

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



BT

DELEGATURA PROPIEDAD INDUSTRIAL

División de Nuevas Creaciones

PCT
SOLICITUD

PATENTE DE INVENCION

ENTRADA EN FASE NACIONAL

21. EXPEDIENTE No.

07-34188

54. TITULO

MODULADORES DE ACTIVADOR DE FACTOR DE CRECIMIENTO HEPATOCITICO

51. CLASIFICACION INTERNACIONAL

71. SOLICITANTE

GENENTECH, INC.

DOMICILIO

SOUTH SAN FRANCISCO, CALIFORNIA, ESTADOS UNIDOS

74. APODERADO

JUAN PABLO CADENA SARMIENTO

22. SANTAFE DE BOGOTA, D.C.

Rev. 04-05-2007

Cesión

Complemento 28 folios 03-07-2007

✓

PETITORIO

AS: 103 309


Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad: 07-034188- -00000-0000
Fec 2007-04-04 15:06 01 Dep. 2020 NUECREACIONES
Tra 11 PATENTEIYU
Eve 376 FASENACIONALI
Act 411 PRESENTACION Folios 473

FORMULARIO ÚNICO DE SOLICITUD DE PATENTE
PCT
ENTRADA EN FASE NACIONAL

1

SOLICITUD DE:

☒ Patente de Invención.☐ Patente de Modelo de Utilidad.☒ Capítulo I.☐ Capítulo II.

2

SOLICITANTE
(71)Nombre: **GENENTECH, INC.**Dirección: 1 DNA Way, South San Francisco, CA
94080-4990, US

Nacionalidad o Domicilio: Estados Unidos

Lugar de Constitución: Estados Unidos

Teléfono:

Fax:

E-mail

IDENTIFICACION

C.C. ☐ NIT ☐C.E. ☐ Otro ☐

Cual _____

Número _____

3

REPRESENTANTE
O APODERADONombre: **JUAN PABLO CADENA SARMIENTO**

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Teléfono: 744-2200

Fax: 744-2700

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IDENTIFICACION

C.C. ☒ NIT ☐C.E. ☐ Otro ☐

Cual _____

Número 80.410.337 TP 57492

4

INVENTOR (ES)
(72)Nombre: **Daniel K. KIRCHHOFER, Yan WU**Dirección: 326 S Springer Road, Los Altos, California 94024, US, 101 Challenge Court, Foster City,
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Nacionalidad o Domicilio: Estados Unidos de América (Respectivamente)

5 PCT (86)

Solicitud Internacional No. PCT/US2005/036300 Fecha 03-10-2005Publicación Internacional No. WO 2006/042173 A3 Fecha 20-04-20066 Título (54): MODULADORES DE ACTIVADOR DE FACTOR DE CRECIMIENTO HEPATOCÍTICO

7 Clasificación Internacional (51)

A61K 39/395

8 Prioridad

SI ☒NO ☐

(33) País de Origen

US

(32) Fecha

04-10-2004

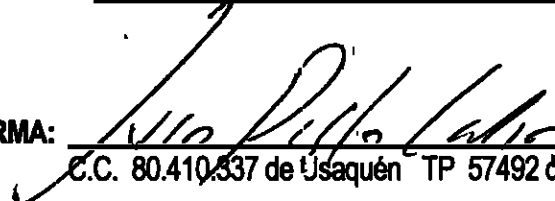
(31) Número de Solicitud

60/615.6579 Comprobante de pago No. 07-13.490Fecha 03-04-2007

Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

2020-F06 (01-11-15)

- ☒ Comprobante de pago de la tasa de presentación de la solicitud. \$482.000
- ☒ Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- ☒ Poderes, si fuere el caso. **Fotocopia Autenticada**
- ☐ Documento de cesión del inventor al solicitante o a su causante.
- ☐ Declaraciones
- ☒ Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud internacional al castellano (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar efectuado por la IPEA.
- ☐ Traducción del examen preliminar efectuado por la IPEA
- ☐ De ser el caso, copia del contrato de acceso.
- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas
- ☐ De ser el caso, certificado de depósito del material biológico.
- ☐ Arte final 12x12.
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.
- ☒ Otros. Listado de Secuencias
Tarjeta de Propietarios
Documentos PCT

11 FIGURA CARACTERÍSTICA:**12** Solicito la concesión de la patente,**NOMBRE:** JUAN PABLO CADENA SARMIENTO**FIRMA:**
C.C. 80.410.337 de Usaquén TP 57492 del C.S.J.

Instrucciones para la presentación de los anexos, ver reverso de esta página.

As 103 209 3

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad: 07-034188-00000-0000
Fec 2007-04-04 15:05:01 Dep 2020 NUECREACIONES
Tra 11 PATENTEYII
Eve. 372 FASENACIONALI
Act 411 PRESENTACION Folios 47E

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 07 - 13,490
FECHA : FEBRERO 14 DE 2007

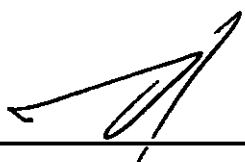
***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	Nº. PAGO	FECHA PGO	Vr. PAGO
BRIGARD & CASTRO LTDA	CONSIGNACION	BANCO POPULAR	050-00110-6	52423538	14/02/2007	17,370,000.00

***** CONCEPTO *****

CANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	482,000.00
	TOTAL :	\$ 482,000.00

SON: CUATROCIENTOS OCHENTA Y DOS MIL PESOS

RESPONSABLE : 

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

I hereby authorize my personal liability insurance policy to be assigned to the above named party.

PCT

(10) International Publication Number
WO 2006/042173 A3

AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY,
MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO,
NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK,
SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,
VC, VN, YU, ZA, ZM, ZW

PCT/US2005/036300

(22) International Filing Date: 3 October 2005 (03 10 2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 60/615,657 4 October 2004 (04 10 2004) US

(71) Applicant (for all designated States except US): GENENTECH, INC. [US/US], 1 DNA Way, South San Francisco, California 94080-4990 (US)

(72) Inventors: and

(75) **Inventors/Applicants (for US only): KIRCHHOFFER, Daniel, K. [US/US]; 326 S. Springer Road, Los Altos, California 94024 (US). WU, Yan [US/US], 101 Challenge Court, Foster City, California 94404 (US)**

(74) Agent: NAIK, Paul, Genentech, Inc , MS 49, 1 DNA Way,
South San Francisco, California 94080 (US)

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GO, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(8b) Date of publication of the International search report:
17 August 2006

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette

(54) Title: MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR

Clone #	Seq	H1					H2										H3										Affinity					
		30	31	32	33		48	50	51	52	53A	53	54	55	56	57	58	65	66	67	68	69	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					N	D	Y	12 nM
35	1	T	G	S	A		G	I	I	N	P	N	S	G	Y	T	N	S	A	R	I	R	G					F	D	Y	12 nM	
37	1	N	S	N	O		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	F	A					F	D	Y	8 nM	
42	1	N	G	T	W		G	G	I	Y	P	A	G	G	A	T	Y	W	R	A	V	F	S					F	D	Y	30 nM	
49	1	T	O	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	A	W	S	V	N	S	V		N	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	H	S	G		G	W	I	Y	P	T	G	G	A	T	D	F	W	W	R	S						F	D	Y	8 nM	
88	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	N	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					N	D	Y	40 nM	
91	2	T	G	N	G		A	W	I	S	P	Y	G	G	S	T	E	G	H	X	V							F	D	Y	100 nM	
95	4	N	N	T	O		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.

WO 2006/042173 A3

5 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/036300

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/40 A61P35/00 A61K39/395
ADD. C12N9/64

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	KATAOKA HIROAKI ET AL: "Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma" CANCER RESEARCH, vol. 60, no. 21, 1 November 2000 (2000-11-01), pages 6148-6159, XP002380896 ISSN: 0008-5472 the whole document ----- -/-	1-17

☒ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents

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

"E" earlier document but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

22 May 2006

Date of mailing of the international search report

13/06/2006

Name and mailing address of the ISA/

European Patent Office, P B 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Tx 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Bumb, P

6 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/036300

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	MIYAZAWA K ET AL: "ACTIVATION OF HEPATOCYTE GROWTH FACTOR IN THE INJURED TISSUES IS MEDIATED BY HEPATOCYTE GROWTH FACTOR ACTIVATOR" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOCHEMICAL BIOLOGISTS, BIRMINGHAM,, US, vol. 271, no. 7, 16 February 1996 (1996-02-16), pages 3615-3618, XP002039579 ISSN: 0021-9258 the whole document	1-17
X	EP 1 213 302 A (MITSUBISHI CHEMICAL CORPORATION) 12 June 2002 (2002-06-12) examples 3-8	1-3,16, 17
A	CAO B ET AL: "Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, US, vol. 98, no. 13, 19 June 2001 (2001-06-19), pages 7443-7448, XP002982804 ISSN: 0027-8424 page 7445 - page 7446	12-15
A	US 6 407 213 B1 (CARTER PAUL J ET AL) 18 June 2002 (2002-06-18) cited in the application the whole document	16,17

7 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/036300

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons

- 1

☒

Claims Nos. 12-15 (with respect to industrial applicability) because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 12-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
- 2

☐

Claims Nos. because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically
- 3

☐

Claims Nos. because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a)

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

- 1

☐

As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims
- 2

☐

As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee
- 3

☐

As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.
- 4

☐

No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.

