0.1mg/ml (0.67μM) antibodies. Aliquots were analyzed by SDS-PAGE under reducing conditions. Soluble HAI-1B (sHAI-1B) was used as a control inhibitor at 1 μM final concentration.A. Lane 1: (t=0) is aliquot taken at beginning of reaction, lane 2: no inhibitor, lane 3: sHAI-1B (1μM), lane 4: #33, lane 5: #35, lane 6: #39, lane 7: #49, lane 8: #74, lane 9: #61. B. Lane 1: #42, lane 2: #91, lane 3: 58, lane 4: #37, lane 5: #75, lane 6: #90, lane 7: #86, lane 8: #95.

Fig. 3. Potent inhibition of HGFA-mediated proHGF conversion by antibody #58. Three different concentrations of the antibody #58 and the non-blocking antibody #49 were used in ^{125}I-labelled proHGF conversion experiments carried out as described in figure 1. Lane 1: (t=0)

is aliquot taken at beginning of reaction, lane 2: no inhibitor, lane 3: sHAI-1B (1μM), lane 4: 0.67μM Ab#49, lane 5: 0.13μM Ab#49, lane 6: 0.03μM Ab#49, lane 7: 0.67μM Ab#58, lane 8: 0.13μM Ab#58, lane 9: 0.03μM Ab#58.

Fig. 4. Concentration-dependent inhibition of HGFA amidolytic activity by anti-HGFA antibodies 58 and 75. Various concentrations of antibodies were incubated with HGFA (5nM final concentration) in HBSA buffer for 20 min at room temperature. After addition of Spectrozyme® fVIIa (200 μM final conc., K_M = 200 μM) the linear rates of substrate activation were measured on a kinetic microplate reader. Inhibition of enzyme activity was expressed as fractional activity (vi/vo) of uninhibited activity.

Fig. 5. Inhibition of HGFA amidolytic activity by IV-49C and a small molecule active site binder/inhibitor. Various concentrations of inhibitors were incubated with HGFA (2.5nM for IV-49C and 5nM for the small molecule, respectively) in HBSA buffer for 20 min at room temperature. Enzyme inhibition of Spectrozyme® fVIIa activation was measured as described in figure 4. A. Inhibition by Kunitz domain inhibitor IV-49C (filled circles) in comparison to the specific factor XIIa inhibitor corn trypsin inhibitor (open circles). B. Inhibition by the small molecule inhibitor (filled triangles).

Fig. 6 Surface plasmon resonance measurements of HGFA binding to anti-HGFA antibodies #58 and #75. Anti-HGFA antibodies (full length IgG1) were immobilized on BIAcore chips and binding data were collected from various concentrations of HGFA. For competition binding studies, HGFA (70 nM) was preincubated with various concentrations of sHAI-1B, IV-49C or small molecule active site binder. A - D: Binding of HGFA to antibody #58 (A) in the absence of inhibitor, or in the presence of (B) sHAI-1B, (C) IV-49C and (D) small molecule active site binder. E - H: Binding of HGFA to antibody #75 (E) in the absence of inhibitor, or in the presence of (F) sHAI-1B, (G) IV-49C and (H) small molecule active site binder.

Fig. 7 Sequences of human (top line; SEQ ID NO:1) and murine (bottom line; SEQ ID NO:2) HGFA protein sequences.

Fig. 8 Table showing data related to inhibition of HGFA enzymatic activity by various anti-HGFA antibodies.

Fig. 9 Table showing data related to binding of HGFA to anti-HGFA antibodies.

MODES FOR CARRYING OUT THE INVENTION

Disclosed herein are methods, compositions, kits and articles of manufacture comprising modulators of hepatocyte growth factor activator function, including methods of using such modulators.

Details of these methods, compositions, kits and articles of manufacture are provided herein.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "Animal Cell Culture" (R. I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Current Protocols in Molecular Biology" (F. M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., ed., 1994); "A Practical Guide to Molecular Cloning" (Perbal Bernard V., 1988); "Phage Display: A Laboratory Manual" (Barbas et al., 2001).

Definitions

The term "hepatocyte growth factor activator" or "HGFA" as used herein encompasses native sequence polypeptides, polypeptide variants and fragments of a native sequence polypeptide and polypeptide variants (which are further defined herein) that is capable of proHGF cleavage in a manner similar to wild type HGFA. The HGFA polypeptide described herein may be that which is isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods. The terms "HGFA", "HGFA polypeptide", "HGFA enzyme", and "HGFA protein" also include variants of a HGFA polypeptide as disclosed herein.

A "native sequence HGFA polypeptide" comprises a polypeptide having the same amino acid sequence as the corresponding HGFA polypeptide derived from nature (e.g., the sequences depicted in Figure 7). In one embodiment, a native sequence HGFA polypeptide comprises the amino acid sequence of SEQ ID NO:1 (see Figure 7; top sequence). Such native sequence HGFA polypeptide can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence HGFA polypeptide" specifically encompasses naturally-occurring truncated or secreted forms of the specific HGFA polypeptide (*e.g.*, an extracellular domain sequence), naturally-occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide.

"HGFA polypeptide variant", or variations thereof, means a HGFA polypeptide, generally an active HGFA polypeptide, as defined herein having at least about 80% amino acid sequence identity with any of the native sequence HGFA polypeptide sequences as disclosed herein. Such HGFA polypeptide variants include, for instance, HGFA polypeptides wherein one or more amino acid residues are added, or deleted, at the N– or C-terminus of a native amino acid sequence.

Ordinarily, a HGFA polypeptide variant will have at least about 80% amino acid sequence identity, alternatively at least about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity, to a native sequence HGFA polypeptide sequence as disclosed herein. Ordinarily, HGFA variant polypeptides are at least about 10 amino acids in length, alternatively at least about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600 amino acids in length, or more. Optionally, HGFA variant polypeptides will have no more than one conservative amino acid substitution as compared to a native HGFA polypeptide sequence, alternatively no more than 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitution as compared to the native HGFA polypeptide sequence.

"Percent (%) amino acid sequence identity" with respect to a peptide or polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific peptide or polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2, wherein the complete source code for the ALIGN-2 program is provided in Table A below. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc. and the source code shown in Table A below has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, California or may be compiled from the source code provided in Figure 8 below. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

Table A

```
/*
 *
 * C-C increased from 12 to 15
 * Z is average of EQ
 * B is average of ND
 * match with stop is _M; stop-stop = 0; J (joker) match = 0
 */
#define _M      -8       /* value of a match with a stop */

int     _day[26][26] = {
/*     A  B  C  D  E  F  G  H  I  J  K  L  M  N  O  P  Q  R  S  T  U  V  W  X  Y  Z */
/* A */  { 2, 0,-2, 0, 0,-4, 1,-1,-1, 0,-1,-2,-1, 0,_M, 1, 0,-2, 1, 1, 0, 0,-6, 0,-3, 0},
/* B */  { 0, 3,-4, 3, 2,-5, 0, 1,-2, 0, 0,-3,-2, 2,_M,-1, 1, 0, 0, 0, 0,-2,-5, 0,-3, 1},
/* C */  {-2,-4,15,-5,-5,-4,-3,-3,-2, 0,-5,-6,-5,-4,_M,-3,-5,-4, 0,-2, 0,-2,-8, 0, 0,-5},
/* D */  { 0, 3,-5, 4, 3,-6, 1, 1,-2, 0, 0,-4,-3, 2,_M,-1, 2,-1, 0, 0, 0,-2,-7, 0,-4, 2},
/* E */  { 0, 2,-5, 3, 4,-5, 0, 1,-2, 0, 0,-3,-2, 1,_M,-1, 2,-1, 0, 0, 0,-2,-7, 0,-4, 3},
/* F */  {-4,-5,-4,-6,-5, 9,-5,-2, 1, 0,-5, 2, 0,-4,_M,-5,-5,-4,-3,-3, 0,-1, 0, 0, 7,-5},
/* G */  { 1, 0,-3, 1, 0,-5, 5,-2,-3, 0,-2,-4,-3, 0,_M,-1,-1,-3, 1, 0, 0,-1,-7, 0,-5, 0},
/* H */  {-1, 1,-3, 1, 1,-2,-2, 6,-2, 0, 0,-2,-2, 2,_M, 0, 3, 2,-1,-1, 0,-2,-3, 0, 0, 2},
/* I */  {-1,-2,-2,-2,-2, 1,-3,-2, 5, 0,-2, 2, 2,-2,_M,-2,-2,-2,-1, 0, 0, 4,-5, 0,-1,-2},
/* J */  { 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,_M, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0},
/* K */  {-1, 0,-5, 0, 0,-5,-2, 0,-2, 0, 5,-3, 0, 1,_M,-1, 1, 3, 0, 0, 0,-2,-3, 0,-4, 0},
/* L */  {-2,-3,-6,-4,-3, 2,-4,-2, 2, 0,-3, 6, 4,-3,_M,-3,-2,-3,-3,-1, 0, 2,-2, 0,-1,-2},
/* M */  {-1,-2,-5,-3,-2, 0,-3,-2, 2, 0, 0, 4, 6,-2,_M,-2,-1, 0,-2,-1, 0, 2,-4, 0,-2,-1},
```

```
    /* N */   { 0, 2,-4, 2, 1,-4, 0, 2,-2, 0, 1,-3,-2, 2,_M,-1, 1, 0, 1, 0, 0,-2,-4, 0,-2, 1},
    /* O */   {_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,
    0,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M},
    /* P */   { 1,-1,-3,-1,-1,-5,-1, 0,-2, 0,-1,-3,-2,-1,_M, 6, 0, 0, 1, 0, 0,-1,-6, 0,-5, 0},
    /* Q */   { 0, 1,-5, 2, 2,-5,-1, 3,-2, 0, 1,-2,-1, 1,_M, 0, 4, 1,-1,-1, 0,-2,-5, 0,-4, 3},
    /* R */   {-2, 0,-4,-1,-1,-4,-3, 2,-2, 0, 3,-3, 0, 0,_M, 0, 1, 6, 0,-1, 0,-2, 2, 0,-4, 0},
    /* S */   { 1, 0, 0, 0, 0,-3, 1,-1,-1, 0, 0,-3,-2, 1,_M, 1,-1, 0, 2, 1, 0,-1,-2, 0,-3, 0},
    /* T */   { 1, 0,-2, 0, 0,-3, 0,-1, 0, 0, 0,-1,-1, 0,_M, 0,-1,-1, 1, 3, 0, 0,-5, 0,-3, 0},
    /* U */   { 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,_M, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0},
    /* V */   { 0,-2,-2,-2,-2,-1,-1,-2, 4, 0,-2, 2, 2,-2,_M,-1,-2,-2,-1, 0, 0, 4,-6, 0,-2,-2},
    /* W */   {-6,-5,-8,-7,-7, 0,-7,-3,-5, 0,-3,-2,-4,-4,_M,-6,-5, 2,-2,-5, 0,-6,17, 0, 0,-6},
    /* X */   { 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,_M, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0},
    /* Y */   {-3,-3, 0,-4,-4, 7,-5, 0,-1, 0,-4,-1,-2,-2,_M,-5,-4,-4,-3,-3, 0,-2, 0, 0,10,-4},
    /* Z */   { 0, 1,-5, 2, 3,-5, 0, 2,-2, 0, 0,-2,-1, 1,_M, 0, 3, 0, 0, 0, 0,-2,-6, 0,-4, 4}
};

/*
 */
#include <stdio.h>
#include <ctype.h>

#define MAXJMP    16    /* max jumps in a diag */
#define MAXGAP    24    /* don't continue to penalize gaps larger than this */
#define JMPS      1024  /* max jmps in an path */
#define MX        4     /* save if there's at least MX-1 bases since last jmp */

#define DMAT      3     /* value of matching bases */
#define DMIS      0     /* penalty for mismatched bases */
#define DINS0     8     /* penalty for a gap */
#define DINS1     1     /* penalty per base */
#define PINS0     8     /* penalty for a gap */
#define PINS1     4     /* penalty per residue */

struct jmp {
        short          n[MAXJMP];  /* size of jmp (neg for dely) */
        unsigned short x[MAXJMP];  /* base no. of jmp in seq x */
```

```
};                                  /* limits seq to 2^16 -1 */

struct diag {
        int             score;      /* score at last jmp */
        long            offset;     /* offset of prev block */
        short           ijmp;       /* current jmp index */
        struct jmp      jp;         /* list of jmps */
};

struct path {
        int     spc;        /* number of leading spaces */
        short   n[JMPS];    /* size of jmp (gap) */
        int     x[JMPS];    /* loc of jmp (last elem before gap) */
};

char            *ofile;             /* output file name */
char            *namex[2];          /* seq names: getseqs() */
char            *prog;              /* prog name for err msgs */
char            *seqx[2];                   /* seqs: getseqs() */
int             dmax;               /* best diag: nw() */
int             dmax0;              /* final diag */
int             dna;                /* set if dna: main() */
int             endgaps;                    /* set if penalizing end gaps */
int             gapx, gapy;         /* total gaps in seqs */
int             len0, len1;         /* seq lens */
int             ngapx, ngapy;       /* total size of gaps */
int             smax;               /* max score: nw() */
int             *xbm;               /* bitmap for matching */
long            offset;             /* current offset in jmp file */
struct  diag    *dx;                /* holds diagonals */
struct  path    pp[2];              /* holds path for seqs */

char            *calloc(), *malloc(), *index(), *strcpy();
char            *getseq(), *g_calloc();
```

```
/* Needleman-Wunsch alignment program
 *
 * usage: progs file1 file2
 *   where file1 and file2 are two dna or two protein sequences.
 *   The sequences can be in upper- or lower-case an may contain ambiguity
 *   Any lines beginning with ';', '>' or '<' are ignored
 *   Max file length is 65535 (limited by unsigned short x in the jmp struct)
 *   A sequence with 1/3 or more of its elements ACGTU is assumed to be DNA
 *   Output is in the file "align.out"
 *
 * The program may create a tmp file in /tmp to hold info about traceback.
 * Original version developed under BSD 4.3 on a vax 8650
 */
#include "nw.h"
#include "day.h"

static  _dbval[26] = {
        1,14,2,13,0,0,4,11,0,0,12,0,3,15,0,0,0,5,6,8,8,7,9,0,10,0
};

static  _pbval[26] = {
        1, 2|(1<<('D'-'A'))|(1<<('N'-'A')), 4, 8, 16, 32, 64,
        128, 256, 0xFFFFFFF, 1<<10, 1<<11, 1<<12, 1<<13, 1<<14,
        1<<15, 1<<16, 1<<17, 1<<18, 1<<19, 1<<20, 1<<21, 1<<22,
        1<<23, 1<<24, 1<<25|(1<<('E'-'A'))|(1<<('Q'-'A'))
};

main(ac, av)                                                    main
        int     ac;
        char    *av[];
{
        prog = av[0];
        if (ac != 3) {
```

```
            fprintf(stderr,"usage: %s file1 file2\n", prog);
            fprintf(stderr,"where file1 and file2 are two dna or two protein sequences.\n");
            fprintf(stderr,"The sequences can be in upper- or lower-case\n");
            fprintf(stderr,"Any lines beginning with ';' or '<' are ignored\n");
            fprintf(stderr,"Output is in the file \"align.out\"\n");
            exit(1);
        }
        namex[0] = av[1];
        namex[1] = av[2];
        seqx[0] = getseq(namex[0], &len0);
        seqx[1] = getseq(namex[1], &len1);
        xbm = (dna)? _dbval : _pbval;

        endgaps = 0;                /* 1 to penalize endgaps */
        ofile = "align.out";        /* output file */

        nw();           /* fill in the matrix, get the possible jmps */
        readjmps();     /* get the actual jmps */
        print();        /* print stats, alignment */

        cleanup(0);     /* unlink any tmp files */
}


/* do the alignment, return best score: main()
 * dna: values in Fitch and Smith, PNAS, 80, 1382-1386, 1983
 * pro: PAM 250 values
 * When scores are equal, we prefer mismatches to any gap, prefer
 * a new gap to extending an ongoing gap, and prefer a gap in seqx
 * to a gap in seq y.
 */
nw()                                                        nw
{
        char        *px, *py;           /* seqs and ptrs */
        int         *ndely, *dely;  /* keep track of dely */
```

```
int             ndelx, delx;    /* keep track of delx */
int             *tmp;           /* for swapping row0, row1 */
int             mis;            /* score for each type */
int             ins0, ins1;     /* insertion penalties */
register            id;             /* diagonal index */
register            ij;             /* jmp index */
register            *col0, *col1;   /* score for curr, last row */
register            xx, yy;         /* index into seqs */

dx = (struct diag *)g_calloc("to get diags", len0+len1+1, sizeof(struct diag));

ndely = (int *)g_calloc("to get ndely", len1+1, sizeof(int));
dely = (int *)g_calloc("to get dely", len1+1, sizeof(int));
col0 = (int *)g_calloc("to get col0", len1+1, sizeof(int));
col1 = (int *)g_calloc("to get col1", len1+1, sizeof(int));
ins0 = (dna)? DINS0 : PINS0;
ins1 = (dna)? DINS1 : PINS1;

smax = -10000;
if (endgaps) {
        for (col0[0] = dely[0] = -ins0, yy = 1; yy <= len1; yy++) {
                col0[yy] = dely[yy] = col0[yy-1] - ins1;
                ndely[yy] = yy;
        }
        col0[0] = 0;      /* Waterman Bull Math Biol 84 */
}
else
        for (yy = 1; yy <= len1; yy++)
                dely[yy] = -ins0;

/* fill in match matrix
 */
for (px = seqx[0], xx = 1; xx <= len0; px++, xx++) {
        /* initialize first entry in col
         */
        if (endgaps) {
```

```
            if (xx == 1)
                col1[0] = delx = -(ins0+ins1);
            else
                col1[0] = delx = col0[0] - ins1;
            ndelx = xx;
        }
        else {
            col1[0] = 0;
            delx = -ins0;
            ndelx = 0;
        }
```

...nw

```
        for (py = seqx[1], yy = 1; yy <= len1; py++, yy++) {
            mis = col0[yy-1];
            if (dna)
                mis += (xbm[*px-'A']&xbm[*py-'A'])? DMAT : DMIS;
            else
                mis += _day[*px-'A'][*py-'A'];

            /* update penalty for del in x seq;
             * favor new del over ongong del
             * ignore MAXGAP if weighting endgaps
             */
            if (endgaps || ndely[yy] < MAXGAP) {
                if (col0[yy] - ins0 >= dely[yy]) {
                    dely[yy] = col0[yy] - (ins0+ins1);
                    ndely[yy] = 1;
                } else {
                    dely[yy] -= ins1;
                    ndely[yy]++;
                }
            } else {
                if (col0[yy] - (ins0+ins1) >= dely[yy]) {
                    dely[yy] = col0[yy] - (ins0+ins1);
```

```
                ndely[yy] = 1;
            } else
                ndely[yy]++;
        }

        /* update penalty for del in y seq;
         * favor new del over ongong del
         */
        if (endgaps || ndelx < MAXGAP) {
            if (col1[yy-1] - ins0 >= delx) {
                delx = col1[yy-1] - (ins0+ins1);
                ndelx = 1;
            } else {
                delx -= ins1;
                ndelx++;
            }
        } else {
            if (col1[yy-1] - (ins0+ins1) >= delx) {
                delx = col1[yy-1] - (ins0+ins1);
                ndelx = 1;
            } else
                ndelx++;
        }

        /* pick the maximum score; we're favoring
         * mis over any del and delx over dely
         */
```