Remark on Protest

- ☐

The additional search fees were accompanied by the applicant's protest
- ☐

No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No
PCT/US2005/036300

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 1213302	A	12-06-2002	CN 1384120 A	11-12-2002
			US 2002165362 A1	07-11-2002
			US 2006073520 A1	06-04-2006
US 6407213	B1	18-06-2002	AT 255131 T	15-12-2003
			AU 675916 B2	27-02-1997
			AU 2250992 A	12-01-1993
			CA 2103059 A1	15-12-1992
			CY 2500 A	02-09-2005
			DE 69233254 D1	08-01-2004
			DE 69233254 T2	16-09-2004
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			DK 590058 T3	29-03-2004
			EP 0590058 A1	06-04-1994
			ES 2206447 T3	16-05-2004
			JP 6508267 T	22-09-1994
			JP 2005000169 A	06-01-2005
			JP 2006083180 A	30-03-2006
			LU 91067 A9	02-04-2004
			NL 300145 I1	01-06-2004
			WO 9222653 A1	23-12-1992
			US 6719971 B1	13-04-2004
			US 6639055 B1	28-10-2003
			US 5821337 A	13-10-1998

103 309(9)
a

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

(19) World Intellectual Property Organization
International Bureau



INTERNATIONAL BUREAU OF PATENT COOPERATION
358, RUE DE LA PATENTE
CH-1202 GENEVE 2, SUISSE

(43) International Publication Date
20 April 2006 (20.04.2006)

PCT

(10) International Publication Number
WO 2006/042173 A2

- (51) International Patent Classification: Not classified
- (21) International Application Number:
PCT/US2005/036300
- (22) International Filing Date: 3 October 2005 (03 10 2005)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/615,657 4 October 2004 (04 10 2004) US
- (71) Applicant (for all designated States except US): GENENTECH, INC. [US/US], 1 DNA Way, South San Francisco, California 94080-4990 (US)
- (72) Inventors; and
- (73) Inventors/Applicants (for US only): KIRCHHOFFER, Daniel, K. [US/US], 326 S Springer Road, Los Altos, California 94024 (US) WU, Yan [US/US], 101 Challenge Court, Foster City, California 94404 (US)
- (74) Agent: NAIK, Paul, Genentech, Inc., MS 49, 1 DNA Way, South San Francisco, California 94080 (US)
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GH, GM, GR, GU, HK, HU, IL, IN, IS, JP, KE, KG, KM, KN, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TI, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EL, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)
- Published:
— without international search report and to be republished upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette

(54) Title. MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR

Clone #	Size	H1	H2	H3	100A	100B	100C	100D	100E	100F	101	102	Affinity
33	2	T E S A	G I I M	P N G	G Y T M	S E R L A G A					M D Y		12 nM
35	1	T O 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	M 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			8 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 O 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
68	1	T O S A	A I I N	P N G G Y T Y	S A R P 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			80 nM
75	1	S N S G	G W I Y	P T G G A T D	F W N R S					F D Y			8 nM
86	1	S D S S	A R I Y	P T S G N T N	G L X 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

WO 2006/042173 A2

PATENT
Attorney Docket P2179R1
Express Mail No EV385655937 US
Mailed 3 October 2005

5

MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR

RELATED APPLICATIONS

10 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

15 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

20 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
25 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
30 variants HAI-1 (16-17) and HAI-1B (15) and by HAI-2 (18). HAI-2 (also known as placental bikunin) (19) also potentially 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.

35 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 specifically 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.

DISCLOSURE OF THE INVENTION

5 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.,

10 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 interfering 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

15 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

20 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

In one aspect, the invention provides antagonists that disrupt the HGF/c-met signaling pathway. For example, the invention provides a molecule that inhibits HGFA cleavage of proHGF

25 (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

30 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 invention does not inhibit HGF binding to c-met. For example, in one embodiment, an antagonist molecule of the invention is not an antibody or

35 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

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HB-11894 (hybridoma 1A3 3 13) or HB-11895 (hybridoma 5D5 11 6). In one embodiment, an antagonist molecule of the invention inhibits biological activities associated with HGF/c-met activation.

5 In one aspect, the invention provides 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. In one aspect, the invention provides 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. In one aspect, the invention provides 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 invention provides 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, 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 invention provides 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, 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 invention provides 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, 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 invention provides 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, 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 aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following:

- 25 (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.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following.

- 30 (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

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following.

- 35 (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.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following.

- 5 (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

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following:

- 10 (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.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following:

- 15 (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.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following:

- 20 (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.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following

- 25 (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

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following:

- 30 (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.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following.

- 35 (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.

In one aspect, the invention provides 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,
- 5 (iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO 35

In one aspect, the invention provides 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,
- 10 (iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO 38.

In one aspect, the invention provides 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;
- 15 (iii) a CDR-H3 sequence comprising the sequence of SEQ ID NO 41

In one aspect, the invention provides 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,
- 20 (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 invention 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.

In one aspect, the invention provides 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.

35 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)

5 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

10 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
15 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,
20 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)
25 (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 κ light chain framework consensus sequence. In one embodiment, these antibodies comprise light chain variable domain sequences of humanized 4D5 antibody (huMAb
30 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
35 D. In one embodiment, an antibody of the invention comprises a light chain variable domain,

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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 of the invention 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 of the invention 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 of the invention 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 of the invention 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.

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

In one aspect, an antagonist molecule of the invention is 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.

The invention also provides methods and compositions useful for modulating disease states associated with dysregulation of the HGF/c-met signaling axis. Thus, in one aspect, the invention provides 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. In one aspect, the invention provides 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 of the invention is an antibody that binds to HGFA.

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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, in another aspect, the invention provides 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 the invention, whereby cell proliferation associated with c-met activation is inhibited. In yet another aspect, the invention provides 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 the invention, whereby angiogenesis is inhibited.

10 In one aspect, the invention provides use of a modulator molecule of the invention 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.

15 In one aspect, the invention provides use of a nucleic acid of the invention 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.

20 In one aspect, the invention provides use of an expression vector of the invention 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.

25 In one aspect, the invention provides use of a host cell of the invention 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.

In one aspect, the invention provides use of an article of manufacture of the invention 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.

30 In one aspect, the invention provides use of a kit of the invention 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.

35 In one aspect, the invention provides a method of inhibiting c-met activated cell proliferation, said method comprising contacting a cell or tissue with an effective amount of a

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modulator molecule of the invention, whereby cell proliferation associated with c-met activation is inhibited

5 In one aspect, the invention provides 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 invention, whereby said condition is treated

10 In one aspect, the invention provides 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 invention 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)

15 In one aspect, the invention provides 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 invention, 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)

20 In one aspect, the invention provides 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 invention, thereby effectively treating or preventing said cell proliferative disorder. In one embodiment, said proliferative disorder is cancer.

25 In one aspect, the invention provides 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 invention, thereby effectively treating or preventing said cell proliferative disorder In one embodiment, said proliferative disorder is cancer.

30 In one aspect, the invention provides 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 invention, 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).

35 In one aspect, the invention provides 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 invention, 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).

5 In one aspect, the invention provides 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 invention, 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).

10 In one aspect, the invention provides 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 invention, 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).

15 Methods of the invention 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 invention 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 invention is a hyperproliferative and/or hyperplastic cell. In one embodiment, a cell that is targeted in a method of the invention is a dysplastic cell. In yet another embodiment, a cell that is targeted in a method of the invention is a metastatic cell.

25 Methods of the invention 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.

30 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 invention, 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 invention causes the death of a targeted cell. For example, contact with a modulator molecule of the invention may result in a cell's inability to signal through the c-met pathway, which results in cell death.

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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 invention comprises targeting a tissue wherein one or more of HGFA, c-met and hepatocyte 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 invention, 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).

In one aspect, the invention provides compositions comprising one or more modulator molecules of the invention and a carrier. In one embodiment, the carrier is pharmaceutically acceptable.

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

In one aspect, the invention provides vectors comprising a nucleic acid of the invention.

In one aspect, the invention provides host cells comprising a nucleic acid or a vector of the invention. 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.

In one aspect, the invention provides methods for making a modulator molecule of the invention. For example, the invention provides 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 invention encoding said antibody (or fragment thereof), and recovering said antibody.

In one aspect, the invention provides 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 invention. In one embodiment, the composition comprises a nucleic acid of the invention. 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 of the invention further comprises instructions for administering the composition (for e.g., the modulator molecule) to a subject.

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In one aspect, the invention provides a kit comprising a first container comprising a composition comprising one or more modulator molecules of the invention, 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.

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In one aspect the invention provides 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 invention, whereby HGFA bound by the antibody indicates presence and/or amount of HGFA in the sample. In another aspect, the invention provides 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 invention 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 invention, 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.

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In one aspect, the invention provides a method of binding an antibody of the invention to HGFA present in a bodily fluid, for example blood.

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In yet another aspect, the invention is directed to a method of binding an antibody of the invention 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.

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In one aspect, the invention provides 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 invention, 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), clone 35 (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 0.1 mg/ml (0.67 µM) 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 (1 µM), 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.

5 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 , $K_M = 200 \mu M$) the linear rates of substrate activation were measured on a kinetic microplate reader. Inhibition of enzyme activity was expressed as fractional activity (vi/v0) of uninhibited activity.

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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)

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20 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.

25

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

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

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

MODES FOR CARRYING OUT THE INVENTION

The invention provides 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.

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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

5 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.

10 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

```
/*
*
15  * 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
    */
20 #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},
25 /* 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},
30 /* 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},
/* 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},
35 /* 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}}
```

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```

/* 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, _M,
0, _M, _M, _M, _M, _M, _M, _M, _M, _M, _M, _M, _M, _M, _M, _M,
5 /* 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, 0},
10 /* 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, 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}
15 );

/*
*/
#include <stdio.h>
20 #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 */
25 #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 */
30 #define DINS1 1 /* penalty per base */
#define PINS0 8 /* penalty for a gap */
#define PINS1 4 /* penalty per residue */

struct jmp {
35 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 */
5       long     offset;     /* offset of prev block */
        short    jmp;        /* current jmp index */
        struct jmp jp;       /* list of jmps */
    },

10   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) */
    };

15   char      *ofile;        /* output file name */
    char      *namex[2],     /* seq names getseqs() */
    char      *prog,         /* prog name for err msgs */
    char      *seqx[2],      /* seqs getseqs() */
20   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 */
25   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 */
30   struct diag *dx;        /* holds diagonals */
    struct path pp[2];       /* holds path for seqs */

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

35

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```

/* Needleman-Wunsch alignment program
5  *
  * usage  progs file1 file2
  * where file1 and file2 are two dna or two protein sequences.
  * The sequences can be in upper- or lower-case and may contain ambiguity
  * Any lines beginning with '.', '>' or '<' are ignored
10  * 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.
15  * Original version developed under BSD 4.3 on a vax 8650
  */
#include "nw.h"
#include "day.h"

20  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] = {
25        1, 2|(1<<('D'-'A'))|(1<<('N'-'A')), 4, 8, 16, 32, 64,
        128, 256, 0xFFFFFFFF, 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'))
30    };

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

```

main

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30