...nw

```
        id = xx - yy + len1 - 1;
```

```
                if (mis >= delx && mis >= dely[yy])
                        col1[yy] = mis;
                else if (delx >= dely[yy]) {
                        col1[yy] = delx;
                        ij = dx[id].ijmp;
                        if (dx[id].jp.n[0] && (!dna || (ndelx >= MAXJMP
                        && xx > dx[id].jp.x[ij]+MX) || mis > dx[id].score+DINS0)) {
                                dx[id].ijmp++;
                                if (++ij >= MAXJMP) {
                                        writejmps(id);
                                        ij = dx[id].ijmp = 0;
                                        dx[id].offset = offset;
                                        offset += sizeof(struct jmp) + sizeof(offset);
                                }
                        }
                        dx[id].jp.n[ij] = ndelx;
                        dx[id].jp.x[ij] = xx;
                        dx[id].score = delx;
                }
                else {
                        col1[yy] = dely[yy];
                        ij = dx[id].ijmp;
        if (dx[id].jp.n[0] && (!dna || (ndely[yy] >= MAXJMP
                        && xx > dx[id].jp.x[ij]+MX) || mis > dx[id].score+DINS0)) {
                                dx[id].ijmp++;
                                if (++ij >= MAXJMP) {
                                        writejmps(id);
                                        ij = dx[id].ijmp = 0;
                                        dx[id].offset = offset;
                                        offset += sizeof(struct jmp) + sizeof(offset);
                                }
                        }
                        dx[id].jp.n[ij] = -ndely[yy];
                        dx[id].jp.x[ij] = xx;
                        dx[id].score = dely[yy];
                }
```

```
                if (xx == len0 && yy < len1) {
                        /* last col
                         */
                        if (endgaps)
                                col1[yy] -= ins0+ins1*(len1-yy);
                        if (col1[yy] > smax) {
                                smax = col1[yy];
                                dmax = id;
                        }
                }
        }
        if (endgaps && xx < len0)
                col1[yy-1] -= ins0+ins1*(len0-xx);
        if (col1[yy-1] > smax) {
                smax = col1[yy-1];
                dmax = id;
        }
        tmp = col0; col0 = col1; col1 = tmp;
    }
    (void) free((char *)ndely);
    (void) free((char *)dely);
    (void) free((char *)col0);
    (void) free((char *)col1);                }

/*
 *
 * print() -- only routine visible outside this module
 *
 * static:
 * getmat() -- trace back best path, count matches: print()
 * pr_align() -- print alignment of described in array p[]: print()
 * dumpblock() -- dump a block of lines with numbers, stars: pr_align()
 * nums() -- put out a number line: dumpblock()
 * putline() -- put out a line (name, [num], seq, [num]): dumpblock()
 * stars() - -put a line of stars: dumpblock()
 * stripname() -- strip any path and prefix from a seqname
```

```
*/

#include "nw.h"

#define SPC   3
#define P_LINE        256     /* maximum output line */
#define P_SPC 3       /* space between name or num and seq */

extern  _day[26][26];
int     olen;         /* set output line length */
FILE    *fx;          /* output file */

print()                                                          print
{
        int     lx, ly, firstgap, lastgap;  /* overlap */

        if ((fx = fopen(ofile, "w")) == 0) {
                fprintf(stderr,"%s: can't write %s\n", prog, ofile);
                cleanup(1);
        }
        fprintf(fx, "<first sequence: %s (length = %d)\n", namex[0], len0);
        fprintf(fx, "<second sequence: %s (length = %d)\n", namex[1], len1);
        olen = 60;
        lx = len0;
        ly = len1;
        firstgap = lastgap = 0;
        if (dmax < len1 - 1) {    /* leading gap in x */
                pp[0].spc = firstgap = len1 - dmax - 1;
                ly -= pp[0].spc;
        }
        else if (dmax > len1 - 1) {       /* leading gap in y */
                pp[1].spc = firstgap = dmax - (len1 - 1);
                lx -= pp[1].spc;
        }
        if (dmax0 < len0 - 1) {  /* trailing gap in x */
                lastgap = len0 - dmax0 -1;
```

```
                lx -= lastgap;
        }
        else if (dmax0 > len0 - 1) {        /* trailing gap in y */
                lastgap = dmax0 - (len0 - 1);
                ly -= lastgap;
        }
        getmat(lx, ly, firstgap, lastgap);
        pr_align();
}



/*
 * trace back the best path, count matches
 */
static
getmat(lx, ly, firstgap, lastgap)                                        getmat
        int     lx, ly;                 /* "core" (minus endgaps) */
        int     firstgap, lastgap;              /* leading trailing overlap */
{
        int             nm, i0, i1, siz0, siz1;
        char            outx[32];
        double          pct;
        register                n0, n1;
        register char   *p0, *p1;

        /* get total matches, score
         */
        i0 = i1 = siz0 = siz1 = 0;
        p0 = seqx[0] + pp[1].spc;
        p1 = seqx[1] + pp[0].spc;
        n0 = pp[1].spc + 1;
        n1 = pp[0].spc + 1;

        nm = 0;
```

```
while ( *p0 && *p1 ) {
        if (siz0) {
                p1++;
                n1++;
                siz0--;
        }
        else if (siz1) {
                p0++;
                n0++;
                siz1--;
        }
        else {
                if (xbm[*p0-'A']&xbm[*p1-'A'])
                        nm++;
                if (n0++ == pp[0].x[i0])
                        siz0 = pp[0].n[i0++];
                if (n1++ == pp[1].x[i1])
                        siz1 = pp[1].n[i1++];
                p0++;
                p1++;
        }
}

/* pct homology:
 * if penalizing endgaps, base is the shorter seq
 * else, knock off overhangs and take shorter core
 */
if (endgaps)
        lx = (len0 < len1)? len0 : len1;
else
        lx = (lx < ly)? lx : ly;
pct = 100.*(double)nm/(double)lx;
fprintf(fx, "\n");
fprintf(fx, "<%d match%s in an overlap of %d: %.2f percent similarity\n",
        nm, (nm == 1)? "" : "es", lx, pct);
```

```
        fprintf(fx, "<gaps in first sequence: %d", gapx);                    ...getmat
        if (gapx) {
                (void) sprintf(outx, " (%d %s%s)",
                        ngapx, (dna)? "base":"residue", (ngapx == 1)? "":"s");
                fprintf(fx,"%s", outx);

        fprintf(fx, ", gaps in second sequence: %d", gapy);
        if (gapy) {
                (void) sprintf(outx, " (%d %s%s)",
                        ngapy, (dna)? "base":"residue", (ngapy == 1)? "":"s");
                fprintf(fx,"%s", outx);
        }
        if (dna)
                fprintf(fx,
                "\n<score: %d (match = %d, mismatch = %d, gap penalty = %d + %d per base)\n",
                smax, DMAT, DMIS, DINS0, DINS1);
        else
                fprintf(fx,
                "\n<score: %d (Dayhoff PAM 250 matrix, gap penalty = %d + %d per residue)\n",
                smax, PINS0, PINS1);
        if (endgaps)
                fprintf(fx,
                "<endgaps penalized. left endgap: %d %s%s, right endgap: %d %s%s\n",
                firstgap, (dna)? "base" : "residue", (firstgap == 1)? "" : "s",
                lastgap, (dna)? "base" : "residue", (lastgap == 1)? "" : "s");
        else
                fprintf(fx, "<endgaps not penalized\n");
}

static          nm;             /* matches in core -- for checking */
static          lmax;           /* lengths of stripped file names */
static          ij[2];          /* jmp index for a path */
static          nc[2];          /* number at start of current line */
```

```
static          ni[2];          /* current elem number -- for gapping */
static          siz[2];
static char     *ps[2];         /* ptr to current element */
static char     *po[2];         /* ptr to next output char slot */
static char     out[2][P_LINE];         /* output line */
static char     star[P_LINE];   /* set by stars() */

/*
 * print alignment of described in struct path pp[]
 */
static
pr_align()                                                      pr_align
{
        int             nn;     /* char count */
        int             more;
        register                i;

        for (i =  0, lmax = 0; i < 2; i++) {
                nn = stripname(namex[i]);
                if (nn > lmax)
                        lmax = nn;

                nc[i] = 1;
                ni[i] = 1;
                siz[i] = ij[i] = 0;
                ps[i] = seqx[i];
                po[i] = out[i];         }


        for (nn = nm = 0, more = 1; more; ) {                   ...pr_align
                for (i = more = 0; i < 2; i++) {
                        /*
                         * do we have more of this sequence?
                         */
                        if (!*ps[i])
                                continue;
```

```
                more++;

                if (pp[i].spc) {   /* leading space */
                        *po[i]++ = ' ';
                        pp[i].spc--;
                }
                else if (siz[i]) {  /* in a gap */
                        *po[i]++ = '-';
                        siz[i]--;
                }
                else {              /* we're putting a seq element
                                     */
                        *po[i] = *ps[i];
                        if (islower(*ps[i]))
                                *ps[i] = toupper(*ps[i]);
                        po[i]++;
                        ps[i]++;

                        /*
                         * are we at next gap for this seq?
                         */
                        if (ni[i] == pp[i].x[ij[i]]) {
                                /*
                                 * we need to merge all gaps
                                 * at this location
                                 */
                                siz[i] = pp[i].n[ij[i]++];
                                while (ni[i] == pp[i].x[ij[i]])
                                        siz[i] += pp[i].n[ij[i]++];
                        }
                        ni[i]++;
                }
        }
        if (++nn == olen || !more && nn) {
                dumpblock();
```

```
                        for (i = 0; i < 2; i++)
                                po[i] = out[i];
                        nn = 0;
                }
        }
}

/*
 * dump a block of lines, including numbers, stars: pr_align()
 */
static
dumpblock()                                                    dumpblock
{
        register        i;

        for (i = 0; i < 2; i++)
                *po[i]-- = '\0';



                                                               ...dumpblock
        (void) putc('\n', fx);
        for (i = 0; i < 2; i++) {
                if (*out[i] && (*out[i] != ' ' || *(po[i]) != ' ')) {
                        if (i == 0)
                                nums(i);
                        if (i == 0 && *out[1])
                                stars();
                        putline(i);
                        if (i == 0 && *out[1])
                                fprintf(fx, star);
                        if (i == 1)
                                nums(i);
                }
        }
}
```

```
/*
 * put out a number line: dumpblock()
 */
static
nums(ix)                                                    nums
        int     ix;     /* index in out[] holding seq line */
{
        char            nline[P_LINE];
        register                i, j;
        register char   *pn, *px, *py;

        for (pn = nline, i = 0; i < lmax+P_SPC; i++, pn++)
                *pn = ' ';
        for (i = nc[ix], py = out[ix]; *py; py++, pn++) {
                if (*py == ' ' || *py == '-')
                        *pn = ' ';
                else {
                        if (i%10 == 0 || (i == 1 && nc[ix] != 1)) {
                                j = (i < 0)? -i : i;
                                for (px = pn; j; j /= 10, px--)
                                        *px = j%10 + '0';
                                if (i < 0)
                                        *px = '-';
                        }
                        else
                                *pn = ' ';
                        i++;
                }
        }
        *pn = '\0';
        nc[ix] = i;
        for (pn = nline; *pn; pn++)
                (void) putc(*pn, fx);
        (void) putc('\n', fx);
}
```

```
/*
 * put out a line (name, [num], seq, [num]): dumpblock()
 */
static
putline(ix)                                                    putline
        int     ix;                     {



                                                               ...putline
        int             i;
        register char   *px;

        for (px = namex[ix], i = 0; *px && *px != ':'; px++, i++)
                (void) putc(*px, fx);
        for (; i < lmax+P_SPC; i++)
                (void) putc(' ', fx);

        /* these count from 1:
         * ni[] is current element (from 1)
         * nc[] is number at start of current line
         */
        for (px = out[ix]; *px; px++)
                (void) putc(*px&0x7F, fx);
        (void) putc('\n', fx);
}


/*
 * put a line of stars (seqs always in out[0], out[1]): dumpblock()
 */
static
stars()                                                        stars
{
        int             i;
        register char   *p0, *p1, cx, *px;
```

```
        if (!*out[0] || (*out[0] == ' ' && *(po[0]) == ' ') ||
           !*out[1] || (*out[1] == ' ' && *(po[1]) == ' '))
                return;
        px = star;
        for (i = lmax+P_SPC; i; i--)
                *px++ = ' ';

        for (p0 = out[0], p1 = out[1]; *p0 && *p1; p0++, p1++) {
                if (isalpha(*p0) && isalpha(*p1)) {
                        if (xbm[*p0-'A']&xbm[*p1-'A']) {
                                cx = '*';
                                nm++;
                        }
                        else if (!dna && _day[*p0-'A'][*p1-'A'] > 0)
                                cx = '.';
                        else
                                cx = ' ';
                }
                else
                        cx = ' ';
                *px++ = cx;
        }
        *px++ = '\n';
        *px = '\0';
}


/*
 * strip path or prefix from pn, return len: pr_align()
 */
static
```

```
stripname(pn)                                                    stripname
        char    *pn;    /* file name (may be path) */
{
        register char   *px, *py;

        py = 0;
        for (px = pn; *px; px++)
                if (*px == '/')
                        py = px + 1;
        if (py)
                (void) strcpy(pn, py);
        return(strlen(pn));

}

/*
 * cleanup() -- cleanup any tmp file
 * getseq() -- read in seq, set dna, len, maxlen
 * g_calloc() -- calloc() with error checkin
 * readjmps() -- get the good jmps, from tmp file if necessary
 * writejmps() -- write a filled array of jmps to a tmp file: nw()
 */
#include "nw.h"
#include <sys/file.h>

char    *jname = "/tmp/homgXXXXXX";             /* tmp file for jmps */
FILE    *fj;

int     cleanup();                      /* cleanup tmp file */
long    lseek();

/*
 * remove any tmp file if we blow
 */
cleanup(i)                                                       cleanup
```

```
        int     i;
{
        if (fj)
                (void) unlink(jname);
        exit(i);
}

/*
 * read, return ptr to seq, set dna, len, maxlen
 * skip lines starting with ';', '<', or '>'
 * seq in upper or lower case
 */
char    *
getseq(file, len)                                                    getseq
        char    *file;  /* file name */
        int     *len;   /* seq len */
{
        char            line[1024], *pseq;
        register char   *px, *py;
        int             natgc, tlen;
        FILE            *fp;

        if ((fp = fopen(file,"r")) == 0) {
                fprintf(stderr,"%s: can't read %s\n", prog, file);
                exit(1);
        }
        tlen = natgc = 0;
        while (fgets(line, 1024, fp)) {
                if (*line == ';' || *line == '<' || *line == '>')
                        continue;
                for (px = line; *px != '\n'; px++)
                        if (isupper(*px) || islower(*px))
                                tlen++;
        }
        if ((pseq = malloc((unsigned)(tlen+6))) == 0) {
                fprintf(stderr,"%s: malloc() failed to get %d bytes for %s\n", prog, tlen+6, file);
```

```
            exit(1);
        }
        pseq[0] = pseq[1] = pseq[2] = pseq[3] = '\0';

                                                                    ...getseq
        py = pseq + 4;
        *len = tlen;
        rewind(fp);

        while (fgets(line, 1024, fp)) {
            if (*line == ';' || *line == '<' || *line == '>')
                continue;
            for (px = line; *px != '\n'; px++) {
                if (isupper(*px))
                    *py++ = *px;
                else if (islower(*px))
                    *py++ = toupper(*px);
                if (index("ATGCU",*(py-1)))
                    natgc++;
            }
        }
        *py++ = '\0';
        *py = '\0';
        (void) fclose(fp);
        dna = natgc > (tlen/3);
        return(pseq+4);
    }

    char    *
    g_calloc(msg, nx, sz)                                           g_calloc
        char    *msg;           /* program, calling routine */
        int     nx, sz;         /* number and size of elements */
    {
        char            *px, *calloc();
```

```
        if ((px = calloc((unsigned)nx, (unsigned)sz)) == 0) {
                if (*msg) {
                        fprintf(stderr, "%s: g_calloc() failed %s (n=%d, sz=%d)\n", prog, msg, nx,
5   sz);
                        exit(1);
                }
        }
        return(px);
10  }

    /*
     * get final jmps from dx[] or tmp file, set pp[], reset dmax: main()
     */
15  readjmps()                                                          readjmps
    {
        int             fd = -1;
        int             siz, i0, i1;
        register        i, j, xx;
20
        if (fj) {
                (void) fclose(fj);
                if ((fd = open(jname, O_RDONLY, 0)) < 0) {
                        fprintf(stderr, "%s: can't open() %s\n", prog, jname);
25                      cleanup(1);
                }
        }
        for (i = i0 = i1 = 0, dmax0 = dmax, xx = len0; ; i++) {
                while (1) {
30                      for (j = dx[dmax].ijmp; j >= 0 && dx[dmax].jp.x[j] >= xx; j--)
                                ;

                                                                        ...readjmps
                        if (j < 0 && dx[dmax].offset && fj) {
35                              (void) lseek(fd, dx[dmax].offset, 0);
                                (void) read(fd, (char *)&dx[dmax].jp, sizeof(struct jmp));
```

```
                (void) read(fd, (char *)&dx[dmax].offset, sizeof(dx[dmax].offset));
                dx[dmax].ijmp = MAXJMP-1;
            }
            else
                break;
        }
        if (i >= JMPS) {
            fprintf(stderr, "%s: too many gaps in alignment\n", prog);
            cleanup(1);
        }
        if (j >= 0) {
            siz = dx[dmax].jp.n[j];
            xx = dx[dmax].jp.x[j];
            dmax += siz;
            if (siz < 0) {              /* gap in second seq */
                pp[1].n[i1] = -siz;
                xx += siz;
                /* id = xx - yy + len1 - 1
                 */
                pp[1].x[i1] = xx - dmax + len1 - 1;
                gapy++;
                ngapy -= siz;
/* ignore MAXGAP when doing endgaps */
                siz = (-siz < MAXGAP || endgaps)? -siz : MAXGAP;
                i1++;
            }
            else if (siz > 0) {         /* gap in first seq */
                pp[0].n[i0] = siz;
                pp[0].x[i0] = xx;
                gapx++;
                ngapx += siz;
/* ignore MAXGAP when doing endgaps */
                siz = (siz < MAXGAP || endgaps)? siz : MAXGAP;
                i0++;
            }
        }
```

```
                else
                        break;
        }

        /* reverse the order of jmps
         */
        for (j = 0, i0--; j < i0; j++, i0--) {
                i = pp[0].n[j]; pp[0].n[j] = pp[0].n[i0]; pp[0].n[i0] = i;
                i = pp[0].x[j]; pp[0].x[j] = pp[0].x[i0]; pp[0].x[i0] = i;
        }
        for (j = 0, i1--; j < i1; j++, i1--) {
                i = pp[1].n[j]; pp[1].n[j] = pp[1].n[i1]; pp[1].n[i1] = i;
                i = pp[1].x[j]; pp[1].x[j] = pp[1].x[i1]; pp[1].x[i1] = i;
        }
        if (fd >= 0)
                (void) close(fd);
        if (fj) {
                (void) unlink(jname);
                fj = 0;
                offset = 0;
        }                                       }


/*
 * write a filled jmp struct offset of the prev one (if any): nw()
 */
writejmps(ix)                                                   writejmps
        int     ix;
{
        char    *mktemp();

        if (!fj) {
                if (mktemp(jname) < 0) {
                        fprintf(stderr, "%s: can't mktemp() %s\n", prog, jname);
                        cleanup(1);
                }
```

```
            if ((fj = fopen(jname, "w")) == 0) {
                fprintf(stderr, "%s: can't write %s\n", prog, jname);
                exit(1);
            }
        }
        (void) fwrite((char *)&dx[ix].jp, sizeof(struct jmp), 1, fj);
        (void) fwrite((char *)&dx[ix].offset, sizeof(dx[ix].offset), 1, fj);
```

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "recombinant vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after synthesis, such as by conjugation with a label. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies,

signal peptides, ply-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, .alpha.-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR.sub.2 ("amidate"), P(O)R, P(O)OR', CO or CH.sub.2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C.) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

"Oligonucleotide," as used herein, generally refers to short, generally single stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat", and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence. Unless indicated otherwise, numbering of all amino acid positions herein is according to the Kabat numbering system.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residue in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat *et al.* In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat *et al.* In one embodiment, for the VH, the subgroup is subgroup III as in Kabat *et al.*

A "VH subgroup III consensus framework" comprises the consensus sequence obtained from the amino acid sequences in variable heavy chain subgroup III of Kabat *et al.* In one embodiment, the VH subgroup III consensus framework amino acid sequence comprises at least a portion or all of each of the following sequences: EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO:46)-H1-WVRQAPGKGLEWV (SEQ ID NO:47)-H2-RFTISRDNSKNTLYLQMNSLRAEDTAVYYC (SEQ ID NO:48)-H3-WGQGTLVTVSS (SEQ ID NO:49).

A "VL subgroup I consensus framework" comprises the consensus sequence obtained from the amino acid sequences in variable light kappa subgroup I of Kabat *et al.* In one embodiment, the VL subgroup I consensus framework amino acid sequence comprises at least a portion or all of each of the following sequences:

DIQMTQSPSSLSASVGDRVTITC (SEQ ID NO:50)-L1-WYQQKPGKAPKLLIY (SEQ ID NO:51)-L2-GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC (SEQ ID NO:52)-L3-FGQGTKVEIK (SEQ ID NO:53).

The term "hepatocyte growth factor" or "HGF", as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether naturally occurring or synthetic) HGF polypeptide that is capable of activating the HGF/c-met signaling pathway under conditions

that permit such process to occur. The term "wild type HGF" generally refers to a polypeptide comprising the amino acid sequence of a naturally occurring HGF protein. Thet term "wild type HGF sequence" generally refers to an amino acid sequence found in a naturally occurring HGF.

The terms "antibody" and "immunoglobulin" are used interchangeably in the broadest sense and include monoclonal antibodies (for *e.g.*, full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies, multispecific antibodies (*e.g.*, bispecific antibodies so long as they exhibit the desired biological activity) and may also include certain antibody fragments (as described in greater detail herein). An antibody can be human, humanized and/or affinity matured.

"Antibody fragments" comprise only a portion of an intact antibody, wherein the portion preferably retains at least one, preferably most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For e.g., such an antibody fragment may comprise on antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigen. Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable

region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994).

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks *et al. Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas *et al. Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994); Schier *et al. Gene* 169:147-155 (1995); Yelton *et al. J. Immunol.* 155:1994-2004 (1995); Jackson *et al., J. Immunol.* 154(7):3310-9 (1995); and Hawkins *et al, J. Mol. Biol.* 226:889-896 (1992).

A "blocking" antibody or an "antagonist" antibody is one which inhibits or reduces biological activity of the antigen it binds. Preferred blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen.

An "agonist antibody", as used herein, is an antibody which mimics at least one of the functional activities of a polypeptide of interest.

A "disorder" is any condition that would benefit from treatment with a substance/molecule or method of the invention. This includes chronic and acute disorders or diseases including those

pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include malignant and benign tumors; non-leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, immunologic and other angiogenesis-related disorders.

The terms "cell proliferative disorder" and "proliferative disorder" refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer.

"Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "cancer", "cancerous", "cell proliferative disorder", "proliferative disorder" and "tumor" are not mutually exclusive as referred to herein.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

Dysregulation of angiogenesis can lead to many disorders that can be treated by compositions and methods of the invention. These disorders include both non-neoplastic and neoplastic conditions. Neoplastics include but are not limited to those described above. Non-neoplastic disorders include but are not limited to undesired or aberrant hypertrophy, arthritis, rheumatoid arthritis (RA), psoriasis, psoriatic plaques, sarcoidosis, atherosclerosis, atherosclerotic plaques, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, arteriovenous malformations (AVM), meningioma, hemangioma, angiofibroma, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, acute lung injury/ARDS, sepsis, primary pulmonary hypertension, malignant pulmonary effusions, cerebral edema (e.g., associated with acute stroke/ closed head injury/ trauma), synovial inflammation, pannus formation in RA, myositis

ossificans, hypertropic bone formation, osteoarthritis (OA), refractory ascites, polycystic ovarian disease, endometriosis, 3rd spacing of fluid diseases (pancreatitis, compartment syndrome, burns, bowel disease), uterine fibroids, premature labor, chronic inflammation such as IBD (Crohn's disease and ulcerative colitis), renal allograft rejection, inflammatory bowel disease, nephrotic syndrome, undesired or aberrant tissue mass growth (non-cancer), hemophilic joints, hypertrophic scars, inhibition of hair growth, Osler-Weber syndrome, pyogenic granuloma retrolental fibroplasias, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

An "autoimmune disease" herein is a non-malignant disease or disorder arising from and directed against an individual's own tissues. The autoimmune diseases herein specifically exclude malignant or cancerous diseases or conditions, especially excluding B cell lymphoma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), Hairy cell leukemia and chronic myeloblastic leukemia. Examples of autoimmune diseases or disorders include, but are not limited to, inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (*e.g.* atopic dermatitis); systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); respiratory distress syndrome (including adult respiratory distress syndrome; ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus (*e.g.* Type I diabetes mellitus or insulin dependent diabetes mellitis); multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjorgen's syndrome; juvenile onset diabetes; and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia (including, but not limited to cryoglobinemia or Coombs positive anemia) ; myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; antiphospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullous; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Behcet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia etc.

As used herein, "treatment" refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or

recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or disorder.

An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

A "therapeutically effective amount" of a substance/molecule of the invention, agonist or antagonist may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, agonist or antagonist to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the substance/molecule, agonist or antagonist are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents e.g. methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and

bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (*e. g.*, calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII (see, *e.g.*, Agnew, *Chem Intl. Ed. Engl.*, 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection (DOXIL®) and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti- adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, *e.g.*, paclitaxel (TAXOL®), albumin-engineered nanoparticle formulation of paclitaxel (ABRAXANE™), and doxetaxel (TAXOTERE®); chloranbucil; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and

carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovovin.

Also included in this definition are anti-hormonal agents that act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer, and are often in the form of systemic, or whole-body treatment. They may be hormones themselves. Examples include anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene (EVISTA®), droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (FARESTON®); anti-progesterones; estrogen receptor down-regulators (ERDs); estrogen receptor antagonists such as fulvestrant (FASLODEX®); agents that function to suppress or shut down the ovaries, for example, leutinizing hormone-releasing hormone (LHRH) agonists such as leuprolide acetate (LUPRON® and ELIGARD®), goserelin acetate, buserelin acetate and tripterelin; other anti-androgens such as flutamide, nilutamide and bicalutamide; and aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, megestrol acetate (MEGASE®), exemestane (AROMASIN®), formestanie, fadrozole, vorozole (RIVISOR®), letrozole (FEMARA®), and anastrozole (ARIMIDEX®). In addition, such definition of chemotherapeutic agents includes bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), etidronate (DIDROCAL®), NE-58095, zoledronic acid/zoledronate (ZOMETA®), alendronate (FOSAMAX®), pamidronate (AREDIA®), tiludronate (SKELID®), or risedronate (ACTONEL®); as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; topoisomerase 1 inhibitor (e.g., LURTOTECAN®); rmRH (e.g., ABARELIX®); lapatinib ditosylate (an ErbB-2 and EGFR dual tyrosine kinase small-molecule inhibitor also known as GW572016); COX-2 inhibitors such as celecoxib (CELEBREX®; 4-(5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl) benzenesulfonamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell whose growth is dependent upon HGF/c-met activation either *in vitro* or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of HGF/c-met-dependent cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

"Doxorubicin" is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexapyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

Vectors, Host Cells and Recombinant Methods

For recombinant production of an antibody of the invention, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The choice of vector depends in part on the host cell to be used. Generally, preferred host cells are of either prokaryotic or eukaryotic (generally mammalian) origin.

Generating antibodies using prokaryotic host cells:

Vector Construction

Polynucleotide sequences encoding polypeptide components of the antibody of the invention can be obtained using standard recombinant techniques. Desired polynucleotide sequences may be isolated and sequenced from antibody producing cells such as hybridoma cells. Alternatively, polynucleotides can be synthesized using nucleotide synthesizer or PCR techniques. Once obtained, sequences encoding the polypeptides are inserted into a recombinant vector capable of replicating and expressing heterologous polynucleotides in prokaryotic hosts. Many vectors that

are available and known in the art can be used for the purpose of the present invention. Selection of an appropriate vector will depend mainly on the size of the nucleic acids to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components, depending on its function (amplification or expression of heterologous polynucleotide, or both) and its compatibility with the particular host cell in which it resides. The vector components generally include, but are not limited to: an origin of replication, a selection marker gene, a promoter, a ribosome binding site (RBS), a signal sequence, the heterologous nucleic acid insert and a transcription termination sequence.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species. pBR322 contains genes encoding ampicillin (Amp) and tetracycline (Tet) resistance and thus provides easy means for identifying transformed cells. pBR322, its derivatives, or other microbial plasmids or bacteriophage may also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of endogenous proteins. Examples of pBR322 derivatives used for expression of particular antibodies are described in detail in Carter et al., U.S. Patent No. 5,648,237.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, bacteriophage such as λGEM.TM.-11 may be utilized in making a recombinant vector which can be used to transform susceptible host cells such as E. coli LE392.

The expression vector of the invention may comprise two or more promoter-cistron pairs, encoding each of the polypeptide components. A promoter is an untranslated regulatory sequence located upstream (5') to a cistron that modulates its expression. Prokaryotic promoters typically fall into two classes, inducible and constitutive. Inducible promoter is a promoter that initiates increased levels of transcription of the cistron under its control in response to changes in the culture condition, e.g. the presence or absence of a nutrient or a change in temperature.

A large number of promoters recognized by a variety of potential host cells are well known. The selected promoter can be operably linked to cistron DNA encoding the light or heavy chain by removing the promoter from the source DNA via restriction enzyme digestion and inserting the isolated promoter sequence into the vector of the invention. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target genes. In some embodiments, heterologous promoters are utilized, as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the PhoA promoter, the β-galactamase and lactose promoter systems, a tryptophan (trp) promoter system and hybrid promoters such as the tac or the trc promoter. However, other promoters that are functional in bacteria (such as other known bacterial or phage promoters) are suitable as well. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to cistrons encoding the target light and heavy chains (Siebenlist et al. (1980) Cell 20: 269) using linkers or adaptors to supply any required restriction sites.

Each cistron within the recombinant vector may comprise a secretion signal sequence component that directs translocation of the expressed polypeptides across a membrane. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. The signal sequence selected for the purpose of this disclosure should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the signal sequences native to the heterologous polypeptides, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA and MBP. In one embodiment of the disclosure, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof.

Also disclosed is production of the immunoglobulins according to the disclosure can occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within each cistron. In that regard, immunoglobulin light and heavy chains are expressed, folded and assembled to form functional immunoglobulins within the cytoplasm. Certain host strains (e.g., the E. coli trxB$^-$ strains) provide cytoplasm conditions that are favorable for disulfide bond formation, thereby permitting proper folding and assembly of expressed protein subunits. Proba and Pluckthun Gene, 159:203 (1995).

Also disclosed is an expression system in which the quantitative ratio of expressed polypeptide components can be modulated in order to maximize the yield of secreted and properly assembled antibodies of the disclosure. Such modulation is accomplished at least in part by simultaneously modulating translational strengths for the polypeptide components.

One technique for modulating translational strength is disclosed in Simmons et al., U.S. Pat. No. 5,840,523. It utilizes variants of the translational initiation region (TIR) within a cistron. For a given TIR, a series of amino acid or nucleic acid sequence variants can be created with a range of translational strengths, thereby providing a convenient means by which to adjust this factor for the desired expression level of the specific chain. TIR variants can be generated by conventional mutagenesis techniques that result in codon changes which can alter the amino acid sequence, although silent changes in the nucleotide sequence are preferred. Alterations in the TIR can include,

for example, alterations in the number or spacing of Shine-Dalgarno sequences, along with alterations in the signal sequence. One method for generating mutant signal sequences is the generation of a "codon bank" at the beginning of a coding sequence that does not change the amino acid sequence of the signal sequence (i.e., the changes are silent). This can be accomplished by changing the third nucleotide position of each codon; additionally, some amino acids, such as leucine, serine, and arginine, have multiple first and second positions that can add complexity in making the bank. This method of mutagenesis is described in detail in Yansura et al. (1992) METHODS: A Companion to Methods in Enzymol. 4:151-158.

Preferably, a set of vectors is generated with a range of TIR strengths for each cistron therein. This limited set provides a comparison of expression levels of each chain as well as the yield of the desired antibody products under various TIR strength combinations. TIR strengths can be determined by quantifying the expression level of a reporter gene as described in detail in Simmons et al. U.S. Pat. No. 5, 840,523. Based on the translational strength comparison, the desired individual TIRs are selected to be combined in the expression vector constructs of the invention.

Prokaryotic host cells suitable for expressing antibodies of the invention include Archaebacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include Escherichia (e.g., E. coli), Bacilli (e.g., B. subtilis), Enterobacteria, Pseudomonas species (e.g., P. aeruginosa), Salmonella typhimurium, Serratia marcescans, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla, or Paracoccus. In one embodiment, gram-negative cells are used. In one embodiment, E. coli cells are used as hosts for the invention. Examples of E. coli strains include strain W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 ΔfhuA (ΔtonA) ptr3 lac Iq lacL8 ΔompTΔ(nmpc-fepE) degP41 kan^R (U.S. Pat. No. 5,639,635). Other strains and derivatives thereof, such as E. coli 294 (ATCC 31,446), E. coli B, E. $coli_\lambda$ 1776 (ATCC 31,537) and E. coli RV308(ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., Proteins, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, E. coli, Serratia, or Salmonella species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

Antibody Production

Host cells are transformed with the above-described expression vectors and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transformation means introducing DNA into the prokaryotic host so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride is generally used for bacterial cells that contain substantial cell-wall barriers. Another method for transformation employs polyethylene glycol/DMSO. Yet another technique used is electroporation.

Prokaryotic cells used to produce the polypeptides of the invention are grown in media known in the art and suitable for culture of the selected host cells. Examples of suitable media include luria broth (LB) plus necessary nutrient supplements. In some embodiments, the media also contains a selection agent, chosen based on the construction of the expression vector, to selectively permit growth of prokaryotic cells containing the expression vector. For example, ampicillin is added to media for growth of cells expressing ampicillin resistant gene.

Any necessary supplements besides carbon, nitrogen, and inorganic phosphate sources may also be included at appropriate concentrations introduced alone or as a mixture with another supplement or medium such as a complex nitrogen source. Optionally the culture medium may contain one or more reducing agents selected from the group consisting of glutathione, cysteine, cystamine, thioglycollate, dithioerythritol and dithiothreitol.

The prokaryotic host cells are cultured at suitable temperatures. For E. coli growth, for example, the preferred temperature ranges from about 20°C to about 39°C, more preferably from about 25°C to about 37°C, even more preferably at about 30°C. The pH of the medium may be any pH ranging from about 5 to about 9, depending mainly on the host organism. For E. coli, the pH is preferably from about 6.8 to about 7.4, and more preferably about 7.0.

If an inducible promoter is used in the expression vector of the invention, protein expression is induced under conditions suitable for the activation of the promoter. In one aspect of the invention, PhoA promoters are used for controlling transcription of the polypeptides. Accordingly, the transformed host cells are cultured in a phosphate-limiting medium for induction. Preferably, the phosphate-limiting medium is the C.R.A.P medium (see, for e.g., Simmons et al., J. Immunol. Methods (2002), 263:133-147). A variety of other inducers may be used, according to the vector construct employed, as is known in the art.

In one embodiment, the expressed polypeptides of the present invention are secreted into and recovered from the periplasm of the host cells. Protein recovery typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis. Once cells are

disrupted, cell debris or whole cells may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity resin chromatography. Alternatively, proteins can be transported into the culture media and isolated therein. Cells may be removed from the culture and the culture supernatant being filtered and concentrated for further purification of the proteins produced. The expressed polypeptides can be further isolated and identified using commonly known methods such as polyacrylamide gel electrophoresis (PAGE) and Western blot assay.

Also disclosed is antibody production is conducted in large quantity by a fermentation process. Various large-scale fed-batch fermentation procedures are available for production of recombinant proteins. Large-scale fermentations have at least 1000 liters of capacity, preferably about 1,000 to 100,000 liters of capacity. These fermentors use agitator impellers to distribute oxygen and nutrients, especially glucose (the preferred carbon/energy source). Small scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, and can range from about 1 liter to about 100 liters.

In a fermentation process, induction of protein expression is typically initiated after the cells have been grown under suitable conditions to a desired density, e.g., an OD_{550} of about 180-220, at which stage the cells are in the early stationary phase. A variety of inducers may be used, according to the vector construct employed, as is known in the art and described above. Cells may be grown for shorter periods prior to induction. Cells are usually induced for about 12-50 hours, although longer or shorter induction time may be used.

To improve the production yield and quality of the polypeptides of the disclosure, various fermentation conditions can be modified. For example, to improve the proper assembly and folding of the secreted antibody polypeptides, additional vectors overexpressing chaperone proteins, such as Dsb proteins (DsbA, DsbB, DsbC, DsbD and or DsbG) or FkpA (a peptidylprolyl cis,trans-isomerase with chaperone activity) can be used to co-transform the host prokaryotic cells. The chaperone proteins have been demonstrated to facilitate the proper folding and solubility of heterologous proteins produced in bacterial host cells. Chen et al. (1999) J Bio Chem 274:19601-19605; Georgiou et al., U.S. Patent No. 6,083,715; Georgiou et al., U.S. Patent No. 6,027,888; Bothmann and Pluckthun (2000) J. Biol. Chem. 275:17100-17105; Ramm and Pluckthun (2000) J. Biol. Chem. 275:17106-17113; Arie et al. (2001) Mol. Microbiol. 39:199-210.

To minimize proteolysis of expressed heterologous proteins (especially those that are proteolytically sensitive), certain host strains deficient for proteolytic enzymes can be used for the present disclosure. For example, host cell strains may be modified to effect genetic mutation(s) in the genes encoding known bacterial proteases such as Protease III, OmpT, DegP, Tsp, Protease I, Protease Mi, Protease V, Protease VI and combinations thereof. Some E. coli protease-deficient strains are available and described in, for example, Joly et al. (1998), supra; Georgiou et al., U.S. Patent No. 5,264,365; Georgiou et al., U.S. Patent No. 5,508,192; Hara et al., Microbial Drug Resistance, 2:63-72 (1996).

In one embodiment, E. coli strains deficient for proteolytic enzymes and transformed with plasmids overexpressing one or more chaperone proteins are used as host cells in the expression system of the disclosure.

Antibody Purification

In one embodiment, the antibody protein produced herein is further purified to obtain preparations that are substantially homogeneous for further assays and uses. Standard protein purification methods known in the art can be employed. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, chromatography on silica or on a cation-exchange resin such as DEAE, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, and gel filtration using, for example, Sephadex G-75.

Protein A immobilized on a solid phase may be used for immunoaffinity purification of the full length antibody products of the disclosure. Protein A is a 41kD cell wall protein from Staphylococcus aureas which binds with a high affinity to the Fc region of antibodies. Lindmark et al (1983) J. Immunol. Meth. 62:1-13. The solid phase to which Protein A is immobilized is preferably a column comprising a glass or silica surface, more preferably a controlled pore glass column or a silicic acid column. In some applications, the column has been coated with a reagent, such as glycerol, in an attempt to prevent nonspecific adherence of contaminants.

As the first step of purification, the preparation derived from the cell culture as described above is applied onto the Protein A immobilized solid phase to allow specific binding of the antibody of interest to Protein A. The solid phase is then washed to remove contaminants non-specifically bound to the solid phase. Finally the antibody of interest is recovered from the solid phase by elution.

Generating antibodies using eukaryotic host cells:

The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal sequence component

A vector for use in a eukaryotic host cell may also contain a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide of interest. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. In mammalian cell expression,

mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the antibody.

(ii) Origin of replication

Generally, an origin of replication component is not needed for mammalian expression vectors. For example, the SV40 origin may typically be used only because it contains the early promoter.

(iii) Selection gene component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, where relevant, or (c) supply critical nutrients not available from complex media.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity (e.g., ATCC CRL-9096).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding an antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

(iv) Promoter component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody polypeptide nucleic acid. Promoter sequences are known for eukaryotes. Virtually alleukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may

be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Antibody polypeptide transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes et al., Nature 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

(v) Enhancer element component

Transcription of DNA encoding the antibody polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody polypeptide-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription termination component

Expression vectors used in eukaryotic host cells will typically also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding an antibody. One useful transcription

termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

(vii) Selection and transformation of host cells

Suitable host cells for cloning or expressing the DNA in the vectors herein include higher eukaryote cells described herein, including vertebrate host cells. Propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol. 36:59 (1977)) ; baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)) ; mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

(viii) Culturing the host cells

The host cells used to produce an antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58:44 (1979), Barnes et al., Anal. Biochem.102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those

previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(ix) Purification of antibody

When using recombinant techniques, the antibody can be produced intracellularly, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., J. Immunol. Meth. 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., EMBO J. 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

Activity Assays

The antibodies of the present invention can be characterized for their physical/chemical properties and biological functions by various assays known in the art.