```
    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"),
5    fprintf(stderr, "Output is in the file 'align out'\n");
    exit(1),
}
    namex[0] = av[1],
    namex[1] = av[2];
10    seqx[0] = getseq(namex[0], &len0),
    seqx[1] = getseq(namex[1], &len1);
    xbm = (dna)? _dbval . _pbval,

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

    nw();                      /* fill in the matrix, get the possible jmps */
    readjmps(),                /* get the actual jmps */
    print(),                   /* print stats, alignment */
20    cleanup(0);               /* unlink any tmp files */
}

25
/* 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
30 * a new gap to extending an ongoing gap, and prefer a gap in seqx
 * to a gap in seq y.
 */
nw()
{
35     char      *px, *py,      /* seqs and ptrs */
    int      *ndely, *dely, /* keep track of dely */
    nw
```

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```

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

10   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)),
15   col1 = (int *)g_calloc("to get col1", len1+1, sizeof(int)),
    ins0 = (dna)? DINS0 . PINS0;
    ins1 = (dna)? DINS1 . PINS1,

    smax = -10000;
20   if (endgaps) {
        for (col0[0] = dely[0] = -ins0, yy = 1, yy <= len1; yy++) {
            col0[yy] = dely[yy] = col0[yy-1] - ins1;
            ndely[yy] = yy;
        }
25   col0[0] = 0,    /* Waterman Bull Math Biol 84 */
    }
    else
        for (yy = 1; yy <= len1; yy++)
            dely[yy] = -ins0,
30

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

```

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

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

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

```

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```

        ndely[yy] = 1,
    } else
        ndely[yy]++,
    }
5
    /* update penalty for del in y seq,
    * favor new del over ongong del
    */
    if (endgaps || ndelx < MAXGAP) {
10        if (col1[yy-1] - ins0 >= delx) {
            delx = col1[yy-1] - (ins0+ins1);
            ndelx = 1,
        } else {
15            delx -= ins1,
            ndelx++,
        }
    } else {
        if (col1[yy-1] - (ins0+ins1) >= delx) {
20            delx = col1[yy-1] - (ins0+ins1);
            ndelx = 1,
        } else
            ndelx++,
    }
25
    /* pick the maximum score; we're favoring
    * mis over any del and delx over dely
    */
30
35
    id = xx - yy + len1 - 1,
    ...nw
```

```

    if (mis >= delx && mis >= dely[yy])
        col1[yy] = mis,
    else if (delx >= dely[yy]) {
        col1[yy] = delx;
5       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) {
10                writejmps(id),
                ij = dx[id] ijmp = 0,
                dx[id] offset = offset;
                offset += sizeof(struct jmp) + sizeof(offset);
            }
15        }
        dx[id].jp n[ij] = ndelx;
        dx[id] jp.x[ij] = xx;
        dx[id].score = delx,
    }
20    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)) {
25            dx[id] ijmp++,
            if (++ij >= MAXJMP) {
                writejmps(id);
                ij = dx[id] ijmp = 0;
                dx[id] offset = offset,
30                offset += sizeof(struct jmp) + sizeof(offset);
            }
        }
        dx[id].jp n[ij] = -ndely[yy],
        dx[id].jp.x[ij] = xx;
35        dx[id].score = dely[yy],
    }
}
```

```

        if (xx == len0 && yy < len1) {
            /* last col
            */
            if (endgaps)
5              col1[yy] -= ins0+ins1*(len1-yy),
              if (col1[yy] > smax) {
                  smax = col1[yy],
                  dmax = id;
              }
10          }
        }
        if (endgaps && xx < len0)
            col1[yy-1] -= ins0+ins1*(len0-xx);
        if (col1[yy-1] > smax) {
15            smax = col1[yy-1];
            dmax = id;
        }
        tmp = col0; col0 = col1, col1 = tmp;
20    }
    (void) free((char *)ndely);
    (void) free((char *)dely);
    (void) free((char *)col0);
    (void) free((char *)col1);
}

25  /*
    *
    * print() -- only routine visible outside this module
    *
    * static:
30  * 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()
35  * stars() -- put a line of stars: dumpblock()
    * stripname() -- strip any path and prefix from a seqname

```


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```
*/

#include "nw h"

5  #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],
10 int olen; /* set output line length */
   FILE *fx, /* output file */

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

        if ((fx = fopen(ofile, "w")) == 0) {
            fprintf(stderr, "%s: can't write %s\n", prog, ofile);
            cleanup(1),
20         }
        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,
25     ly = len1,
        firstgap = lastgap = 0;
        if (dmax < len1 - 1) { /* leading gap in x */
            pp[0] spc = firstgap = len1 - dmax - 1,
            ly -= pp[0] spc,
30         }
        else if (dmax > len1 - 1) { /* leading gap in y */
            pp[1] spc = firstgap = dmax - (len1 - 1),
            lx -= pp[1].spc,
            }
35     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),
5        ly -= lastgap,
    }
    getmat(lx, ly, firstgap, lastgap),
    pr_align();
}
10

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

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

```

getmat

```

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),

```

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

10     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),
15     }
      if (dna)
          fprintf(fx,
              "\n<score: %d (match = %d, mismatch = %d, gap penalty = %d + %d per base)\n",
              smax, DMAT, DMIS, DINS0, DINS1);
20     else
          fprintf(fx,
              "\n<score: %d (Dayhoff PAM 250 matrix, gap penalty = %d + %d per residue)\n",
              smax, PINS0, PINS1);
      if (endgaps)
25         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
30         fprintf(fx, "<endgaps not penalized\n");
    }

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

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```
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 */
5  static char out[2][P_LINE], /* output line */
static char  star[P_LINE]; /* set by stars() */

/*
 * print alignment of described in struct path pp[]
10 */
static
pr_align()                                pr_align
{
    int      nn,          /* char count */
15    int      more;
    register      i;

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

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

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

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```
more++;

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

20     /*
      * are we at next gap for this seq?
      */
      if (ni[i] == pp[i].x[ij[i]]) {
          /*
25         * we need to merge all gaps
          * at this location
          */
          siz[i] = pp[i].n[ij[i]++];
          while (ni[i] == pp[i].x[ij[i]])
30             siz[i] += pp[i].n[ij[i]++];
          }
          ni[i]++;
      }
}

35 if (++nn == olen || !more && nn) {
    dumpblock(),
```

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```

    for (i = 0, i < 2; i++)
        po[i] = out[i],
        nn = 0;
    }
5      }
    }

    /*
    * dump a block of lines, including numbers, stars pr_align()
10  */
    static
    dumpblock()
    {
        register      i,
15
        for (i = 0, i < 2; i++)
            *po[i]-- = '\0';

20
        (void) putc('\n', fx);
        for (i = 0; i < 2; i++) {
            if (*out[i] && (*out[i] != ' ' || *(po[i]) != ' ')) {
                if (i == 0)
25                    nums(i);
                if (i == 0 && *out[1])
                    stars(),
                    putline(i);
                if (i == 0 && *out[1])
30                    fprintf(fx, star),
                    if (i == 1)
                        nums(i),
                }
            }
35  }
}
```

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

        for (pn = nline, i = 0; i < lmax+P_SPC, i++, pn++)
            *pn = ' ',
15     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,
20             for (px = pn; j, j /= 10, px--)
                *px = j%10 + '0',
                if (i < 0)
                    *px = '-';
            }
25             else
                *pn = ' ';
            i++;
        }
    }
30     *pn = '\0';
    nc[ix] = i,
    for (pn = nline; *pn; pn++)
        (void) putc(*pn, fx),
        (void) putc('\n', fx);
35 }
```


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

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

    for (px = namex[ix], i = 0; *px && *px != ' ', px++, i++)
        (void) putc(*px, fx),
15    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
     */
20    for (px = out[ix]; *px; px++)
        (void) putc(*px&0x7F, fx);
    (void) putc('\n', fx),
25 }

/*
 * put a line of stars (seqs always in out[0], out[1]) dumpblock()
30 */
static
stars()
{
    int    i,
35    register char  *p0, *p1, cx, *px;
```

45

```

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

    for (p0 = out[0], p1 = out[1], *p0 && *p1, p0++, p1++) {
        if (isalpha(*p0) && isalpha(*p1)) {
10
            if (xbm[*p0-'A']&xbm[*p1-'A']) {
                cx = '*';
                nm++;
            }
15            else if (ldna && _day[*p0-'A'][*p1-'A'] > 0)
                cx = '.';
            else
                cx = ' ',
        }
20        else
            cx = ' ',
        *px++ = cx,
    }
    *px++ = '\n',
25    *px = '\0',
}

30

/*
* strip path or prefix from pn, return len' pr_align()
35 */
static

```

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stripname(pn)

stripname

```
char *pn; /* file name (may be path) */
{
    register char *px, *py,
5
    py = 0;
    for (px = pn, *px, px++)
        if (*px == '/')
            py = px + 1,
10
    if (py)
        (void) strcpy(pn, py),
        return(strlen(pn)),
    }
15
```

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

```
char *jname = "/tmp/homgXXXXXX", /* tmp file for jumps */
FILE *fj;

30 int cleanup(); /* cleanup tmp file */
long lseek(),
```

```
/*
 * remove any tmp file if we blow
35 */
cleanup(i)
```

cleanup

```

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

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

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

```

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```

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

5

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

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

30 char *
    g_calloc(msg, nx, sz)
        char *msg, /* program, calling routine */
        int nx, sz; /* number and size of elements */
35 {
    char *px, *calloc();

    ...getseq
    g_calloc

```

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```
        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].jmp; 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)),

```

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50

```
(void) read(fd, (char *)&dx[dmax] offset, sizeof(dx[dmax] offset));
dx[dmax] jmp = MAXJMP-1,
    }
    else
5         break;
    }
    if (i >= JMPS) {
        fprintf(stderr, "%s. too many gaps in alignment\n", prog),
        cleanup(1);
10    }
    if (j >= 0) {
        siz = dx[dmax].jp n[j],
        xx = dx[dmax].jp x[j],
        dmax += siz,
15        if (siz < 0) {            /* gap in second seq */
            pp[1].n[i1] = -siz;
            xx += siz;
            /* id = xx - yy + len1 - 1
            */
20            pp[1].x[i1] = xx - dmax + len1 - 1,
            gapy++,
            ngapy -= siz,
        /* ignore MAXGAP when doing endgaps */
            siz = (-siz < MAXGAP || endgaps)? -siz : MAXGAP;
25            i1++,
        }
        else if (siz > 0) {            /* gap in first seq */
            pp[0].n[i0] = siz,
            pp[0].x[i0] = xx;
30            gapx++;
            ngapx += siz,
        /* ignore MAXGAP when doing endgaps */
            siz = (siz < MAXGAP || endgaps)? siz : MAXGAP,
            i0++,
35        }
    }
}
```

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```

        else
            break;
    }

5    /* 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;
10    }
    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,
15    }
    if (fd >= 0)
        (void) close(fd);
    if (fj) {
        (void) unlink(jname);
        fj = 0;
20    offset = 0,
    }

/*
25    * write a filled jmp struct offset of the prev one (if any): nw()
    */
    writejmps(1x)
        int    ix;
    {
30        char    *mktemp();

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

writejmps
```


53
52

```

        if ((fj = fopen(jname, "w")) == 0) {
            fprintf(stderr, "%s: can't write %s\n", prog, jname),
            exit(1),
        }
5      }
      (void) fwrite((char *)&dx[ix] jp, sizeof(struct jmp), 1, fj),
      (void) fwrite((char *)&dx[ix] offset, sizeof(dx[ix] offset), 1, fj);

```

10 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.