The purified immunoglobulins can be further characterized by a series of assays including, but not limited to, N-terminal sequencing, amino acid analysis, non-denaturing size exclusion high

pressure liquid chromatography (HPLC), mass spectrometry, ion exchange chromatography and papain digestion.

In certain embodiments of the invention, the immunoglobulins produced herein are analyzed for their biological activity. In some embodiments, the immunoglobulins of the present invention are tested for their antigen binding activity. The antigen binding assays that are known in the art and can be used herein include without limitation any direct or competitive binding assays using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immnosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, and protein A immunoassays. An illustrative antigen binding assay is provided below in the Examples section.

In one embodiment, the present invention contemplates an altered antibody that possesses some but not all effector functions, which make it a desired candidate for many applications in which the half life of the antibody in vivo is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. In certain embodiments, the Fc activities of the produced immunoglobulin are measured to ensure that only the desired properties are maintained. In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). An example of an in vitro assay to assess ADCC activity of a molecule of interest is described in US Patent No. 5,500,362 or 5,821,337. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. PNAS (USA) 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. To assess complement activation, a CDC assay, for e.g. as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), may be performed. FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art.

Humanized Antibodies

The present invention encompasses humanized antibodies. Various methods for humanizing non-human antibodies are known in the art. For example, a humanized antibody can have one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al. (1986) Nature 321:522-525; Riechmann et al. (1988)

Nature 332:323-327; Verhoeyen et al. (1988) Science 239:1534-1536), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework for the humanized antibody (Sims et al. (1993) J. Immunol. 151:2296; Chothia et al. (1987) J. Mol. Biol. 196:901. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al. (1992) Proc. Natl. Acad. Sci. USA, 89:4285; Presta et al. (1993) J. Immunol., 151:2623.

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to one method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

Antibody Variants

Also disclosed is an antibody comprising modifications in the interface of Fc polypeptides comprising the Fc region, wherein the modifications facilitate and/or promote heterodimerization. These modifications comprise introduction of a protuberance into a first Fc polypeptide and a cavity into a second Fc polypeptide, wherein the protuberance is positionable in the cavity so as to promote complexing of the first and second Fc polypeptides. Methods of

generating antibodies with these modifications are known in the art, for e.g., as described in U.S. Pat. No. 5,731,168.

In some embodiments, amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid alterations may be introduced in the subject antibody amino acid sequence at the time that sequence is made.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) Science, 244:1081-1085. Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed immunoglobulins are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino acid substitutions at each site. The antibodies thus generated are displayed from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

It may be desirable to introduce one or more amino acid modifications in an Fc region of the immunoglobulin polypeptides of the invention, thereby generating a Fc region variant. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions including that of a hinge cysteine.

In accordance with this description and the teachings of the art, it is contemplated that in some embodiments, an antibody used in methods of the invention may comprise one or more alterations as compared to the wild type counterpart antibody, for e.g. in the Fc region. These antibodies would nonetheless retain substantially the same characteristics required for therapeutic utility as compared to their wild type counterpart. For e.g., it is thought that certain alterations can be made in the Fc region that would result in altered (i.e., either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), for e.g., as described in WO99/51642. See also Duncan & Winter Nature 322:738-40 (1988); US Patent No. 5,648,260; US Patent No. 5,624,821; and WO94/29351 concerning other examples of Fc region variants.

Immunoconjugates

The invention also pertains to immunoconjugates, or antibody-drug conjugates (ADC), comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

The use of antibody-drug conjugates for the local delivery of cytotoxic or cytostatic agents, i.e. drugs to kill or inhibit tumor cells in the treatment of cancer (Syrigos and Epenetos (1999) Anticancer Research 19:605-614; Niculescu-Duvaz and Springer (1997) Adv. Drg Del. Rev. 26:151-172; U.S. patent 4,975,278) theoretically allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein, where systemic administration of these unconjugated drug agents may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Baldwin et al., (1986) Lancet pp. (Mar. 15, 1986):603-05; Thorpe, (1985) "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in Monoclonal Antibodies '84: Biological And Clinical Applications, A. Pinchera et al. (ed.s), pp. 475-506). Maximal efficacy with minimal toxicity is sought thereby. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies (Rowland et al., (1986) Cancer Immunol. Immunother., 21:183-87). Drugs used in these methods include daunomycin, doxorubicin, methotrexate, and vindesine (Rowland et al., (1986) supra). Toxins used in antibody-toxin conjugates include bacterial toxins such as diphtheria toxin, plant toxins such as ricin, small molecule toxins such as geldanamycin (Mandler et al (2000) Jour. of the Nat. Cancer Inst. 92(19):1573-1581; Mandler et al (2000) Bioorganic & Med. Chem. Letters 10:1025-1028; Mandler et al (2002) Bioconjugate Chem. 13:786-791), maytansinoids (EP 1391213; Liu et al., (1996) Proc. Natl. Acad. Sci. USA 93:8618-

8623), and calicheamicin (Lode et al (1998) Cancer Res. 58:2928; Hinman et al (1993) Cancer Res. 53:3336-3342). The toxins may effect their cytotoxic and cytostatic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands.

ZEVALIN® (ibritumomab tiuxetan, Biogen/Idec) is an antibody-radioisotope conjugate composed of a murine IgG1 kappa monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes and ^{111}In or ^{90}Y radioisotope bound by a thiourea linker-chelator (Wiseman et al (2000) Eur. Jour. Nucl. Med. 27(7):766-77; Wiseman et al (2002) Blood 99(12):4336-42; Witzig et al (2002) J. Clin. Oncol. 20(10):2453-63; Witzig et al (2002) J. Clin. Oncol. 20(15):3262-69). Although ZEVALIN has activity against B-cell non-Hodgkin's Lymphoma (NHL), administration results in severe and prolonged cytopenias in most patients. MYLOTARG™ (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), an antibody drug conjugate composed of a hu CD33 antibody linked to calicheamicin, was approved in 2000 for the treatment of acute myeloid leukemia by injection (Drugs of the Future (2000) 25(7):686; US Patent Nos. 4970198; 5079233; 5585089; 5606040; 5693762; 5739116; 5767285; 5773001). Cantuzumab mertansine (Immunogen, Inc.), an antibody drug conjugate composed of the huC242 antibody linked via the disulfide linker SPP to the maytansinoid drug moiety, DM1, is advancing into Phase II trials for the treatment of cancers that express CanAg, such as colon, pancreatic, gastric, and others. MLN-2704 (Millennium Pharm., BZL Biologics, Immunogen Inc.), an antibody drug conjugate composed of the anti-prostate specific membrane antigen (PSMA) monoclonal antibody linked to the maytansinoid drug moiety, DM1, is under development for the potential treatment of prostate tumors. The auristatin peptides, auristatin E (AE) and monomethylauristatin (MMAE), synthetic analogs of dolastatin, were conjugated to chimeric monoclonal antibodies cBR96 (specific to Lewis Y on carcinomas) and cAC10 (specific to CD30 on hematological malignancies) (Doronina et al (2003) Nature Biotechnology 21(7):778-784) and are under therapeutic development.

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re. Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl),

active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science, 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, maytansinoids, a trichothecene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

Maytansine and maytansinoids

In one embodiment, an antibody (full length or fragments) of the invention is conjugated to one or more maytansinoid molecules.

Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub Maytenus serrata (U.S. Patent No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Patent No. 4,151,042). Synthetic maytansinol and derivatives and analogues thereof are disclosed, for example, in U.S. Patent Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533, the disclosures of which are hereby expressly incorporated by reference.

Maytansinoid-antibody conjugates

In an attempt to improve their therapeutic index, maytansine and maytansinoids have been conjugated to antibodies specifically binding to tumor cell antigens. Immunoconjugates containing maytansinoids and their therapeutic use are disclosed, for example, in U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of which are hereby expressly incorporated by reference. Liu et al., Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic towards cultured colon cancer cells, and showed antitumor activity in an in vivo tumor growth assay. Chari et al., Cancer Research 52:127-131 (1992) describe immunoconjugates in which a maytansinoid was conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/neu oncogene. The cytotoxicity of the TA.1-maytansonoid conjugate was tested in vitro on the human breast cancer cell line SK-BR-3, which expresses 3 x 10^5 HER-2 surface antigens per

cell. The drug conjugate achieved a degree of cytotoxicity similar to the free maytansinoid drug, which could be increased by increasing the number of maytansinoid molecules per antibody molecule. The A7-maytansinoid conjugate showed low systemic cytotoxicity in mice.

Antibody-maytansinoid conjugates (immunoconjugates)

Antibody-maytansinoid conjugates are prepared by chemically linking an antibody to a maytansinoid molecule without significantly diminishing the biological activity of either the antibody or the maytansinoid molecule. An average of 3-4 maytansinoid molecules conjugated per antibody molecule has shown efficacy in enhancing cytotoxicity of target cells without negatively affecting the function or solubility of the antibody, although even one molecule of toxin/antibody would be expected to enhance cytotoxicity over the use of naked antibody. Maytansinoids are well known in the art and can be synthesized by known techniques or isolated from natural sources. Suitable maytansinoids are disclosed, for example, in U.S. Patent No. 5,208,020 and in the other patents and nonpatent publications referred to hereinabove. Preferred maytansinoids are maytansinol and maytansinol analogues modified in the aromatic ring or at other positions of the maytansinol molecule, such as various maytansinol esters.

There are many linking groups known in the art for making antibody-maytansinoid conjugates, including, for example, those disclosed in U.S. Patent No. 5,208,020 or EP Patent 0 425 235 B1, and Chari et al., Cancer Research 52:127-131 (1992). The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred.

Conjugates of the antibody and maytansinoid may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Particularly preferred coupling agents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Carlsson et al., Biochem. J. 173:723-737 [1978]) and N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with a

hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

Calicheamicin

Another immunoconjugate of interest comprises an antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, see U.S. patents 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296 (all to American Cyanamid Company). Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ^I_1 (Hinman et al., Cancer Research 53:3336-3342 (1993), Lode et al., Cancer Research 58:2925-2928 (1998) and the aforementioned U.S. patents to American Cyanamid). Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through antibody mediated internalization greatly enhances their cytotoxic effects.

Other cytotoxic agents

Other antitumor agents that can be conjugated to the antibodies of the invention include BCNU, streptozoicin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex described in U.S. patents 5,053,394, 5,770,710, as well as esperamicins (U.S. patent 5,877,296).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g., a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the conjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc^{99m} or I^{123}, or a spin label for nuclear magnetic resonance

(NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as tc^{99m} or I^{123}, .Re^{186}, Re^{188} and In^{111} can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al (1978) Biochem. Biophys. Res. Commun. 80: 49-57 can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal,CRC Press 1989) describes other methods in detail.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., Cancer Research 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

The compounds of the invention expressly contemplate, but are not limited to, ADC prepared with cross-linker reagents: BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL., U.S.A). See pages 467-498, 2003-2004 Applications Handbook and Catalog.

Preparation of antibody drug conjugates

In the antibody drug conjugates (ADC) of the invention, an antibody (Ab) is conjugated to one or more drug moieties (D), e.g. about 1 to about 20 drug moieties per antibody, through a linker (L). The ADC of Formula I may be prepared by several routes, employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent, to form Ab-L, via a covalent bond,

followed by reaction with a drug moiety D; and (2) reaction of a nucleophilic group of a drug moiety with a bivalent linker reagent, to form D-L, via a covalent bond, followed by reaction with the nucleophilic group of an antibody.

$$Ab-(L-D)_p \qquad I$$

Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, e.g. lysine, (iii) side chain thiol groups, e.g. cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, i.e. cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol). Each cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through the reaction of lysines with 2-iminothiolane (Traut's reagent) resulting in conversion of an amine into a thiol.

Antibody drug conjugates of the invention may also be produced by modification of the antibody to introduce electrophilic moieties, which can react with nucleophilic subsituents on the linker reagent or drug. The sugars of glycosylated antibodies may be oxidized, e.g. with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, e.g. by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either glactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the protein that can react with appropriate groups on the drug (Hermanson, Bioconjugate Techniques). In another embodiment, proteins containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan & Stroh, (1992) Bioconjugate Chem. 3:138-146; US 5362852). Such aldehyde can be reacted with a drug moiety or linker nucleophile.

Likewise, nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters,

haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, e.g., by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

In yet another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g., avidin) which is conjugated to a cytotoxic agent (e.g., a radionucleotide).

Antibody Derivatives

The antibodies of the present invention can be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. Preferably, the moieties suitable for derivatization of the antibody are water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymers are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

Pharmaceutical Formulations

Therapeutic formulations comprising an antibody of the invention are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of aqueous solutions, lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as

octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the immunoglobulin of the invention, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated immunoglobulins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to

be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

In certain embodiments, an immunoconjugate comprising an antibody conjugated with a cytotoxic agent is administered to the patient. In some embodiments, the immunoconjugate and/or antigen to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate in killing the target cell to which it binds. In one embodiment, the cytotoxic agent targets or interferes with nucleic acid in the target cell. Examples of such cytotoxic agents include any of the chemotherapeutic agents noted herein (such as a maytansinoid or a calicheamicin), a radioactive isotope, or a ribonuclease or a DNA endonuclease.

Antibodies of the invention can be used either alone or in combination with other compositions in a therapy. For instance, an antibody of the invention may be co-administered with another antibody, chemotherapeutic agent(s) (including cocktails of chemotherapeutic agents), other cytotoxic agent(s), anti-angiogenic agent(s), cytokines, and/or growth inhibitory agent(s). Where an antibody of the invention inhibits tumor growth, it may be particularly desirable to combine it with one or more other therapeutic agent(s) which also inhibits tumor growth. For instance, an antibody of the invention may be combined with anti-VEGF antibodies blocking VEGF activities and/or anti-ErbB antibodies (e.g. HERCEPTIN® anti-HER2 antibody) in a treatment of metastatic breast cancer. Alternatively, or additionally, the patient may receive combined radiation therapy (e.g. external beam irradiation or therapy with a radioactive labeled agent, such as an antibody). Such combined therapies noted above include combined administration (where the two or more agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody of the invention can occur prior to, and/or following, administration of the adjunct therapy or therapies.

The antibody of the invention (and adjunct therapeutic agent) is/are administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Dosing can be by any suitable route, for e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

The antibody composition of the invention will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibodies of the disclosure present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

For the prevention or treatment of disease, the appropriate dosage of an antibody of the invention (when used alone or in combination with other agents such as chemotherapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1mg/kg-10mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.05mg/kg to about 10mg/kg. Thus, one or more doses of about 0.5mg/kg, 2.0mg/kg, 4.0mg/kg or 10mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, e.g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the antibody. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Articles of Manufacture

Also disclosed is an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture may comprise a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or when combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in

the composition is an antibody of the invention. The label or package insert indicates that the composition is used for treating the condition of choice, such as cancer. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the first and second antibody compositions can be used to treat a particular condition, for e.g. cancer. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

The following are examples of the methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

EXAMPLES

Materials & Methods

Reagents

Corn trypsin inhibitor was from Haematologic Technologies (Essex Junction, VT) and the chromogenic substrate for HGFA, Spectrozyme® fVIIa, was from American Diagnostica (Stamford, CT). Soluble HAI-1B (sHAI-1B) was expressed in Chinese Hamster Ovary cells and purified as previously described (1). The Kunitz domain inhibitor IV-49C was previously described (2) (Genentech, Inc., South San Francisco). Human recombinant HGFA (HGFA) was expressed in a baculovirus expression system as previously described (1).

ProHGF activation assays

ProHGF activation assays and proHGF labeling with Iodogen were carried out as previously described (1,3). Briefly, HGFA was preincubated with anti-HGFA antibodies or sHAI-1B in HNC buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$) for 15 min at room temperature, after which ^{125}I-labeled proHGF in HNC buffer was added and incubated for 4hrs at 37°C. The reactant concentrations in the final mixture were as follows: 2 nM HGFA, 0.05mg/ml ^{125}I-labeled proHGF, 0.1 mg/ml anti-HGFA antibodies, 1 μM sHAI-1B. After 4hrs aliquots were removed and added to sample buffer (Bio-Rad Laboratories, Hercules, CA) with reducing agent dithiotreitol (BIO-Rad). After a brief heating, samples (approx. 10^6cpm/lane) were loaded onto a 4-20% gradient polyacrylamide gel (Invitrogen Corp., Carlsbad, CA). After electrophoresis, the dried gels were exposed on x-ray films (X-OMAT AR, Eastman Kodak Company, Rochester, NY) for 10-20 min.

Films were developed (Kodak M35A X-OMAT Processor), scanned (Umax S-12, Umax Data Systems, Inc., Fremont, CA) and further processed with Adobe V.6.0 Photoshop software (Adobe Systems Inc., San Jose, CA).

BIAcore experiments

Binding affinities of anti-HGFA antibodies to HGFA were determined by surface plasmon resonance measurements on a BIAcore 3000 instrument (Biacore, Inc.) The reformatted full length anti-HGFA IgG1 was immobilized at a density of 300 resonance units (RU) on the flow cells of a Pioneer CM5 sensor chip. Immobilization was achieved by random coupling through amino groups using a protocol provided by the manufacturer. Sensorgrams were recorded for binding of HGFA to these surfaces by injection of a series of solutions ranging from 1 μM to 8 nM in 2-fold increments. The signal from the reference cell was subtracted from the observed sensorgram. Kinetic constants were calculated by nonlinear regression analysis of the data according to a 1:1 Languir binding model using software supplied by the manufacturer. In competition experiments, HGFA (70 nM) was preincubated with various concentrations of sHAI-1B (4nM-300nM) or IV-49C (11nM-300nM) or a small molecule HGFA active site binder (220nM-10uM). After incubation for 60 min at room temperature, the enzyme-inhibitor mixture was injected into the flow cells and sensorgrams recorded.

HGFA enzyme inhibition assay

The antibodies or sHAI-1B were incubated with HGFA (final concentration 5nM) in HBSA buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.5 mg/ml BSA, 5 mM $CaCl_2$) for 20 min at room temperature. Spectrozyme® fVIIa (200 μM final conc., K_M = 200 μM) was added and the linear rates of the increase in absorbance at 405 nm measured on a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Inhibition of enzyme activity was expressed as fractional activity (v_i/v_o) of uninhibited activity.

Results & Discussion

Identification of anti-HGFA antibodies by phage display

One method of identifying antibodies is through the use of a phage antibody library. See, for example, Lee et al. (4). To identify antibodies against HGFA, we carried out four rounds of panning using a previously reported human synthetic phagemid antibody library (a $F(ab')_2$ library). Plates were coated with 5 μg/well of HGFA. We increased stringency of washing after each round, from 10-40 times of washes. We observed enrichment after three rounds of panning. After four rounds of panning, 95 clones were picked for ELISA assays. After sequencing, 67 unique clones were found to specifically bind to HGFA. After spot competition ELISA, 24 clones were further characterized using purified phage to measure IC_{50} values, which were determined using a standard phage competition ELISA. 14 unique clones with IC_{50} values < 100 nM were sub-cloned into PRK-human IgG1 vector. CDR sequences for these clones are listed in Fig. 1. Heavy and light chains

(from the humanized 4D5 antibody as described in Lee et al. (4)) of anti-HGFA clones were co-transfected into mammalian 293 cells. After one week, the serum-free supernatants were harvested and the antibodies purified using protein A affinity chromatography.

Inhibition of HGFA enzymatic activity by full-length anti-HGFA antibodies

The selected antibodies were reformatted as full-length antibodies (IgG) by standard recombinant techniques. These full length antibodies were examined in a macromolecular substrate activation assay using ^{125}I-labelled proHGF. During the 4 hr experiment, HGFA completely converted proHGF into 2-chain HGF, and this reaction could be inhibited by 1μM sHAI-1B (Fig.2A) consistent with previous reports (1). With the exception of antibody #49 (Fig. 1), all tested anti-HGFA antibodies at the tested concentration of 0.67μM significantly inhibited proHGF conversion (Fig. 2). Additional experiments showed that #58 inhibited proHGF conversion at concentrations as low as 0.03 μM (Fig. 3). Consistent with these results, antibody #58 most potently inhibited HGFA enzymatic activity towards the small synthetic substrate Spectrozyme® fVIIa, having an IC_{50} of 1.3 nM, whereas antibody #49 did not inhibit at 500nM (Fig. 8). Furthermore, in agreement with their relatively weaker inhibitory activities in proHGF activation assays, the antibodies #39, #86, #90 and #95 had comparably weaker activities in the chromogenic substrate assay, having IC_{50} > 500nM (Fig. 8). The 3 antibodies #42, #61, and #74 also showed relatively weak inhibition (IC_{50} >500nM) despite almost complete inhibition of proHGF conversion at 0.67μM (Fig. 8). Interestingly, antibody #75 displayed unusual inhibition kinetics in that its inhibitory activity reached a plateau at about 70% inhibition as compared to the complete inhibition achieved by antibody #58 (Fig. 4).

Inhibitory mechanisms of antibodies #75 and #58

In light of the complete inhibition of macromolecular substrate processing by antibody #75, its inability to completely neutralize HGFA enzymatic activity towards the small synthetic substrate suggested that antibody #75 binds to a functionally important HGFA region located outside, or in proximity to, the active site. In contrast, antibody #58 strongly inhibited both the macromolecular and small substrate processing by HGFA. To gain more detailed insight into the antibodies' inhibitory mechanisms, competition binding studies with various known active site inhibitors were carried out. The three HGFA active site inhibitors used were the previously described bi-Kunitz domain inhibitor sHAI-1B (1), the single Kunitz domain inhibitor IV-49C (2) and the small molecule HGFA active site binder. IV-49C is a 62 amino acid Kunitz domain derived from Alzheimer's β-protein precursor inhibitor (APPI) and is a specific inhibitor of the tissue factor/factor VIIa complex (2). We found that IV-49C is also a potent inhibitor of HGFA enzymatic activity, having an IC_{50} of 0.079 μM, whereas the small molecule HGFA active site binder inhibited with an IC_{50} of 0.8 μM (K_i = 0.4 μM) as shown in Fig. 5.

The K_D of HGFA to immobilized antibody #58 was 1.3 nM (Fig. 9), similar to the affinity determined by amidolytic assays (Fig. 8). BIAcore measurements showed that sHAI-1B, IV-49C and the small molecule HGFA active site binder inhibited HGFA binding to #58. This suggested that #58 either binds directly to the active site of HGFA or exerts allosteric influences on the active site.

Antibody #75 had weaker binding to HGFA (Fig. 6E; Fig. 9) than #58. Moreover, the small molecule HGFA active site binder had no effect on HGFA binding to antibody #75, indicating that antibody #75 does not bind to the 'core' region of the active site. Interestingly, antibody #75 partially inhibited HGFA amidolytic activity, suggesting that even though the #75 epitope lies outside the active site, there must be a molecular linkage between these two sites. This would explain the partial effects of sHAI-1B and IV-49C on antibody #75 binding (Fig. 9; Fig. 6F,G).

Similar to antibody #75, the two antibodies #74 and #61 also bound to HGFA in the presence of the small molecule active site binder (Fig. 9), while the Kunitz domain inhibitors interfered with HGFA binding. These results suggested that the epitopes of #74 and #61 lie outside the active site of HGFA. It is conceivable that the antibodies #61, #74 and #75 bind to an HGFA exosite region that is important for macromolecular substrate interaction or that they allosterically influence the conformation of the active site region. In the structurally related serine protease factor VIIa an important exosite is located between the active site and the calcium binding loop (5). Antibodies as well as peptides which bind to the factor VIIa exosite are potent inhibitors of macromolecular substrate processing (6,7). For instance, binding of the peptidic inhibitor E76 effects conformational changes in one of the 'activation domain' loops thereby disrupting a substrate interaction site (7). In addition, these changes induce allosteric effects at the active site, which explains the observation that E-76 peptide inhibits amidolytic activity despite binding outside the active site region (7).

Additional competition binding experiments with biotinylated antibodies #75 and #58 indicated that #75 and #58 have overlapping epitopes on HGFA (data not shown). Enzyme kinetic studies further demonstrated that #58 is a competitive inhibitor and that #75 is a partial competitive inhibitor (i.e. simple intersecting hyperbolic competitive inhibitor) (data not shown). Together, these results suggest that both antibodies bind outside the HGFA active site and that they are allosteric inhibitors of HGFA enzymatic activity.

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CLAIMS

1. An isolated antibody that binds human hepatocyte growth factor activator (HGFA) and blocks HGFA proteolytic activity, wherein the antibody blocks HGFA proteolysis of the small molecule substrate methanesulphonyl-D-cyclohexylalanyl-butyl-arginine-paranitroanilide (FVIIa).

2. The antibody of claim 1, wherein the antibody is an allosteric inhibitor of HGFA enzymatic activity.

3. The antibody of claim 1, wherein the antibody is a complete competitive inhibitor or a partial competitive inhibitor of HGFA enzymatic activity inhibitor.

4. The antibody of any one of claims 1 to 3, which blocks HGFA proteolysis of single chain HGF.

5. The antibody of any one of claims 1 to 4, which inhibits HGF/c-met signaling.

6. The antibody of claim 5, which inhibits cell proliferation.

7. The antibody of claim 5, which inhibits angiogenesis.

8. The isolated antibody of any one of claims 1 to 7, wherein the antibody comprises a heavy chain CDR sequence comprising the respective H1, H2 and/or H3 sequences from a group selected from SEQ ID NOs: 3 to 5, SEQ ID NOs: 6 to 8, SEQ ID NOs: 9 to 11, SEQ ID NOs: 21 to 23, SEQ ID NOs: 30 to 32, SEQ ID NOs: 36 to 38 or SEQ ID NOs: 39 to 41.

9. The isolated antibody of claim 8, wherein the H1, H2 and/or H3 sequences are as depicted as SEQ ID NOs: 71, 85 and 99, SEQ ID NOs: 72, 86 and 100, SEQ ID NOs: 73, 87 and 101, SEQ ID NOs: 77, 91 and 105, SEQ ID NOs: 80, 94 and 108, SEQ ID NOs: 82, 96 and 110 or SEQ ID NOs: 83, 97 and 111.

10. The isolated antibody of claim 8, wherein the antibody comprises a heavy chain CDR sequence comprising the respective H1, H2 and H3 sequences from a group selected from SEQ ID NOs: 21 to 23 or SEQ ID NOs: 30 to 32.

11. The isolated antibody of claim 9, wherein the H1, H2 and H3 sequences are as depicted as SEQ ID NOs: 77, 91 and 105 or SEQ ID NOs: 80, 94 and 108.

12. The antibody of any one of claims 1 to 11, wherein the antibody further comprises a light chain variable domain comprising the sequence as shown in SEQ ID NO:45 or SEQ ID NO:54.

13. A method of treating a disease associated with dysregulation of HGF/c-met signaling in a subject, comprising administering to the subject an effective amount of the antibody of any one of claims 1 to 12.

14. Use of the antibody of any one of claims 1 to 12 in the manufacture of a medicament for treating a disease associated with dysregulation of HGF/c-Met signaling in a subject.

15. The method of claim 13 or use of claim 14, wherein the disease is cancer.

16. The method of claim 13 or use of claim 14, wherein the disease is associated with dysregulation of angiogenesis.

17. The method of claim 13 or use of claim 14, wherein the disease is immune related.

18. An isolated antibody according to claim 1, a method according to claim 13, or use according to claim 14, substantially as hereinbefore described with reference to any one of the examples or figures.

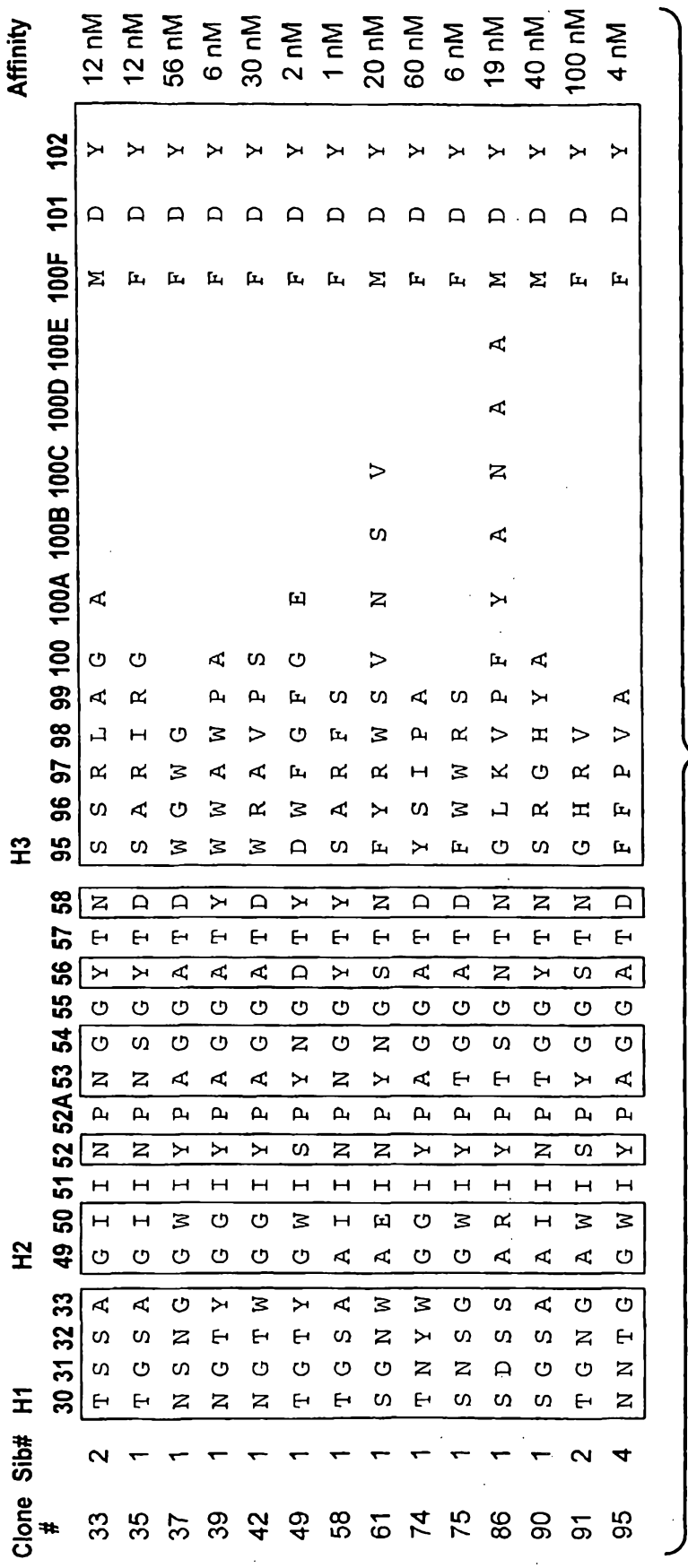
Clone # Sib# H1 H2 H3 Affinity
30 31 32 33 49 50 51 52 52A 53 54 55 56 57 58 95 96 97 98 99 100 100A 100B 100C 100D 100E 100F 101 102
33 2 T S S A G I I N P N G G Y T N S S R L A G A M D Y 12 nM
35 1 T G S A G I I N P N S G Y T D S A R I R G F D Y 12 nM
37 1 N S N G G W I Y P A G G A T D W G W G F D Y 56 nM
39 1 N G T Y G G I Y P A G G A T Y W W A W P A F D Y 6 nM
42 1 N G T W G G I Y P A G G A T D W R A V P S F D Y 30 nM
49 1 T G T Y G W I S P Y N G D T Y D W F G F G E F D Y 2 nM
58 1 T G S A A I I N P N G G Y T Y S A R F S F D Y 1 nM
61 1 S G N W A E I N P Y N G S T N F Y R W S V N S V M D Y 20 nM
74 1 T N Y W G G I Y P A G G A T D Y S I P A F D Y 60 nM
75 1 S N S G G W I Y P T G G A T D F W W R S F D Y 6 nM
86 1 S D S S A R I Y P T S G N T N G L K V P F Y A N A M D Y 19 nM
90 1 S G S A A I I N P T G G Y T N S R G H Y A M D Y 40 nM
91 2 T G N G A W I S P Y G G S T N G H R V F D Y 100 nM
95 4 N N T G G W I Y P A G G A T D F F P V A F D Y 4 nM

FIG. 1A

Clone #	H1												SEQ ID NO:
	26	27	28	29	30	31	32	33	34	35			
33	G	F	N	I	T	S	S	A	I	H			71
35	G	F	N	I	T	G	S	A	I	H			72
37	G	F	N	I	N	S	N	G	I	H			73
39	G	F	N	I	N	G	T	Y	I	H			74
42	G	F	N	I	N	G	T	W	I	H			75
49	G	F	N	I	T	G	T	Y	I	H			76
58	G	F	N	I	T	G	S	A	I	H			77
61	G	F	N	I	S	G	N	W	I	H			78
74	G	F	N	I	T	N	Y	W	I	H			79
75	G	F	N	I	S	N	S	G	I	H			80
86	G	F	N	I	S	D	S	S	I	H			81
90	G	F	N	I	S	G	S	A	I	H			82
91	G	F	N	I	T	G	N	G	I	H			83
95	G	F	N	I	N	N	T	G	I	H			84

FIG. 1B

Clone #	H2																				SEQ ID NO:
	49	50	51	52	52a	53	54	55	56	57	58	59	60	61	62	63	64	65			
33	G	I	I	N	P	N	G	G	Y	T	N	Y	A	D	S	V	K	G			85
35	G	I	I	N	P	N	S	G	Y	T	D	Y	A	D	S	V	K	G			86
37	G	W	I	Y	P	A	G	G	A	T	D	Y	A	D	S	V	K	G			87
39	G	G	I	Y	P	A	G	G	A	T	Y	Y	A	D	S	V	K	G			88
42	G	G	I	Y	P	A	G	G	A	T	D	Y	A	D	S	V	K	G			89
49	G	W	I	S	P	Y	N	G	D	T	Y	Y	A	D	S	V	K	G			90
58	A	I	I	N	P	N	G	G	Y	T	Y	Y	A	D	S	V	K	G			91
61	A	E	I	N	P	Y	N	G	S	T	N	Y	A	D	S	V	K	G			92
74	G	G	I	Y	P	A	G	G	A	T	D	Y	A	D	S	V	K	G			93
75	G	W	I	Y	P	T	G	G	A	T	D	Y	A	D	S	V	K	G			94
86	A	R	I	Y	P	T	S	G	N	T	N	Y	A	D	S	V	K	G			95
90	A	I	I	N	P	T	G	G	Y	T	N	Y	A	D	S	V	K	G			96
91	A	W	I	S	P	Y	G	G	S	T	N	Y	A	D	S	V	K	G			97
95	G	W	I	Y	P	A	G	G	A	T	D	Y	A	D	S	V	K	G			98

FIG. 1C

Clone #	H3																					SEQ ID NO:
	93	94	95	96	97	98	99	100	a	b	c	d	e	f		101	102					
33	A	R	S	S	R	L	A	G	A					M		D	Y					99
35	A	R	S	A	R	I	R	G						F		D	Y					100
37	A	R	W	G	W	G								F		D	Y					101
39	A	R	W	W	A	W	P	A						F		D	Y					102
42	A	R	W	R	A	V	P	S						F		D	Y					103
49	A	R	D	W	F	G	F	G	E					F		D	Y					104
58	A	R	S	A	R	F	S							F		D	Y					105
61	A	R	F	Y	R	W	S	V	N	S	V			M		D	Y					106
74	A	R	Y	S	I	P	A							F		D	Y					107
75	A	R	F	W	W	R	S							F		D	Y					108
86	A	R	G	L	K	V	P	F	Y	A	N	A	A	M		D	Y					109
90	A	R	S	R	G	H	Y	A						M		D	Y					110
91	A	R	G	H	R	V								F		D	Y					111
95	A	R	F	F	P	V	A							F		D	Y					112

FIG. 1D

Framework Sequences of Light Chain

LC-FR1 (1) Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr (23) Cys (SEQ ID NO: 55)

LC-FR2 (35) Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile (49) Tyr (SEQ ID NO: 56)

LC-FR3 (57) Gly Val Pro Ser Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr (88) Cys (SEQ ID NO: 57)

LC-FR4 (98) Phe Gly Gln Gly Thr Lys Val Glu Ile (107) Lys (SEQ ID NO: 58)

Framework Sequences of Heavy Chain

HC-FR1 (1) Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala (25) Ser (SEQ ID NO: 59)

HC-FR2 (36) Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp (48) Val (SEQ ID NO: 60)

HC-FR3 (66) Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr Leu Gln Met (83) Asn (83a) Ser (83b) Leu (83c) Arg Ala Glu Asp Thr Ala Val Tyr Tyr (92) Cys (SEQ ID NO: 61)

HC-FR4 (103) Trp Gly Gln Gly Thr Leu Val Thr Val Ser (113) Ser (SEQ ID NO: 62)

FIG. 1E

Framework Sequences of Light Chain

LC-FR1 1 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
Gly Asp Arg Val Thr Ile Thr 23 Cys (SEQ ID NO: 63)

LC-FR2 35 Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile 49 Tyr (SEQ ID NO: 64)

LC-FR3 57 Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr 88 Cys (SEQ ID NO: 65)

LC-FR4 98 Phe Gly Gln Gly Thr Lys Val Glu Ile 107 Lys (SEQ ID NO: 66)

Framework Sequences of Heavy Chain

HC-FR1 1 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu
Arg Leu Ser Cys Ala Ala 25 Ser (SEQ ID NO: 67)

HC-FR2 36 Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp 48 Val (SEQ ID NO: 68)

HC-FR3 66 Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln Met 83 Asn
83a 83b 83c
Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr 92 Cys (SEQ ID NO: 69)

HC-FR4 103 Trp Gly Gln Gly Thr Leu Val Thr Val Ser 113 Ser (SEQ ID NO: 70)