25 "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, poly-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("amide")", 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-RFTISRDN SKNTLYLQMNSLRAEDTAVYYC (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-GVPSRFGSGSGTDFLTISLQPEDFATYYC (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. The 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 an 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

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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.*, *BioTechnology* 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

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ossificans, hypertrophic 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 mellitus); 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 cryoglobulinemia 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

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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

5 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
10 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
15 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),
20 chemotherapeutic agents e.g. methotrexate, adriamycin, 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
25 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® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such
30 as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide 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
35 (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopoletin, and 9-aminocamptothecin), bryostatin; calystatin, 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 CBI-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 ranimustine, 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 antibiotic chromophores), aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycins, 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, epitio stanol, mepitio stanol, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane, folic acid replenisher such as frolinic acid, aceglatone, aldophosphamide glycoside; aminolevulinic acid, enturacil, amsacrine, bestabucil; bisantrene, edatraxate, defofamine, demecolcine, diaziquone; elfornithine; elliptinium acetate, etoglucid, gallium nitrate, hydroxyurea, lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins, mitoguazone; mitoxantrone, mopidanmol; nitracrine, 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 doxorubicin (TAXOTERE®), chlorambucil, 6-thioguanine; mercaptopurine, methotrexate, platinum analogs such as cisplatin and

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carboplatin, vinblastine (VELBAN®), platinum, etoposide (VP-16), ifosfamide, mitoxantrone, vincristine (ONCOVIN®), oxaliplatin; leucovorin; 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 leucovorin

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 triptorelin; 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®), formestane, 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 aberrant 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 I 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.

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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 λ GEM 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.

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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
5 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

In one aspect of the invention, each cistron within the recombinant vector comprises a secretion signal sequence component that directs translocation of the expressed polypeptides across
10 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 invention 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
15 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 invention, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof

In another aspect, the production of the immunoglobulins according to the invention can
20 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
25 subunits. Proba and Pluckthun Gene, 159:203 (1995)

The present invention provides 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 invention. 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
30 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
35 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 Archaeobacteria 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 marcescens, 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 Δ fhvA (Δ tonA) ptr3 lacIq lacL8 Δ ompTA(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 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

5 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.

10 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.

15 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.

20 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.

25 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.

30 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

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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.

In one aspect of the invention, 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 OD₅₅₀ 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 invention, 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, Ane 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 invention. 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 invention.

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

In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the full length antibody products of the invention. Protein A is a 41kD cell wall protein from Staphylococcus aureus 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

5 (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

10 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

15 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

20 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

25 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).

30 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

35 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 all eukaryotic 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

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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 $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark et al., J. Immunol. Meth. 62 1-13 (1983)). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss et al., EMBO J. 5 1567-1575 (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(styrene-divinyl)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 immunosorbent 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)

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Nature 332 323-327, Verhoeven 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.

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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.

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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

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In one aspect, the invention provides 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

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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. (eds), 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 10:1025-1023; 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.

5 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 ¹¹¹In or ⁹⁰Y 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, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ²¹²Bi, ¹³¹I, ¹³¹In, ⁹⁰Y, and ¹⁸⁶Re. 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),

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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
5 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
10 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.

15 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,
20 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
25 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 Lnu et al., Proc. Natl. Acad. Sci. USA 93 8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal antibody
30 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. Chan 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
35 HER-2/neu oncogene The cytotoxicity of the TA.1-maytansinoid conjugate was tested in vitro on the human breast cancer cell line SK-BR-3, which expresses 3×10^5 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

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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
5 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 (Hinman et al., Cancer Research 53:3336-3342 (1993), Lode et al., Cancer Research
10 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
15 enhances their cytotoxic effects.

Other cytotoxic agents

Other antitumor agents that can be conjugated to the antibodies of the invention include BCNU, streptozotocin, 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.
20 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, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia
25 inhibitor, curcumin, crotin, saponaria 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
30 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
35 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 ^{99m}Tc or ^{123}I , ^{186}Re , ^{188}Re and ^{111}In 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.

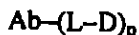
Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such 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 Vitella et al, Science 238 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminopentaacetic 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



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5 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
10 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,
15 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 substituents on the
20 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 galactose oxidase or
25 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
30 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 as 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, propylene glycol homopolymers, polypropylene 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

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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; 5 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 dextrans, 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 10 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.

15 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-(methylmethacrylate) 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 20 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 25 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 30 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 35 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

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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.

5 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
10 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
15 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
20 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.

25 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
30 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
35 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.

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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 invention 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 $\mu\text{g/kg}$ to 15 mg/kg (e.g. 0.1 mg/kg -10 mg/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 $\mu\text{g/kg}$ 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 0.05 mg/kg to about 10 mg/kg . Thus, one or more doses of about 0.5 mg/kg , 2.0 mg/kg , 4.0 mg/kg or 10 mg/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

In another aspect of the invention, 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 comprises 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

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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 (BWFJ), 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 CaCl₂) for 15 min at room temperature, after which ¹²⁵I-labeled proHGF in HNC buffer was added and incubated for 4 hrs at 37°C. The reactant concentrations in the final mixture were as follows: 2 nM HGFA, 0.05 mg/ml ¹²⁵I-labeled proHGF, 0.1 mg/ml anti-HGFA antibodies, 1 μM sHAI-1B. After 4 hrs aliquots were removed and added to sample buffer (Bio-Rad Laboratories, Hercules, CA) with reducing agent dithiothreitol (BIO-Rad). After a brief heating, samples (approx. 10⁶ cpm/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)

BLAcore experiments

5 Binding affinities of anti-HGFA antibodies to HGFA were determined by surface plasmon resonance measurements on a BLAcore 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
10 these surfaces by injection of a series of solutions ranging from 1 μ M 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 Langmuir binding model using software supplied by the manufacturer. In competition experiments, HGFA (70 nM) was preincubated with various concentrations of sHA1-1B (4nM-300nM) or IV-49C (11nM-300nM)
15 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 sHA1-1B were incubated with HGFA (final concentration 5nM) in HBSA
20 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 μ M final conc, $K_M = 200 \mu$ M) 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/v_0) of uninhibited activity.

25 **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
30 were coated with 5 μ g/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
35 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

5 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 $1\mu\text{M}$ sHAI-1B (Fig 2A) consistent with previous reports (1). With the exception of antibody #49 (Fig. 1), all tested
10 anti-HGFA antibodies at the tested concentration of $0.67\mu\text{M}$ significantly inhibited proHGF conversion (Fig 2). Additional experiments showed that #58 inhibited proHGF conversion at concentrations as low as $0.03\mu\text{M}$ (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 500 nM (Fig. 8). Furthermore, in
15 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 $\text{IC}_{50} > 500\text{ nM}$ (Fig 8). The 3 antibodies #42, #61, and #74 also showed relatively weak inhibition ($\text{IC}_{50} > 500\text{ nM}$) despite almost complete inhibition of proHGF conversion at $0.67\mu\text{M}$ (Fig. 8). Interestingly, antibody #75 displayed unusual inhibition kinetics in that its
20 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
25 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
30 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 $0.079\mu\text{M}$, whereas the small molecule HGFA active site binder inhibited with an
35 IC_{50} of $0.8\mu\text{M}$ ($K_i = 0.4\mu\text{M}$) 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 allesteric 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)
- 2 The antibody of claim 1, wherein the antibody binds to an active site of HGFA
- 5 3. The antibody of claim 1, wherein the antibody binds to HGFA at a position other than an active site of HGFA
- 4 The antibody of claim 1 which is a blocking antibody
5. The antibody of claim 4 which blocks HGFA proteolytic activity
- 6 The antibody of claim 5 which blocks HGFA proteolysis of single chain HGF
- 10 7 The antibody of claim 1 which inhibits HGF/c-met signaling.
8. The antibody of claim 7 which inhibits cell proliferation.
- 9 The antibody of claim 7 which inhibits angiogenesis
- 10 The antibody of claim 1, wherein the antibody is an allosteric inhibitor of HGFA enzymatic activity.
- 15 11. The antibody of claim 1, wherein the antibody is a complete or partial competitive inhibitor of HGFA enzymatic activity.
- 12 A method 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 that binds HGFA in accordance with any of the preceding claims.
- 20 13. The method of claim 10 wherein the disease is cancer
- 14 The method of claim 10 wherein the disease is associated with dysregulation of angiogenesis
- 15 The method of claim 10 wherein the disease is immune related.
16. An isolated antibody that specifically binds human hepatocyte growth factor activator, wherein the antibody comprises a heavy chain CDR sequence comprising the respective H1, H2 and/or H3 sequences depicted in Figure 1A
- 25 17. The antibody of claim 16, wherein the H1, H2 and/or H3 sequences are as depicted in Figures 1B, 1C and/or 1D.

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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	

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FIG. 1A

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Clone #	H1											
	26	27	28	29	30	31	32	33	34	35	SEQ ID NO:	
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

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Clone #	H3																	
	93	94	95	96	97	98	99	100	a	b	c	d	e	f	101	102	SEQ ID NO:	
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

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FIG. 1E

Framework Sequences of Light Chain

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

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

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

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

Framework Sequences of Heavy Chain

	1	Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu	
HC-FR1		Arg Leu Ser Cys Ala Ala Ser (SEQ ID NO: 67)	25
	36		48
HC-FR2	Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val	(SEQ ID NO: 68)	
	66		83
HC-FR3	Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln Met Asn		
	83a 83b 83c	Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys (SEQ ID NO: 69)	92
	103		113
HC-FR4	Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser	(SEQ ID NO: 70)	

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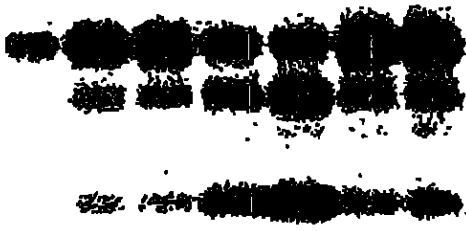
FIG. 1F

2

103
102

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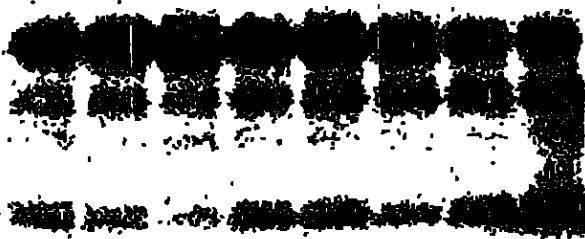
1 2 3 4 5 6 7 8 9



— Pro-HGF
— HGF-α
— HGF-β

FIG. 2A

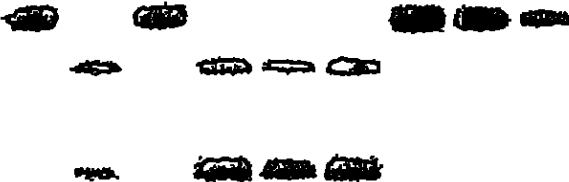
1 2 3 4 5 6 7 8



— Pro-HGF
— HGF-α
— HGF-β

FIG. 2B

1 2 3 4 5 6 7 8 9



— Pro-HGF
— HGF-α
— HGF-β

FIG. 3

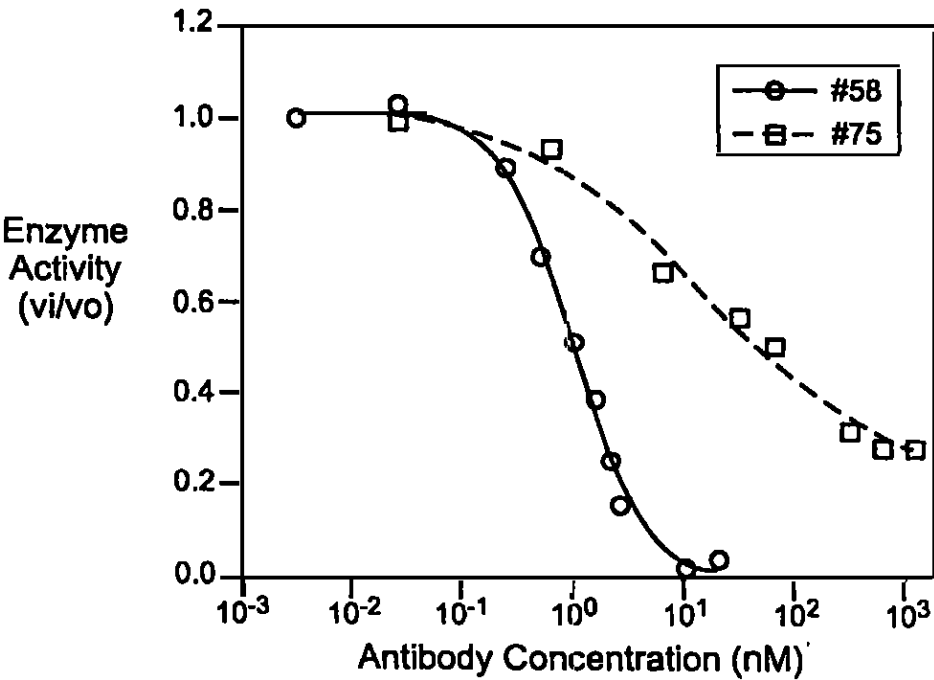


FIG. 4

COT
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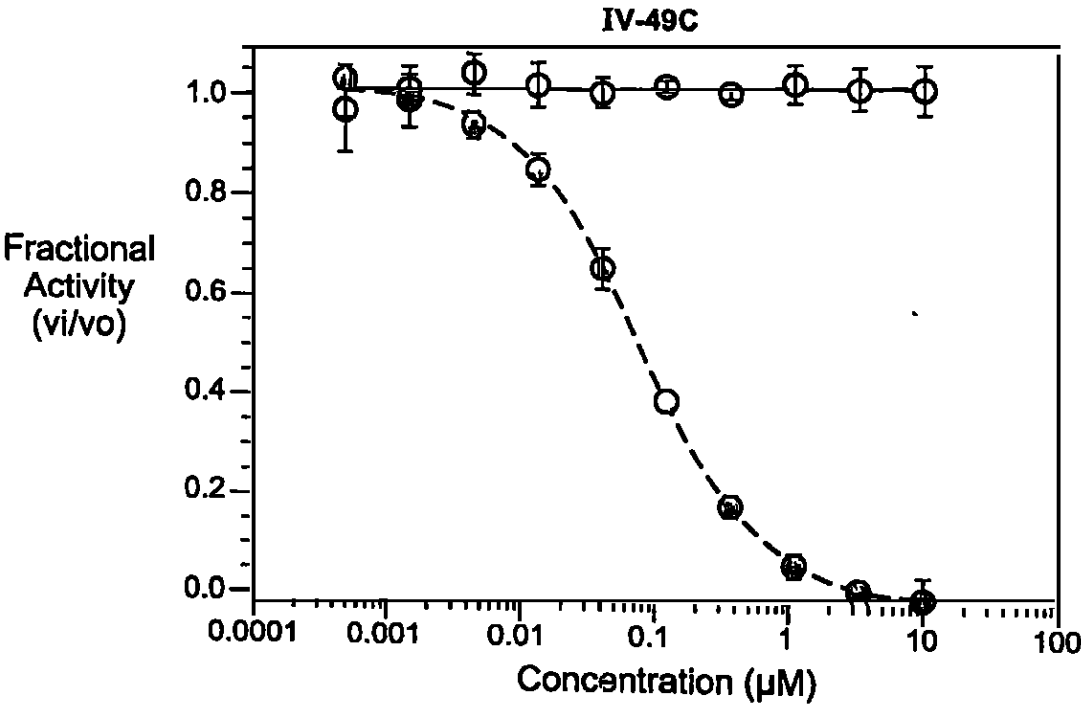