FIG. 1F

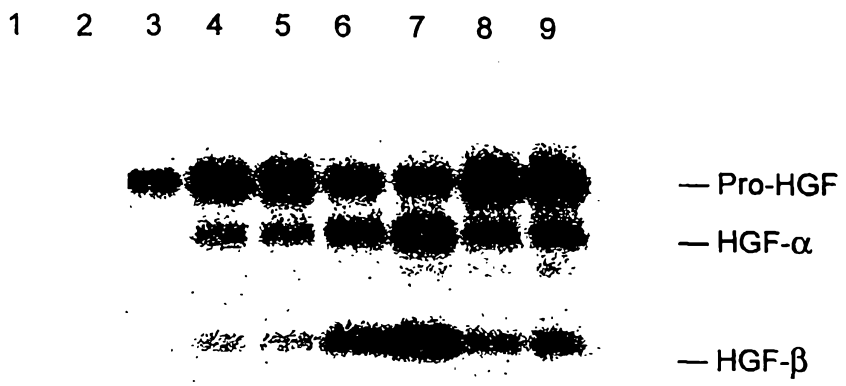

FIG. 2A

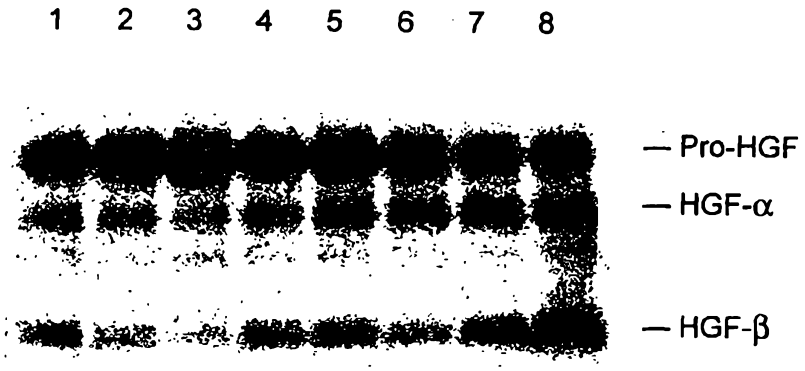

FIG. 2B

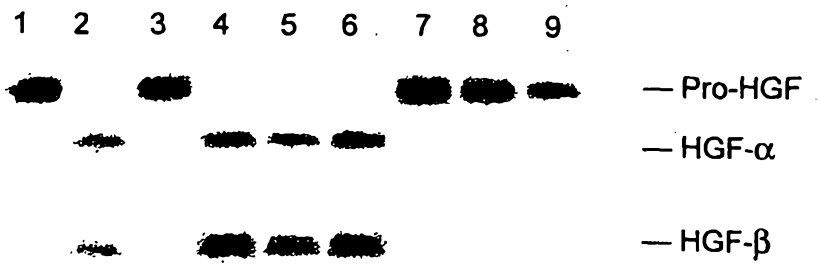

FIG. 3

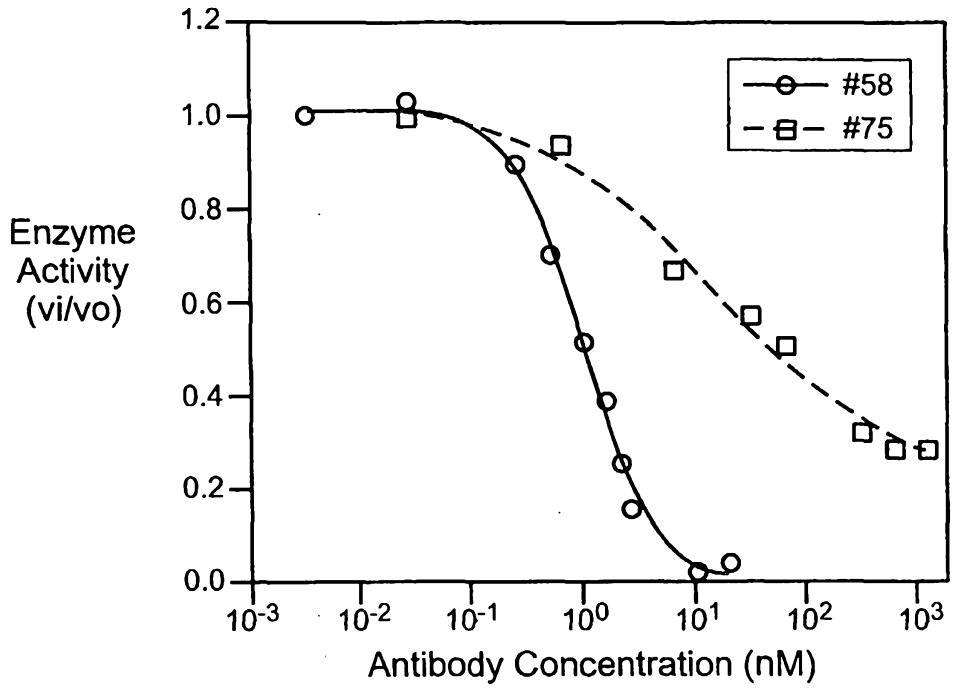
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Enzyme Activity (vi/vo)
#58
#75
10-3
10-2
10-1
100
101
102
103
Antibody Concentration (nM)

FIG. 4

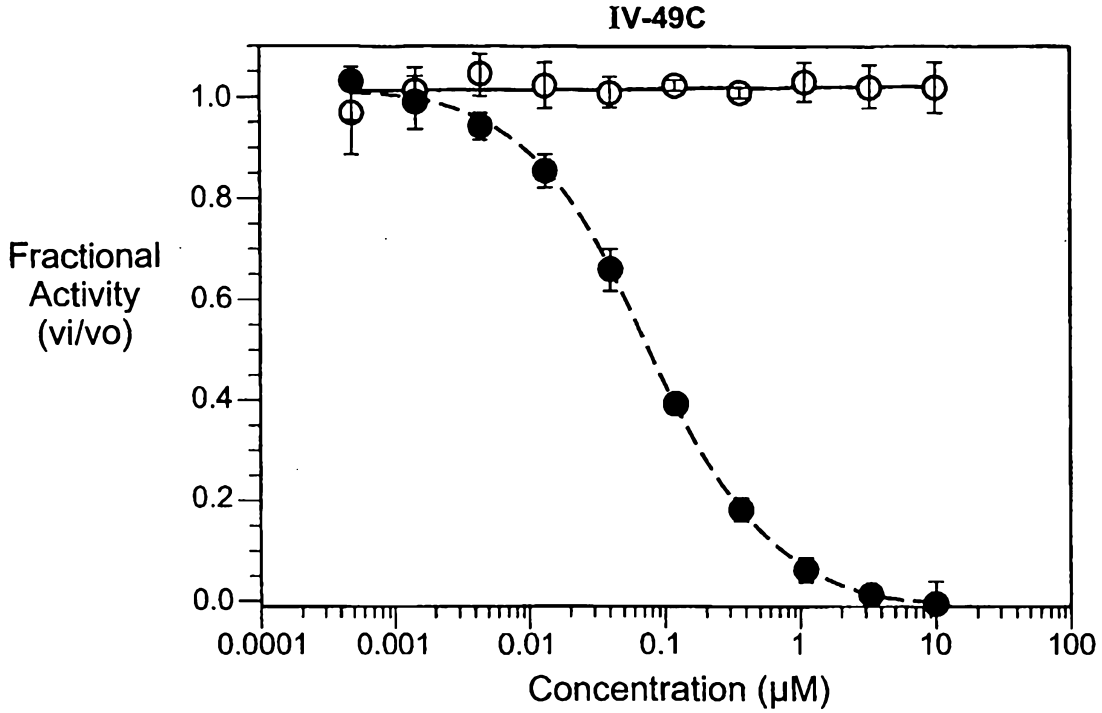
IV-49C
Fractional Activity (vi/vo)
1.0
0.8
0.6
0.4
0.2
0.0
0.0001
0.001
0.01
0.1
1
10
100
Concentration (μM)

FIG. 5A

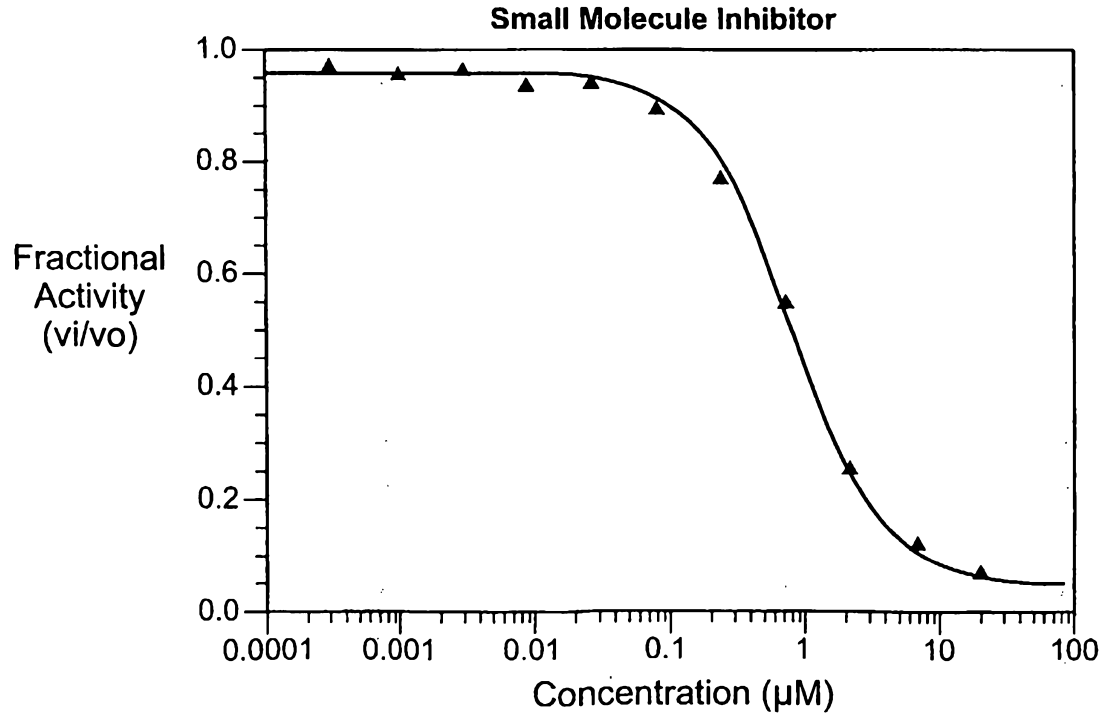
Small Molecule Inhibitor
Fractional Activity (vi/vo)
1.0
0.8
0.6
0.4
0.2
0.0
0.0001
0.001
0.01
0.1
1
10
100
Concentration (μM)

FIG. 5B

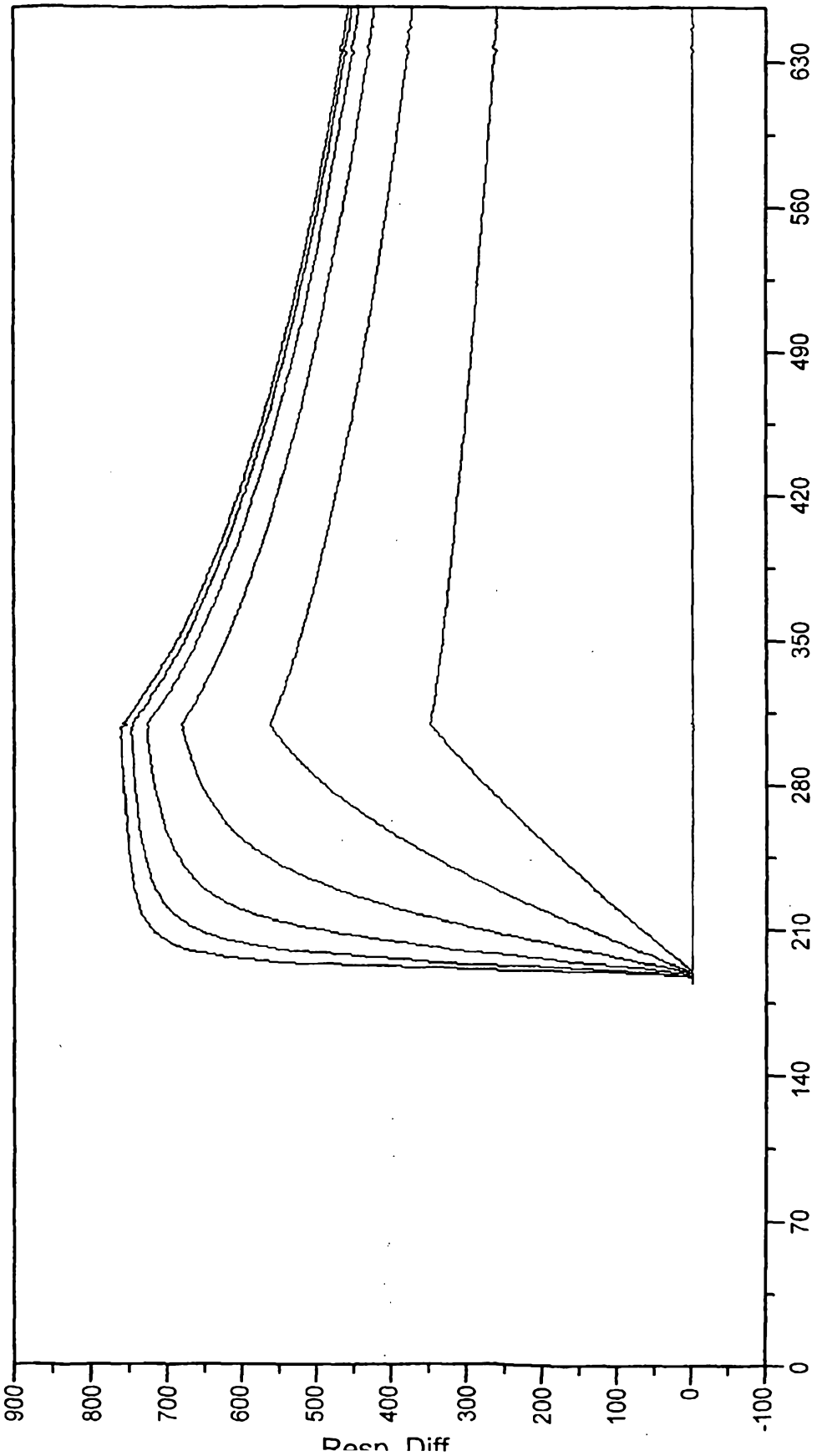
Resp. Diff
900
800
700
600
500
400
300
200
100
0
-100
Time
0
70
140
210
280
350
420
490
560
630

FIG. 6A

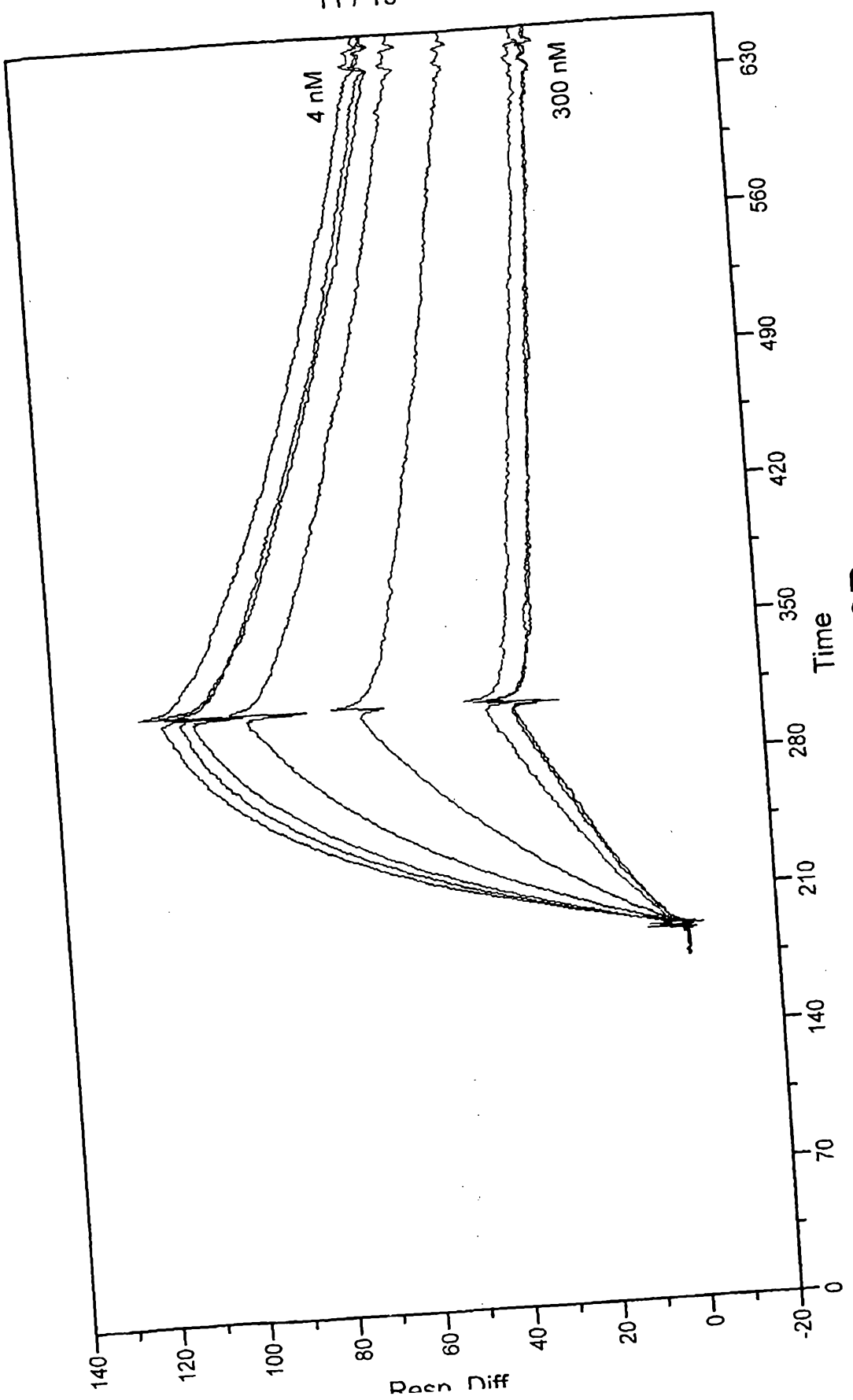
4 nM
300 nM
Time
Resp Diff
630
560
490
420
350
280
210
140
70
0
-20
140
120
100
80
60
40
20
0

FIG. 6B

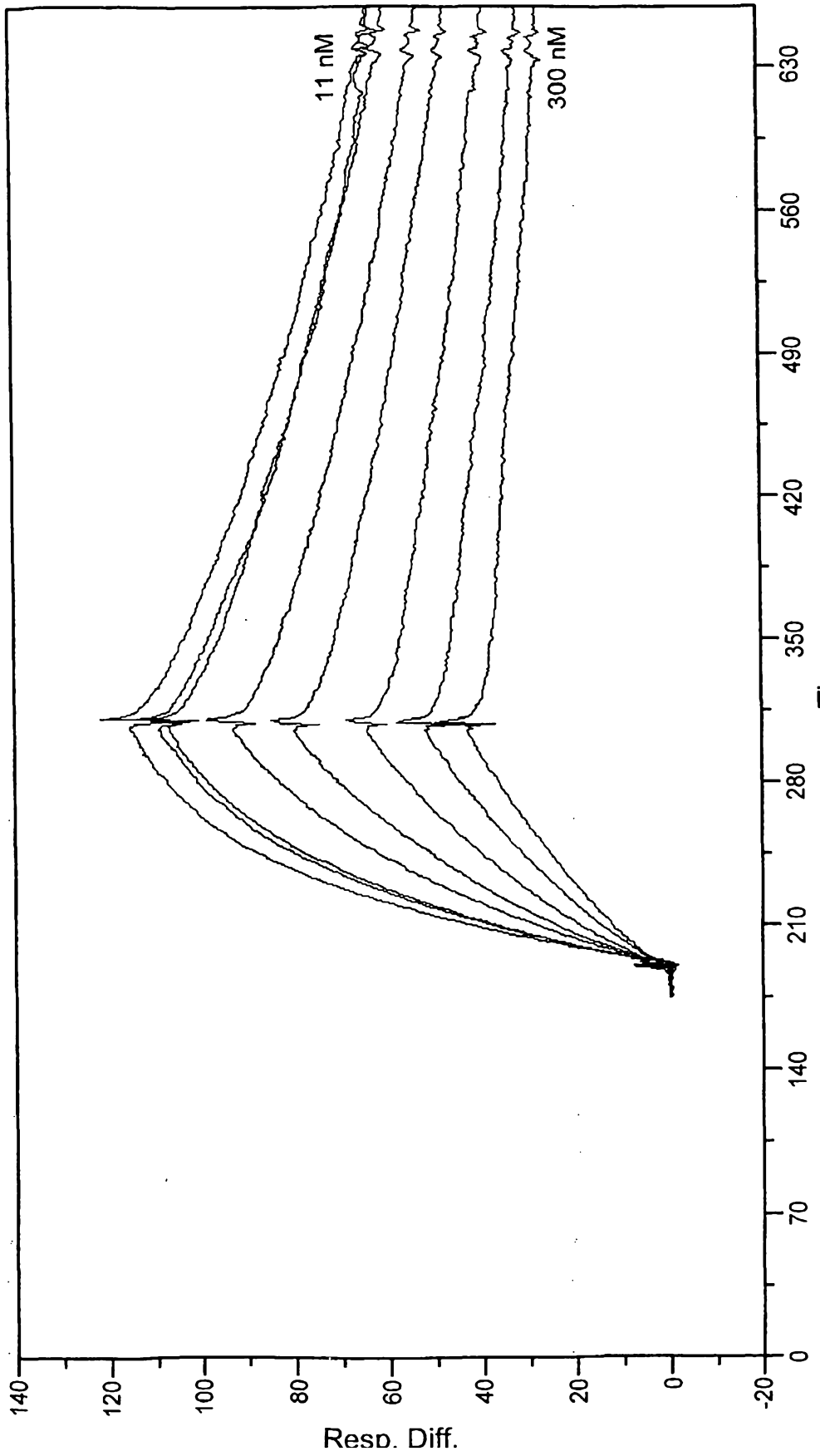
11 nM
300 nM
Resp. Diff.
Time
140
120
100
80
60
40
20
0
-20
0
70
140
210
280
350
420
490
560
630

FIG. 6C

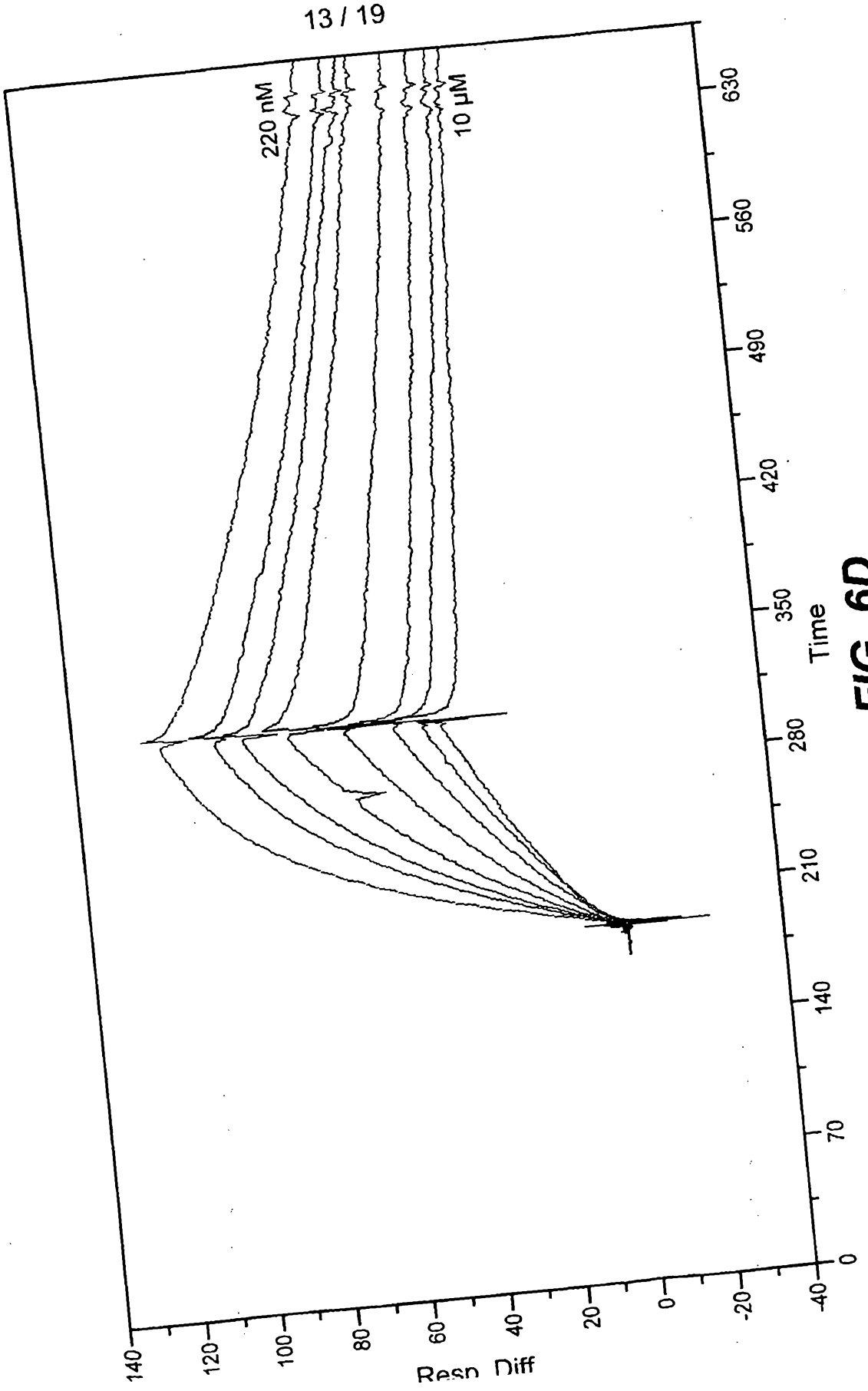
220 nM
10 µM
Resp Diff
140
120
100
80
60
40
20
0
-20
-40
Time
0
70
140
210
280
350
420
490
560
630

FIG. 6D

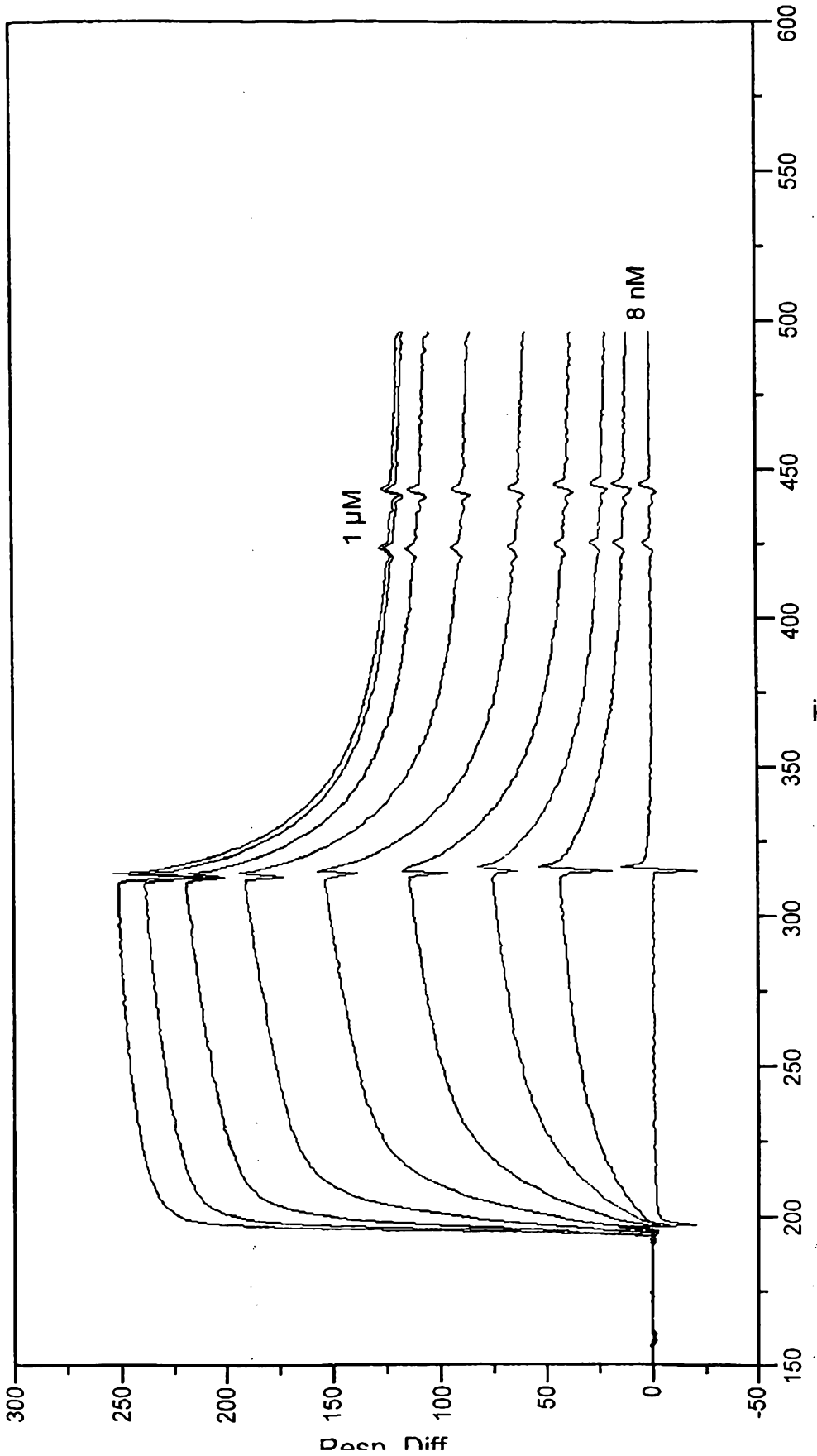
1 µM
8 nM
Time
Resp. Diff.
600
550
500
450
400
350
300
250
200
150
-50
0
50
100
150
200
250
300

FIG. 6E

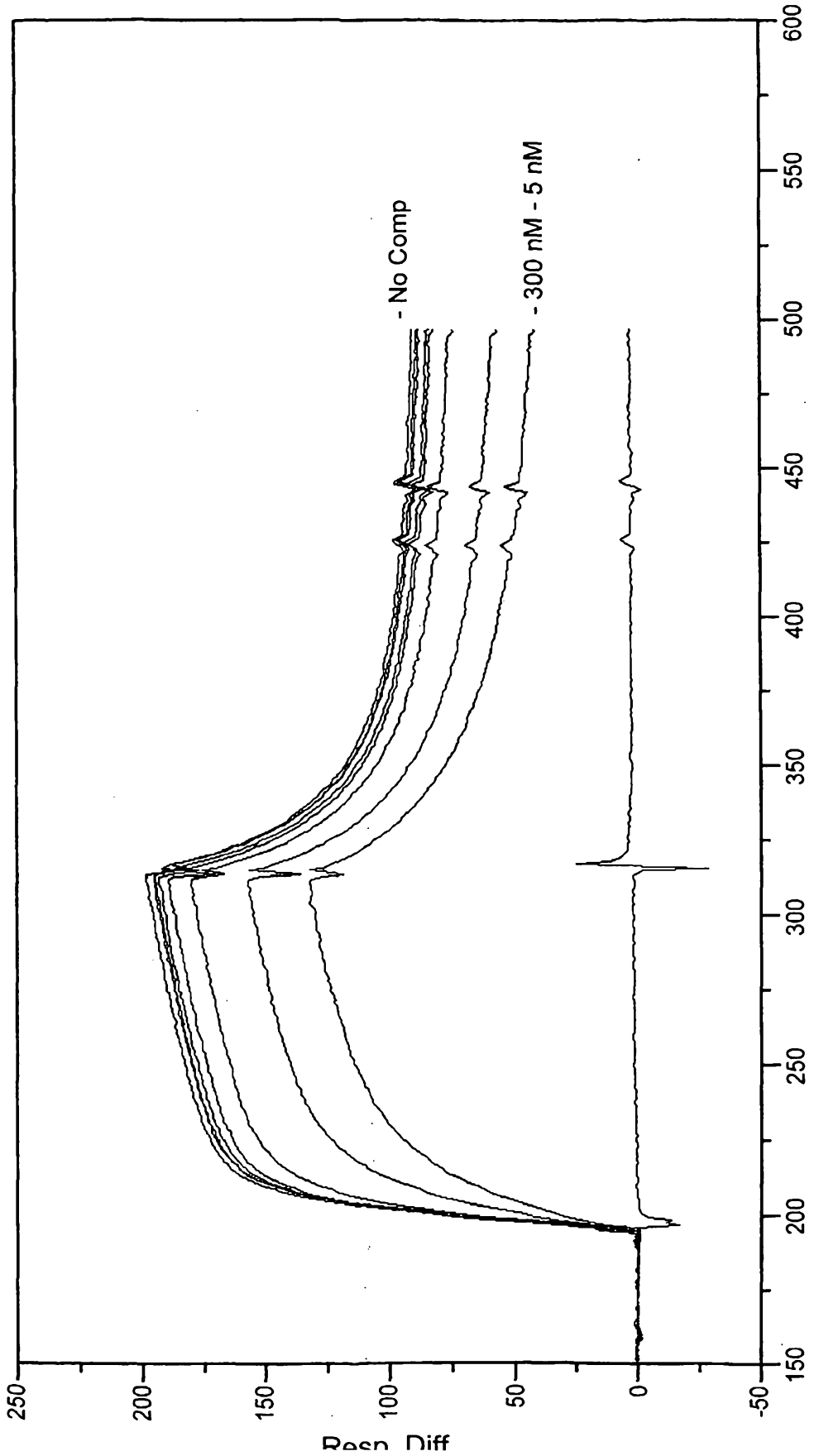
- No Comp
- 300 nM - 5 nM
Time
Resp Diff
250
200
150
100
50
0
-50
150
200
250
300
350
400
450
500
550
600

FIG. 6F

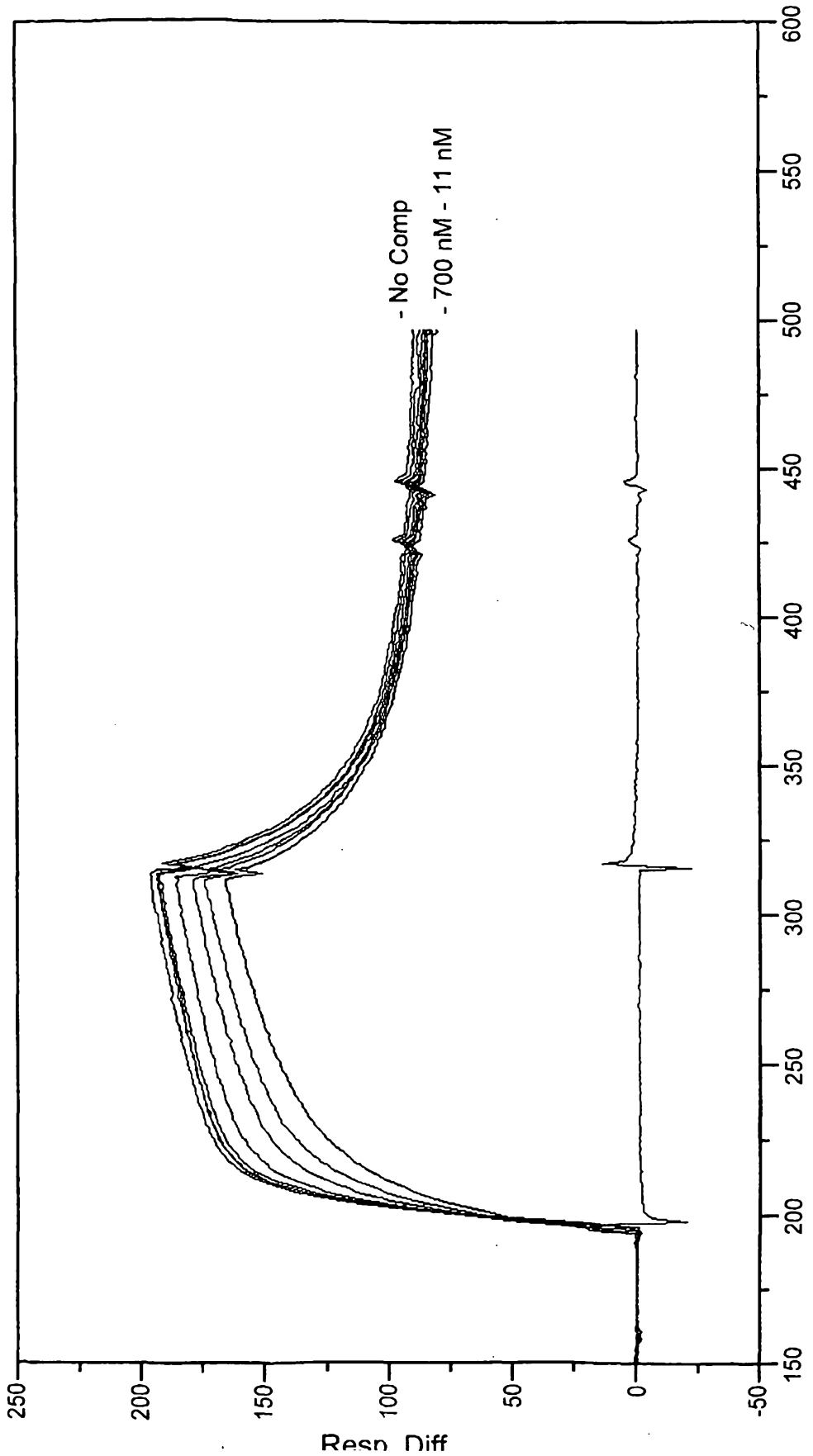
- No Comp
- 700 nM - 11 nM
Resp. Diff
Time
250
200
150
100
50
0
-50
150
200
250
300
350
400
450
500
550
600

FIG. 6G

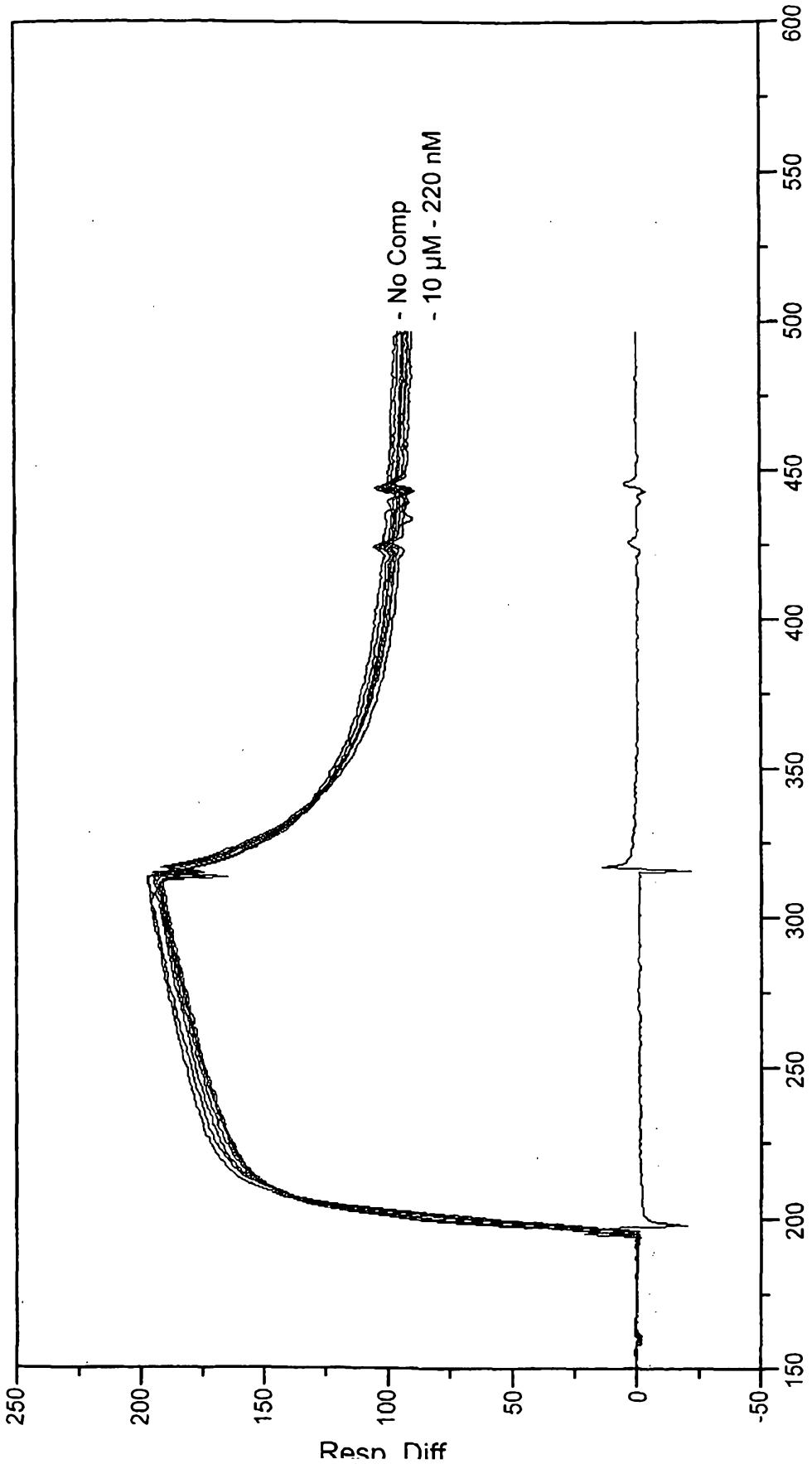
- No Comp
- 10 µM - 220 nM
Resp. Diff
Time
250
200
150
100
50
0
-50
150
200
250
300
350
400
450
500
550
600