FIG. 5A

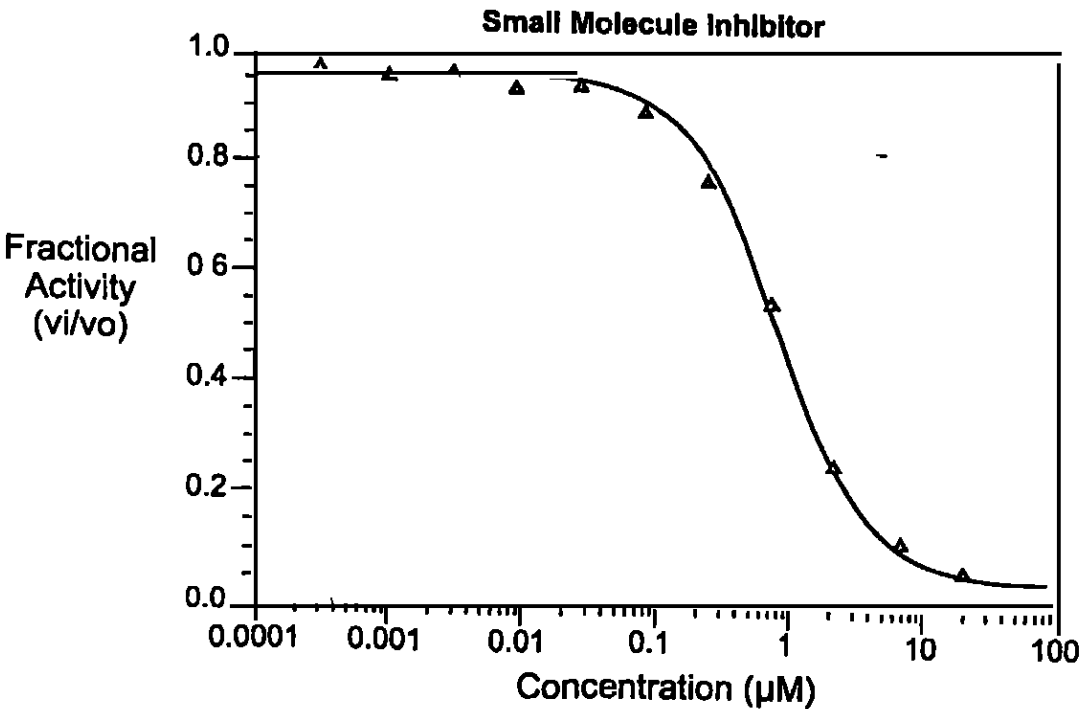


FIG. 5B

105

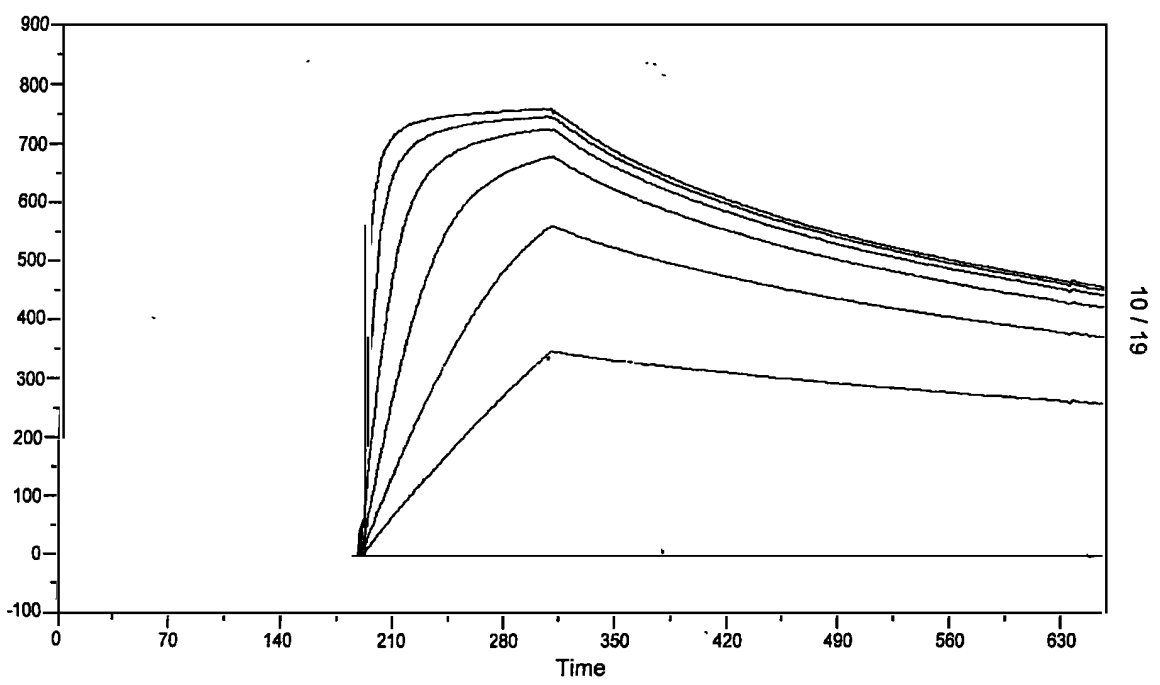


FIG. 6A

[Handwritten signature]

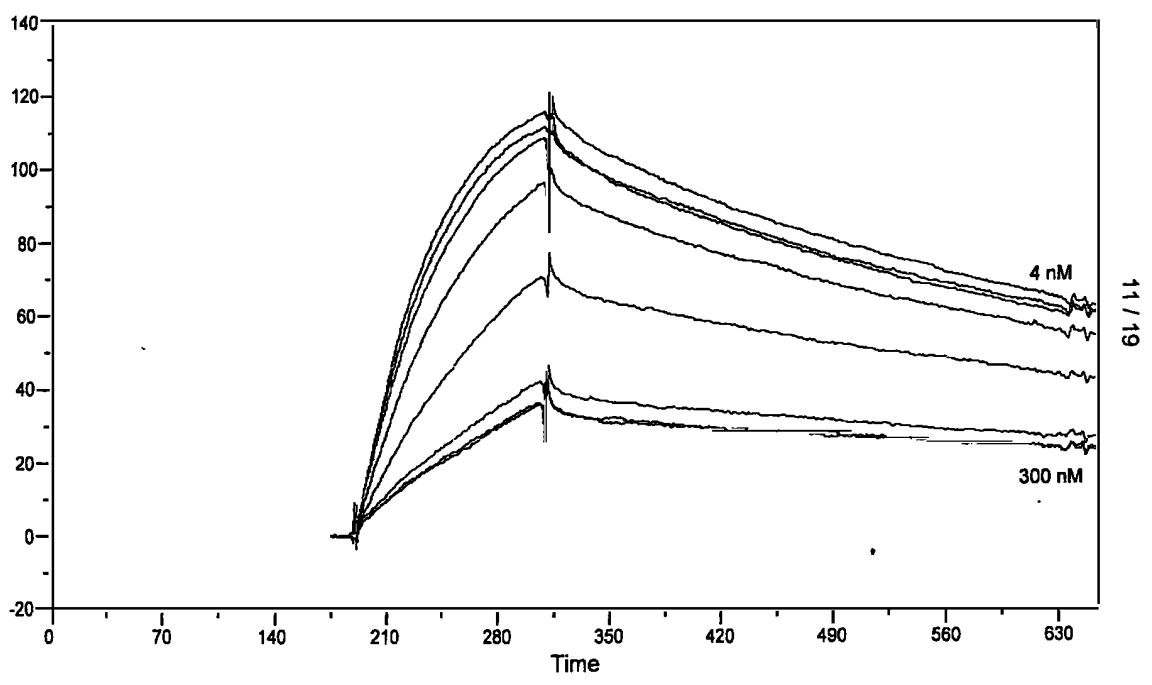


FIG. 6B

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[Handwritten signature]

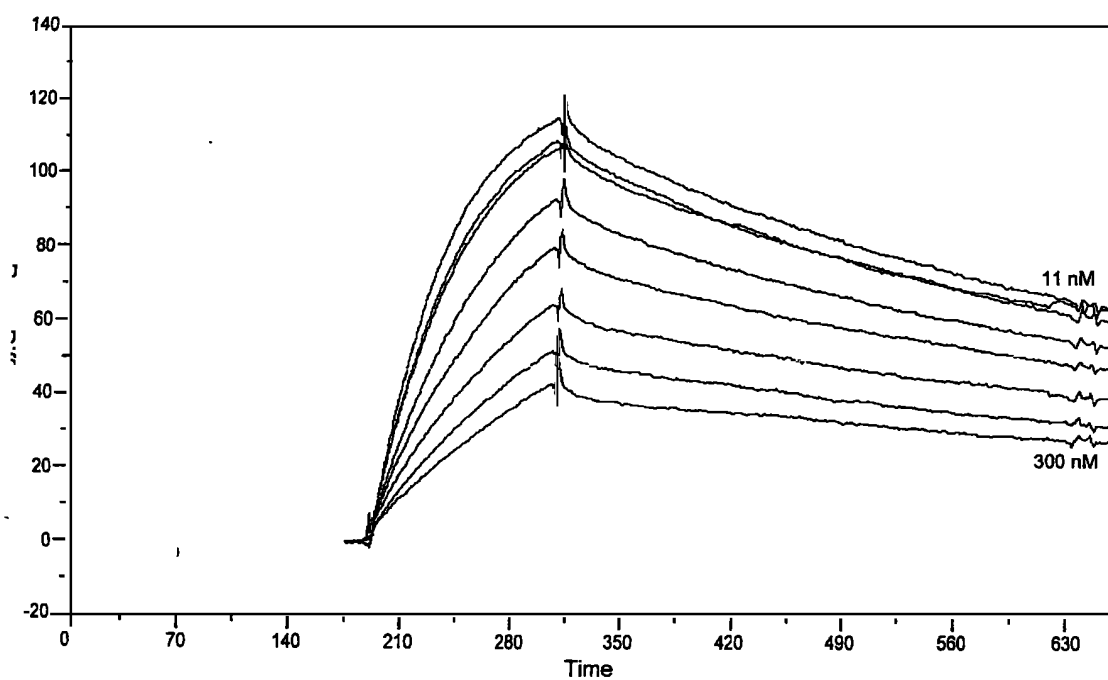


FIG. 6C

108

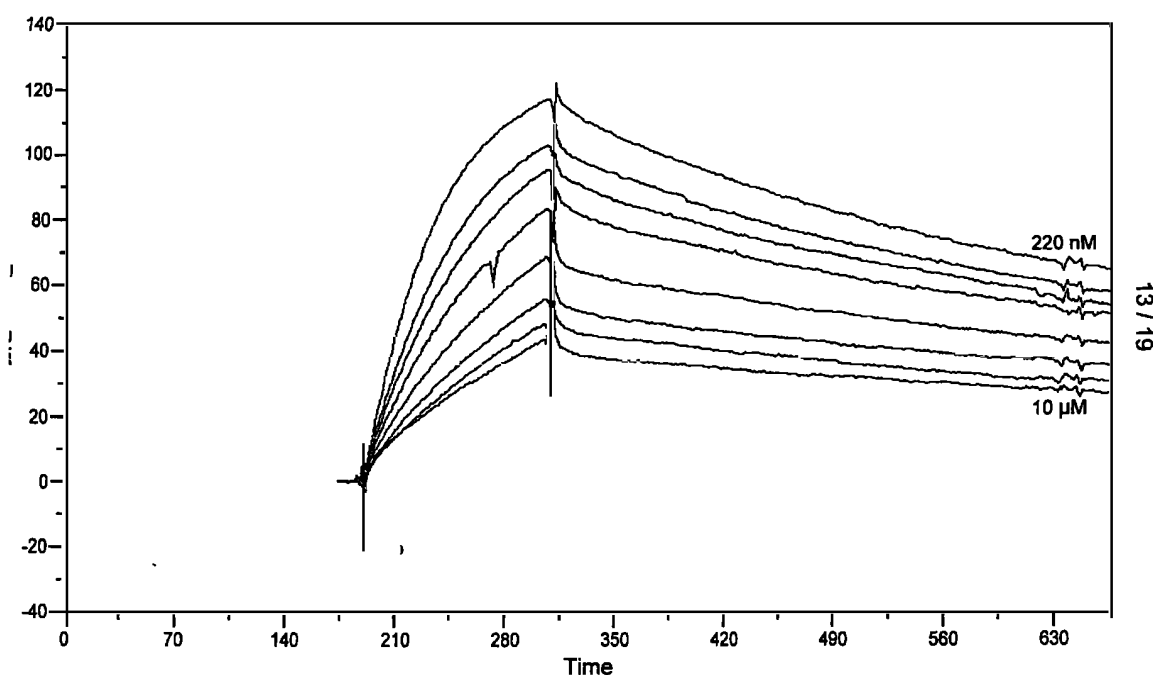


FIG. 6D

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[Handwritten signature]