FIG. 6H

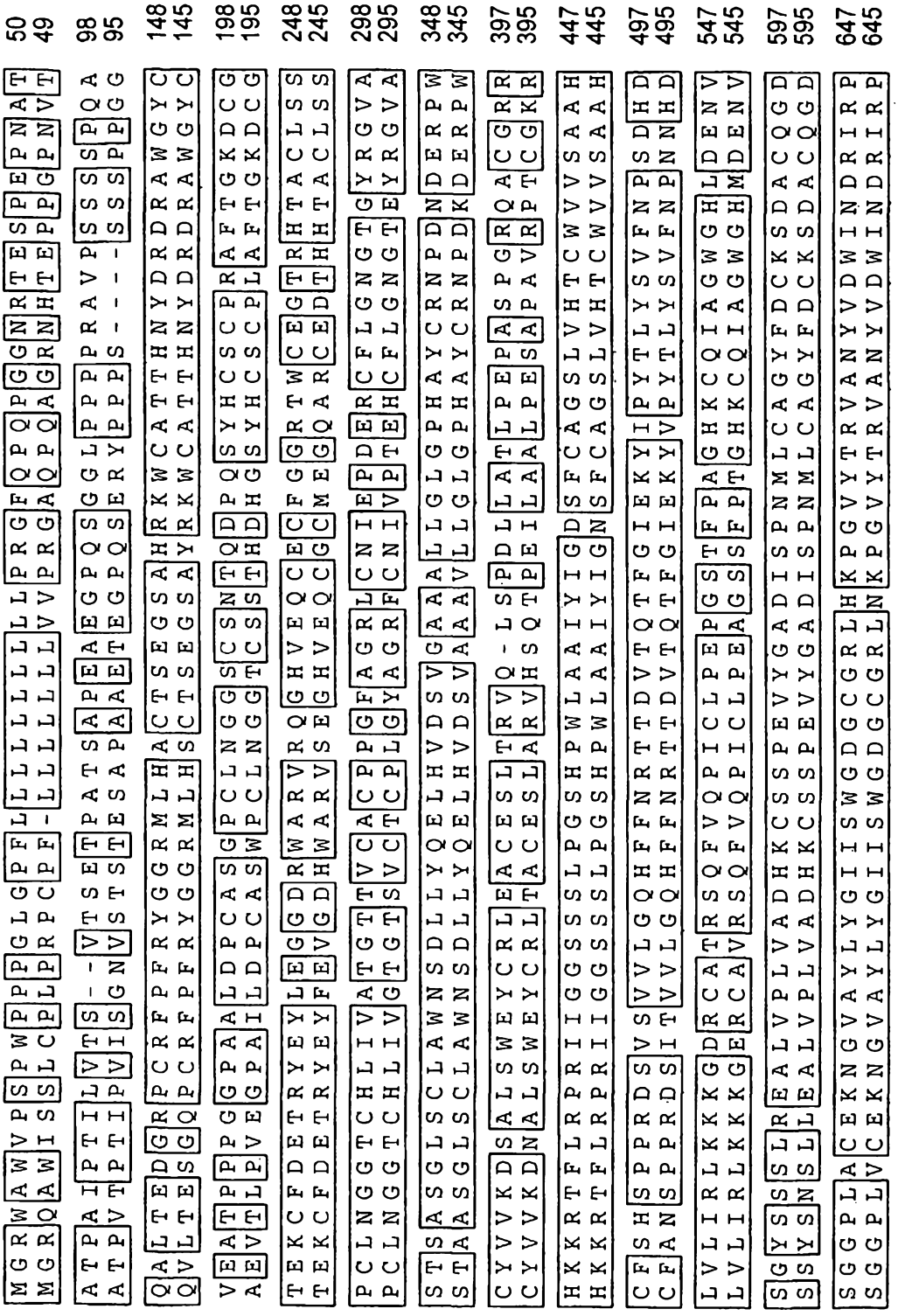

FIG. 7

Inhibition of HGFA Enzymatic Activity by Anti-HGFA Antibodies

Antibody #	Chromogenic Substrate Assay[a] IC_{50} (nM)	Macromolecular Substrate proHGF Inhibition at 670nM Antibody[b]
33	15.3	**
35	21.3	**
37	334	**
39	>500	*
42	>500	**
49	>500	N.I.
58	1.3	***
61	>500	**
74	>500	**
75	32.6	**
86	>500	*
90	203	**
91	169	**
95	>500	*

[a]Substrate was Spectrozyme® fVIIa at 0.2mM (~K_M)

[b]Qualitative assessment of inhibition based on disappearance of single chain HGF band: *, weak inhibition, ** strong inhibition, *** very strong inhibition; N.I., no inhibition.

FIG. 8

Binding of HGFA to Anti-HGFA Antibodies

Anti-HGFA Phage Antibody	Affinity by Phage ELISA IC_{50} (nM)	Affinity by BIAcore K_D (nM)	Inhibition of HGFA Binding by IV49	Inhibition of HGFA Binding by Small Molecule	Inhibition of HGFA Binding by sHAI-1B
58	2	1.3	++	++	++
75	6	100	+/-	-	+/-
37	56	ND[a]	+/-	+/-	ND[a]
74	60	ND[a]	+/-	-	+/-
42	30	ND[a]	+	+/-	+
61	20	ND[a]	+	-	+

++, +: >50% Inhibition

+/-: 20-50% Inhibition

-: <20% Inhibition

[HGFA] : 70 nM

[Competitor]: start at 10 fold above reported KD, and decrease by 2-fold dilution

[a]ND, Not Determined

FIG. 9

<110> Genentech, Inc.

<120> MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR

<130> P2179R1 PCT

<140> PCT/US2005/036300
<141> 2005-10-03

<150> US 60/615,657
<151> 2004-10-04

<160> 112

<210> 1
<211> 655
<212> PRT
<213> Homo sapiens

<400> 1

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Met Gly Arg Trp Ala Trp Val Pro Ser Pro Trp Pro Pro Pro Gly
  1               5                  10                  15

Leu Gly Pro Phe Leu Leu Leu Leu Leu Leu Leu Leu Leu Leu Pro
             20                  25                  30

Arg Gly Phe Gln Pro Gln Pro Gly Gly Asn Arg Thr Glu Ser Pro
             35                  40                  45

Glu Pro Asn Ala Thr Ala Thr Pro Ala Ile Pro Thr Ile Leu Val
             50                  55                  60

Thr Ser Val Thr Ser Glu Thr Pro Ala Thr Ser Ala Pro Glu Ala
             65                  70                  75

Glu Gly Pro Gln Ser Gly Gly Leu Pro Pro Pro Pro Arg Ala Val
             80                  85                  90

Pro Ser Ser Ser Ser Pro Gln Ala Gln Ala Leu Thr Glu Asp Gly
             95                 100                 105

Arg Pro Cys Arg Phe Pro Phe Arg Tyr Gly Gly Arg Met Leu His
            110                 115                 120

Ala Cys Thr Ser Glu Gly Ser Ala His Arg Lys Trp Cys Ala Thr
            125                 130                 135

Thr His Asn Tyr Asp Arg Asp Arg Ala Trp Gly Tyr Cys Val Glu
            140                 145                 150

Ala Thr Pro Pro Pro Gly Gly Pro Ala Ala Leu Asp Pro Cys Ala
            155                 160                 165

Ser Gly Pro Cys Leu Asn Gly Gly Ser Cys Ser Asn Thr Gln Asp
            170                 175                 180

Pro Gln Ser Tyr His Cys Ser Cys Pro Arg Ala Phe Thr Gly Lys
            185                 190                 195

Asp Cys Gly Thr Glu Lys Cys Phe Asp Glu Thr Arg Tyr Glu Tyr
            200                 205                 210

Leu Glu Gly Gly Asp Arg Trp Ala Arg Val Arg Gln Gly His Val
            215                 220                 225

Glu Gln Cys Glu Cys Phe Gly Gly Arg Thr Trp Cys Glu Gly Thr
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230 235 240

Arg His Thr Ala Cys Leu Ser Ser Pro Cys Leu Asn Gly Gly Thr
245 250 255

Cys His Leu Ile Val Ala Thr Gly Thr Thr Val Cys Ala Cys Pro
260 265 270

Pro Gly Phe Ala Gly Arg Leu Cys Asn Ile Glu Pro Asp Glu Arg
275 280 285

Cys Phe Leu Gly Asn Gly Thr Gly Tyr Arg Gly Val Ala Ser Thr
290 295 300

Ser Ala Ser Gly Leu Ser Cys Leu Ala Trp Asn Ser Asp Leu Leu
305 310 315

Tyr Gln Glu Leu His Val Asp Ser Val Gly Ala Ala Ala Leu Leu
320 325 330

Gly Leu Gly Pro His Ala Tyr Cys Arg Asn Pro Asp Asn Asp Glu
335 340 345

Arg Pro Trp Cys Tyr Val Val Lys Asp Ser Ala Leu Ser Trp Glu
350 355 360

Tyr Cys Arg Leu Glu Ala Cys Glu Ser Leu Thr Arg Val Gln Leu
365 370 375

Ser Pro Asp Leu Leu Ala Thr Leu Pro Glu Pro Ala Ser Pro Gly
380 385 390

Arg Gln Ala Cys Gly Arg Arg His Lys Lys Arg Thr Phe Leu Arg
395 400 405

Pro Arg Ile Ile Gly Gly Ser Ser Ser Leu Pro Gly Ser His Pro
410 415 420

Trp Leu Ala Ala Ile Tyr Ile Gly Asp Ser Phe Cys Ala Gly Ser
425 430 435

Leu Val His Thr Cys Trp Val Val Ser Ala Ala His Cys Phe Ser
440 445 450

His Ser Pro Pro Arg Asp Ser Val Ser Val Val Leu Gly Gln His
455 460 465

Phe Phe Asn Arg Thr Thr Asp Val Thr Gln Thr Phe Gly Ile Glu
470 475 480

Lys Tyr Ile Pro Tyr Thr Leu Tyr Ser Val Phe Asn Pro Ser Asp
485 490 495

His Asp Leu Val Leu Ile Arg Leu Lys Lys Lys Gly Asp Arg Cys
500 505 510

Ala Thr Arg Ser Gln Phe Val Gln Pro Ile Cys Leu Pro Glu Pro
515 520 525

Gly Ser Thr Phe Pro Ala Gly His Lys Cys Gln Ile Ala Gly Trp
530 535 540

Gly His Leu Asp Glu Asn Val Ser Gly Tyr Ser Ser Ser Leu Arg
545 550 555

Glu Ala Leu Val Pro Leu Val Ala Asp His Lys Cys Ser Ser Pro
560 565 570

Glu Val Tyr Gly Ala Asp Ile Ser Pro Asn Met Leu Cys Ala Gly
575 580 585

Tyr Phe Asp Cys Lys Ser Asp Ala Cys Gln Gly Asp Ser Gly Gly
590 595 600

Pro Leu Ala Cys Glu Lys Asn Gly Val Ala Tyr Leu Tyr Gly Ile
605 610 615

Ile Ser Trp Gly Asp Gly Cys Gly Arg Leu His Lys Pro Gly Val
620 625 630

Tyr Thr Arg Val Ala Asn Tyr Val Asp Trp Ile Asn Asp Arg Ile
635 640 645

Arg Pro Pro Arg Arg Leu Val Ala Pro Ser
650 655

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Pro Cys Pro Phe Leu Leu Leu Leu Leu Leu Leu Val Val Pro Arg
20 25 30

Gly Ala Gln Pro Gln Ala Gly Arg Asn His Thr Glu Pro Pro Gly
35 40 45

Pro Asn Val Thr Ala Thr Pro Val Thr Pro Thr Ile Pro Val Ile
50 55 60

Ser Gly Asn Val Ser Thr Ser Thr Glu Ser Ala Pro Ala Ala Glu
65 70 75

Thr Glu Gly Pro Gln Ser Glu Arg Tyr Pro Pro Pro Ser Ser Ser
80 85 90

Ser Pro Pro Gly Gly Gln Val Leu Thr Glu Ser Gly Gln Pro Cys
95 100 105

Arg Phe Pro Phe Arg Tyr Gly Gly Arg Met Leu His Ser Cys Thr
110 115 120

Ser Glu Gly Ser Ala Tyr Arg Lys Trp Cys Ala Thr Thr His Asn
125 130 135

Tyr Asp Arg Asp Arg Ala Trp Gly Tyr Cys Ala Glu Val Thr Leu
140 145 150

Pro Val Glu Gly Pro Ala Ile Leu Asp Pro Cys Ala Ser Trp Pro
155 160 165

Cys Leu Asn Gly Gly Thr Cys Ser Ser Thr His Asp His Gly Ser
170 175 180

Tyr His Cys Ser Cys Pro Leu Ala Phe Thr Gly Lys Asp Cys Gly
185 190 195

Thr Glu Lys Cys Phe Asp Glu Thr Arg Tyr Glu Tyr Phe Glu Val
200 205 210

Gly Asp His Trp Ala Arg Val Ser Glu Gly His Val Glu Gln Cys
215 220 225

Gly Cys Met Glu Gly Gln Ala Arg Cys Glu Asp Thr His His Thr
230 235 240

Ala Cys Leu Ser Ser Pro Cys Leu Asn Gly Gly Thr Cys His Leu
245 250 255

Ile Val Gly Thr Gly Thr Ser Val Cys Thr Cys Pro Leu Gly Tyr
260 265 270

Ala Gly Arg Phe Cys Asn Ile Val Pro Thr Glu His Cys Phe Leu
275 280 285

Gly Asn Gly Thr Glu Tyr Arg Gly Val Ala Ser Thr Ala Ala Ser
290 295 300

Gly Leu Ser Cys Leu Ala Trp Asn Ser Asp Leu Leu Tyr Gln Glu
305 310 315

Leu His Val Asp Ser Val Ala Ala Ala Val Leu Leu Gly Leu Gly
320 325 330

Pro His Ala Tyr Cys Arg Asn Pro Asp Lys Asp Glu Arg Pro Trp
335 340 345

Cys Tyr Val Val Lys Asp Asn Ala Leu Ser Trp Glu Tyr Cys Arg
350 355 360

Leu Thr Ala Cys Glu Ser Leu Ala Arg Val His Ser Gln Thr Pro
365 370 375

Glu Ile Leu Ala Ala Leu Pro Glu Ser Ala Pro Ala Val Arg Pro
380 385 390

Thr Cys Gly Lys Arg His Lys Lys Arg Thr Phe Leu Arg Pro Arg
395 400 405

Ile Ile Gly Gly Ser Ser Ser Leu Pro Gly Ser His Pro Trp Leu
410 415 420

Ala Ala Ile Tyr Ile Gly Asn Ser Phe Cys Ala Gly Ser Leu Val
425 430 435

His Thr Cys Trp Val Val Ser Ala Ala His Cys Phe Ala Asn Ser
440 445 450

Pro Pro Arg Asp Ser Ile Thr Val Val Leu Gly Gln His Phe Phe
455 460 465

Asn Arg Thr Thr Asp Val Thr Gln Thr Phe Gly Ile Glu Lys Tyr
470 475 480

Val Pro Tyr Thr Leu Tyr Ser Val Phe Asn Pro Asn Asn His Asp
485 490 495

Leu Val Leu Ile Arg Leu Lys Lys Lys Gly Glu Arg Cys Ala Val
500 505 510

Arg Ser Gln Phe Val Gln Pro Ile Cys Leu Pro Glu Ala Gly Ser
515 520 525

Ser Phe Pro Thr Gly His Lys Cys Gln Ile Ala Gly Trp Gly His
530 535 540

Met Asp Glu Asn Val Ser Ser Tyr Ser Asn Ser Leu Leu Glu Ala
545 550 555

Leu Val Pro Leu Val Ala Asp His Lys Cys Ser Ser Pro Glu Val

560 565 570

Tyr Gly Ala Asp Ile Ser Pro Asn Met Leu Cys Ala Gly Tyr Phe
575 580 585

Asp Cys Lys Ser Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu
590 595 600

Val Cys Glu Lys Asn Gly Val Ala Tyr Leu Tyr Gly Ile Ile Ser
605 610 615

Trp Gly Asp Gly Cys Gly Arg Leu Asn Lys Pro Gly Val Tyr Thr
620 625 630

Arg Val Ala Asn Tyr Val Asp Trp Ile Asn Asp Arg Ile Arg Pro
635 640 645

Pro Lys Arg Pro Val Ala Thr Ser
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<220>
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Gly Ile Ile Asn Pro Asn Gly Gly Tyr Thr Asn
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<212> PRT
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<400> 5
Ser Ser Arg Leu Ala Gly Ala Met Asp Tyr
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Gly Trp Ile Tyr Pro Ala Gly Gly Ala Thr Asp
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Trp Gly Trp Gly Phe Asp Tyr
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Asn Gly Thr Tyr

<210> 13
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<400> 13
Gly Gly Ile Tyr Pro Ala Gly Gly Ala Thr Tyr
5 10

<210> 14
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Trp Trp Ala Trp Pro Ala Phe Asp Tyr
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Asn Gly Thr Trp

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<400> 16
Gly Gly Ile Tyr Pro Ala Gly Gly Ala Thr Asp
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Trp Arg Ala Val Pro Ser Phe Asp Tyr
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Thr Gly Thr Tyr

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Gly Trp Ile Ser Pro Tyr Asn Gly Asp Thr Tyr
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Thr Gly Ser Ala

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Ala Ile Ile Asn Pro Asn Gly Gly Tyr Thr Tyr
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Ser Ala Arg Phe Ser Phe Asp Tyr
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Ser Gly Asn Trp

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Ala Glu Ile Asn Pro Tyr Asn Gly Ser Thr Asn
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Thr Asn Tyr Trp

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Gly Gly Ile Tyr Pro Ala Gly Gly Ala Thr Asp
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Tyr Ser Ile Pro Ala Phe Asp Tyr
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Ser Asn Ser Gly

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<400> 31
Gly Trp Ile Tyr Pro Thr Gly Gly Ala Thr Asp
5 10

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Phe Trp Trp Arg Ser Phe Asp Tyr
5

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<400> 33
Ser Asp Ser Ser

<210> 34
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Ala Arg Ile Tyr Pro Thr Ser Gly Asn Thr Asn
5 10

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Gly Leu Lys Val Pro Phe Tyr Ala Asn Ala Ala Met Asp Tyr
5 10

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Ser Gly Ser Ala

<210> 37
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Ala Ile Ile Asn Pro Thr Gly Gly Tyr Thr Asn
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Ser Arg Gly His Tyr Ala Met Asp Tyr
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Thr Gly Asn Gly

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<400> 41
Gly His Arg Val Phe Asp Tyr
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Asn Asn Thr Gly

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<400> 43
Gly Trp Ile Tyr Pro Ala Gly Gly Ala Thr Asp
5 10

<210> 44
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<400> 44
Phe Phe Pro Val Ala Phe Asp Tyr
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<210> 45
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Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
20 25 30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Glu Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys Arg Thr

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser
20 25

<210> 47
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Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
5 10

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<400> 48
Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu
1 5 10 15

Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
20 25 30

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5 10

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1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys
20

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<400> 51
Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
1 5 10 15

<210> 52
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Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe
1 5 10 15

Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr
20 25 30

Tyr Cys

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<400> 53
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
5 10

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Ser
20 25 30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser
50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
65 70 75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
80 85 90

Ser Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

Ile Lys

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1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys
20

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1 5 10 15

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Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr
20 25 30

Tyr Cys

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Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
5 10

<210> 59
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1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser
20 25

<210> 60
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Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
5 10

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<220>
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Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr Leu
1 5 10 15

Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
20 25 30

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5 10

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<220>
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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15

Gly Asp Arg Val Thr Ile Thr Cys
20

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<220>
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<400> 64
Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
1 5 10 15

<210> 65
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<400> 65
Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe
1 5 10 15

Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr
20 25 30

Tyr Cys

<210> 66
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<220>
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<400> 66
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
5 10

<210> 67
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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser
20 25

<210> 68
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<210> 69
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<400> 69
Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu
1 5 10 15

Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
20 25 30

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Gly Phe Asn Ile Thr Ser Ser Ala Ile His
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5 10

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Gly Phe Asn Ile Asn Gly Thr Trp Ile His
5 10

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Gly Phe Asn Ile Thr Gly Thr Tyr Ile His
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Gly Phe Asn Ile Thr Gly Ser Ala Ile His
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2005294146 29 Oct 2008

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2005294146 29 Oct 2008

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