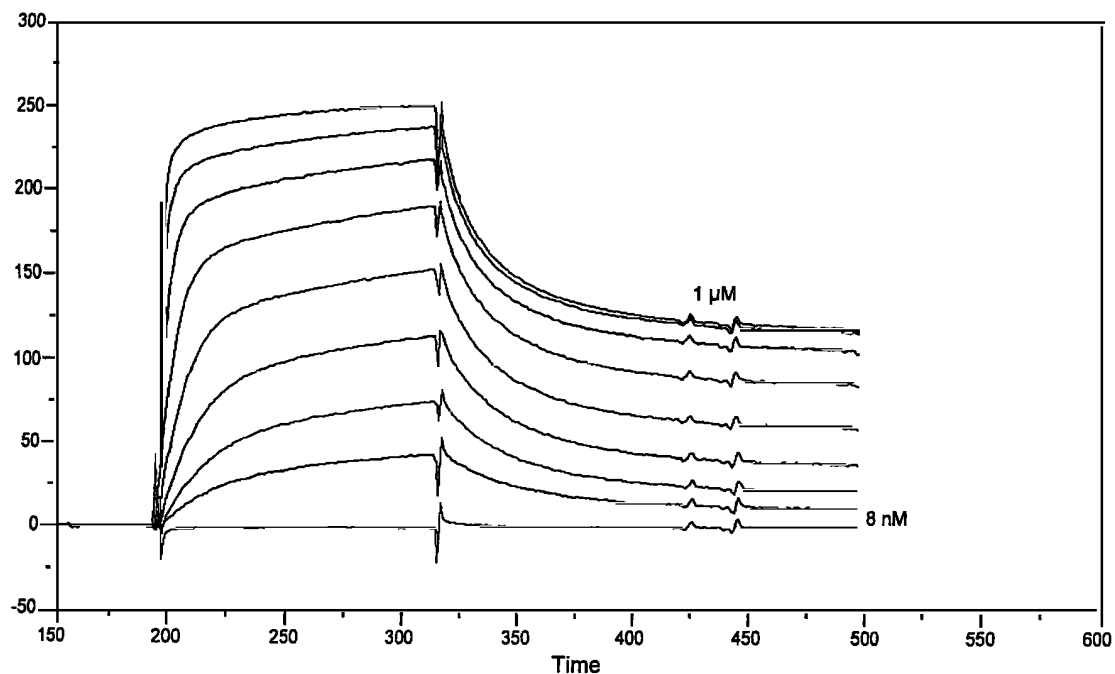


FIG. 6E

110

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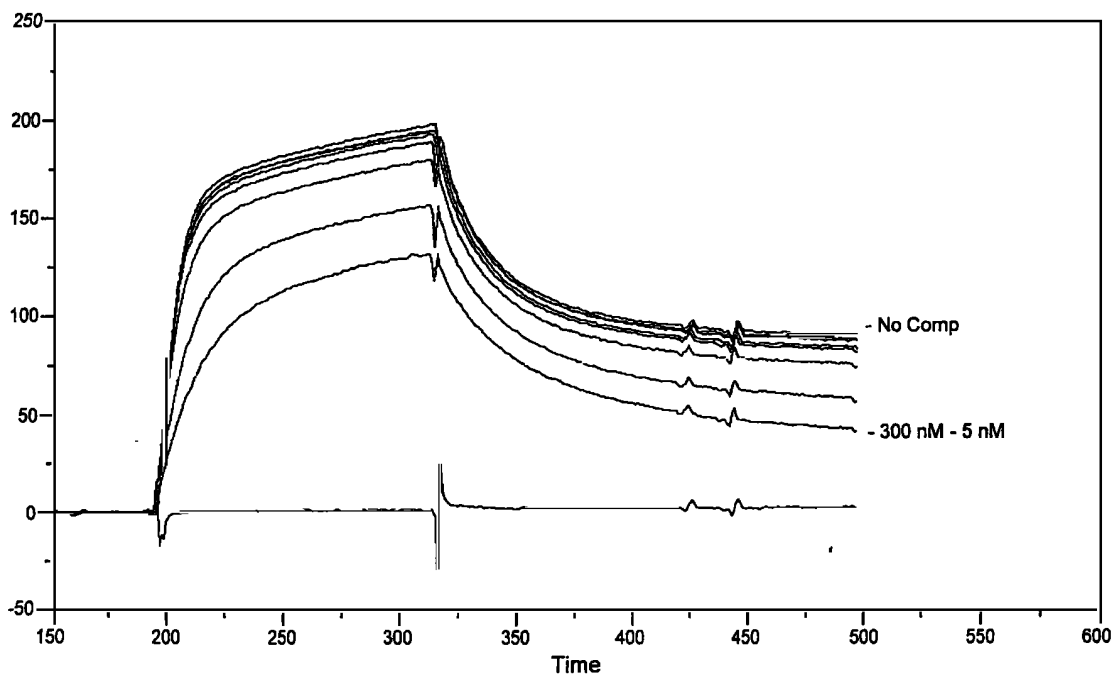


FIG. 6F

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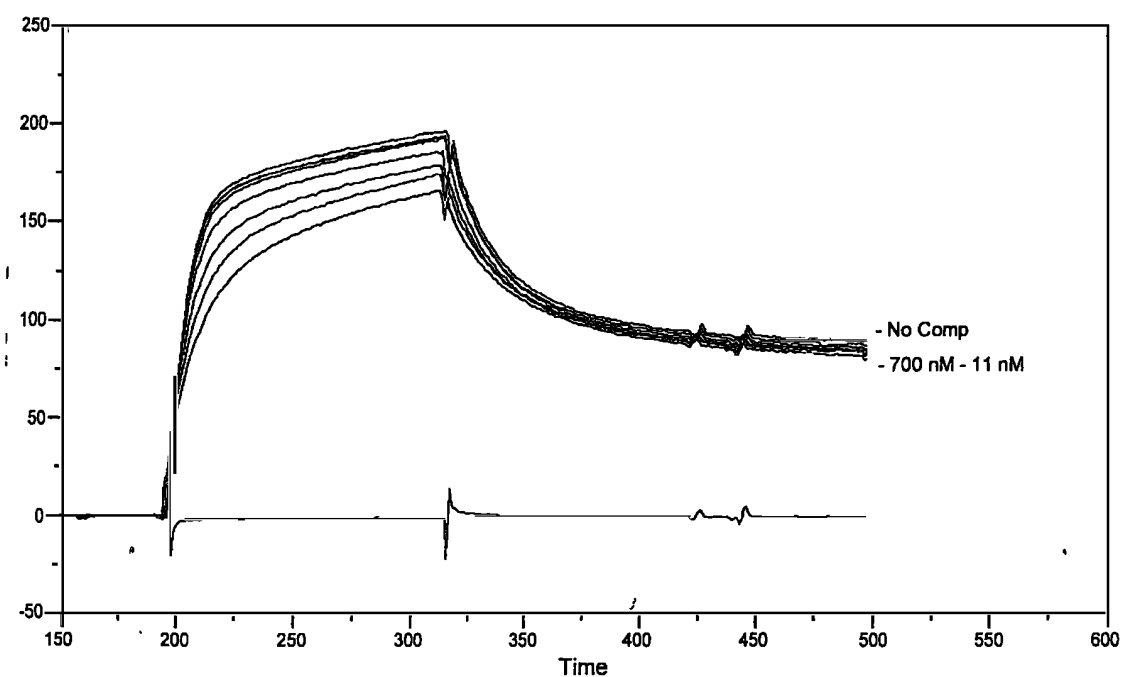


FIG. 6G

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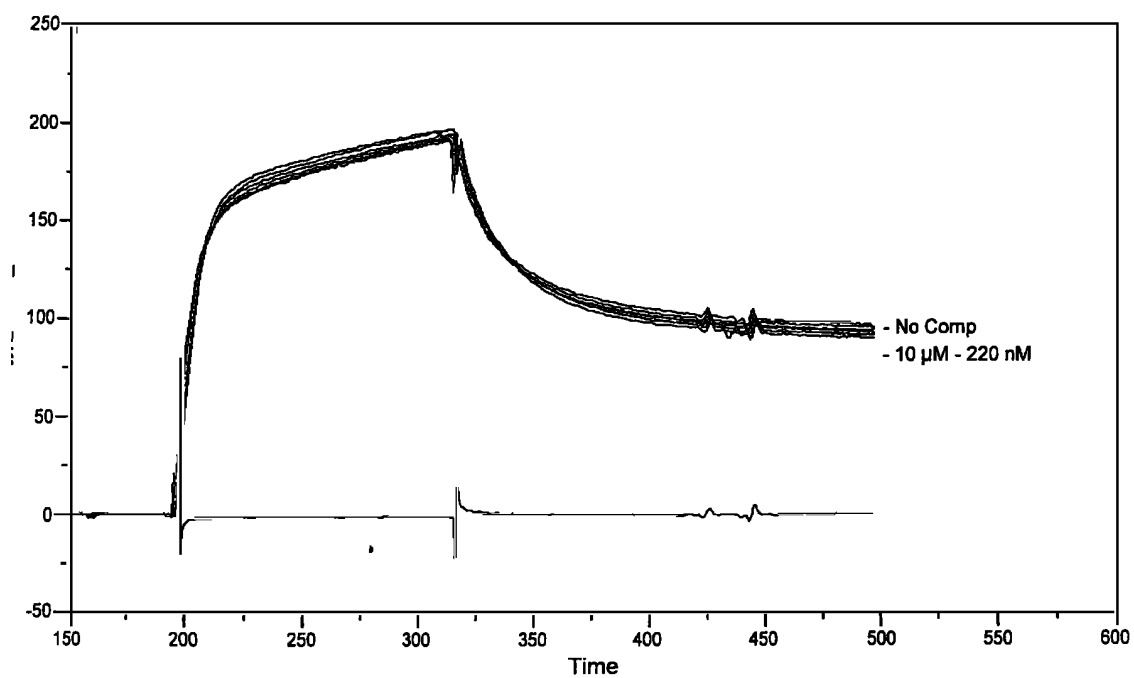


FIG. 6H

huHGFA	1	MGRWAWVPSPWPFPGLGPFLLLLLLLLLFLFRGFQPPQPGGNRTESPENAT	50
muHGFA	1	MGRQAWISSLCPLPFRPCPF-LLLLLLLVLVFRGAQPQAGRNHTEPFGPNVT	49
huHGFA	51	ATPAIPTTILVITGS- -VTSETPTATSAPEAEGPQSGGLPPPRAVPSSSSSPQA	98
muHGFA	50	ATPVTPPTIFVITSGNVISTSTESAPAAETEGPQSERYPSPS- - -SSSSPPGG	95
huHGFA	99	QALTEDGRPCRFPFRYGGRLHSACTSEGSAYHRKWCATTNNYDRDRAWGYC	148
muHGFA	96	QVLTESGQPCRFPFRYGGRLHSACTSEGSAYHRKWCATTNNYDRDRAWGYC	145
huHGFA	149	VEATPTPPGGGPAALDPCASGFCLNGGSCCSNTQDPQSYHCSCPRAFATGKDCG	198
muHGFA	146	AEVTLPEVEGPAILDPCASWFCLNGGTCCSSTHDHGSYHCSCPLAFATGKDCG	195
huHGFA	199	TEKCFDETRYEYLEEGGDRWARVRQGHVEQCECFGGRTWCETGTRHTACLSS	248
muHGFA	196	TEKCFDETRYEYFEVGDHWARVSEGHVEQCGCMEGQARCEDTGHHTACLSS	245
huHGFA	249	PCLNGGTCHLIVATGTTVCACPLPGFVAGRLCNIEPDEHCFGLNGTGYRGVA	298
muHGFA	246	PCLNGGTCHLIVGTGTSVCTCPLGFAAGRCFNIEPTEHCFLNGTGYRGVA	295
huHGFA	299	STSAAGLSCLAWNSDLLYQELHVDVSVAAGAAALLGLGPHAYCRNPDNDERPW	348
muHGFA	296	STAAAGLSCLAWNSDLLYQELHVDVSVAAGAAVLLGLGPHAYCRNPDKNDERPW	345
huHGFA	349	CYVVKDSALSWEYCRLEACESLTHRVQ-LSPLDLAATLPEPAPASPGRQAACGRR	397
muHGFA	346	CYVVKDNALSWEYCRLEACESLARVHSQTPEILAAALPEPAPAFVRPTCGKR	395
huHGFA	398	HKKRTFLRPRIIGSSSLPGSHPWLAATYIGD-SFCAGSLVHTCWVVSAAH	447
muHGFA	396	HKKRTFLRPRIIGSSSLPGSHPWLAATYIGD-SFCAGSLVHTCWVVSAAH	445
huHGFA	448	CFSSHSPPRDSVSVVLGQHFFNRTTDDVTQTFGIEKYIPTYLTVSVFNPSDHD	497
muHGFA	446	CFIANSPPRDSITTVVLGQHFFNRTTDDVTQTFGIEKYVPTYLTVSVFNPNNDHD	495
huHGFA	498	LVLIRLKKKGDERCAVTRSQFVQPICLPEPGSTTFPA GHKQCQIAGWGHLDENV	547
muHGFA	496	LVLIRLKKKGDERCAVTRSQFVQPICLPEAGSSTFPTGHKQCQIAGWGHMDENV	545
huHGFA	548	SGYSLSLEALVPLVADHKCSSPEVYGADISPMLCAGFYDCKSDACQGD	597
muHGFA	546	SSYSNLSLEALVPLVADHKCSSPEVYGADISPMLCAGFYDCKSDACQGD	595
huHGFA	598	SGGPLACEKNGVAYLYGIISWGDGCGRLHKPGVYTRVANYVDWINDRIRP	647
muHGFA	596	SGGPLVCEKNGVAYLYGIISWGDGCGRLNKPVGUYTRVANYVDWINDRIRP	645
huHGFA	648	PRRLVAPSS 655	
muHGFA	646	PKRPVATSS 653	

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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	*

^aSubstrate was Spectrozyme® fVIIa at 0.2mM (~K_M)

^bQualitative 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	58	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

^aND, Not Determined

FIG. 9

(12) SOLICITUD INTERNACIONAL PUBLICADA SEGÚN EL TRATADO DE COOPERACIÓN INTERNACIONAL EN MATERIA DE PATENTES (PCT)

(19) rganización Mundial de Propiedad
Intelectual
Oficina Internacional



(43) Fecha de Publicación Internacional
20 de abril de 2006 (20.04.2006)

PCT

(10) Número de Publicación Internacional
WO 2006/042173 A3

(51) Clasificación Internacional de Patentes⁷
C07K 16/40 (2006 01) A61K 39/395 (2006 01)
A61P 35/00 (2006 01) C12N 9/64 (2006 01)

(21) No. Solicitud Internacional: PCT/US2005/036300

(22) Fecha de Presentación Internacional:
3 de octubre de 2005 (03 10 2005)

(25) Idioma de Presentación: Inglés

(26) Idioma de Publicación: Inglés

(30) Datos relativos a la Prioridad:
60/615,657 4 de octubre de 2004 (04 10 2004) US

(71) Solicitante (para todos los países destinatarios con excepción de US) GENENTECH, INC. [US/US], 1 DNA Way, South San Francisco, California 94080-4990 (US)

AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW

(84) Países designados (a menos que se indique lo contrario para cada clase de protección disponible) ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasiática (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Europea (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

Publicado:
— con informe internacional de búsqueda
— antes del vencimiento del tiempo límite para modificar las reivindicaciones y para volverse a publicar en el caso de recibo de modificaciones

(88) Fecha de publicación del informe de búsqueda internacional: 17 de agosto de 2006

Para códigos de dos letras y otras abreviaturas, remítase a las "Notas Guía sobre Códigos y Abreviaturas" que aparece al comienzo de cada edición regular del Boletín Oficial PCT

(54) Título: MODULADORES DE ACTIVADOR DE FACTOR DE CRECIMIENTO HEPATOCÍTICO

Clen #	Sib#	H1				H2				H3												Affinity									
		30	31	32	33	49	50	51	52	53A	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	M	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	Q	W	G							F	D	Y	58 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	P	Q	P	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	P	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	Q	L	X	V	P	P	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	P	P	V	A						F	D	Y	4 nM	

(57) Extracto: La invención provee métodos y composiciones para modular la función del activador del factor de crecimiento hepatocítico

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PATENTE

Expediente P2179R1

Correo expreso No. EV385655937 US

Fecha de entrega al correo: 3 de octubre de 2005

MODULADORES DE ACTIVADOR DE FACTOR DE CRECIMIENTO HEPATOCÍTICO

SOLICITUDES RELACIONADAS

Esta solicitud es una solicitud no provisional presentada bajo 37 CFR 1.53(b)(1), que reivindica prioridad bajo 35 USC 119(e) a la solicitud provisional número 60/616,104 presentada el 4 de octubre de 2004, cuyos contenidos se incorporan aquí por referencia.

CAMPO TÉCNICO

La presente invención se refiere generalmente a los campos de la biología molecular y a la regulación del factor de crecimiento. Más específicamente, la invención concierne a moduladores de la función del activador del factor de crecimiento hepatocítico, y a los usos de dichos moduladores.

ANTECEDENTES

El factor de crecimiento hepatocítico (HGF) promueve la proliferación celular, la migración, la angiogénesis, la supervivencia y la morfogénesis activando la tirosina cinasa del receptor Met (reseñado en 8, 9). Además de su importancia en la fisiología normal, la vía HGF/Met ha estado implicada en crecimiento tumoral invasivo y metástasis tumoral (8). HGF posee una alta similitud con el plasminógeno de serina proteasa y se compone de una cadena α que contiene un dominio N y cuatro dominios de Kringle y una cadena β con homología con proteasas tipo quimiotripsina. Se segrega en la matriz extracelular como un precursor inactivo de cadena única (proHGF) y requiere corte proteolítico de activación en Arg494 -

Val495 para formar el heterodimero α/β enlazado a disulfuro, biológicamente competente (10-13). Este paso es mediado por serina proteasas convertidoras de proHGF, tales como el activador del factor de crecimiento hepatocítico (HGFA) (14). El HGFA es inhibido por inhibidores tipo Kunitz expresados en la superficie celular, tales como las dos variantes de empalme del inhibidor del factor de crecimiento hepatocítico HAM (16-17) y HAI-1B (15) y por HAI-2 (18). HAI-2 (también conocido como bicornina placentaria) (19) inhibe también potentemente el factor XIa y la calicreína plasmática (20), mientras que HAI-1B posee poca o ninguna actividad inhibidora (15). Por lo tanto, la disponibilidad biológica del grupo proHGF en la matriz extracelular se regula por las actividades de las proHGF convertasas tales como HGFA y sus inhibidores.

Puesto que la activación de proHGF requiere corte proteolítico por una convertasa tal como HGFA, la modulación de la función HGFA y/o su interacción con su sustrato podría demostrar ser un enfoque terapéutico eficaz. En este aspecto, existe una clara necesidad de identificara agentes clínicamente relevantes capaces de modular la actividad de, y/o interactuar específicamente con, HGFA. La invención satisface esta necesidad y ofrece otras ventajas.

Todas las referencias citadas en esta solicitud, incluidas las solicitudes de patente y publicaciones, se incorporan por referencia en su totalidad.

DIVULGACIÓN DE LA INVENCION

La invención proporciona métodos, composiciones, kits y artículos de fabricación para modular la función del activador del factor de crecimiento hepatocítico (HGFA), modulando en esa forma los efectos fisiológicos de la actividad HGFA.

La modulación de la función HGFA puede efectuarse por el uso de anticuerpos como se describe en esta solicitud. La invención provee moléculas moduladoras susceptibles de usarse para modular la función

HGFA. En una incorporación, la función HGFA se modula a través de la inhibición de la actividad HGFA (por ejemplo, actividad proteolítica). Generalmente, las moléculas moduladoras comprenden un anticuerpo como se describe en esta solicitud. Las moléculas moduladoras son capaces de efectuar la modulación directamente (por ejemplo, uniéndose a HGFA e interfiriendo la actividad proteolítica HGFA) o indirectamente (por ejemplo, dirigiendo un agente activo a HGFA en un tejido o célula, en donde el agente activo es capaz de interferir la actividad proteolítica HGFA). En una incorporación, la invención provee una molécula antagonista que comprende un anticuerpo que se une a HGFA. En una incorporación, la unión del antagonista a HGFA interfiere la actividad proteolítica HGFA. En una incorporación, la unión del antagonista a HGFA interfiere la activación de HGF por HGFA. En una incorporación, el anticuerpo se une al sitio activo de HGFA. En una incorporación, el anticuerpo se une a HGFA en una posición distinta del sitio activo HGFA (por ejemplo, un exosite). En una incorporación, la unión del anticuerpo a HGFA en una posición distinta del sitio activo HGFA inhibe la interacción de HGFA con su molécula sustrato. En una incorporación, la unión del anticuerpo a HGFA en una posición distinta del sitio activo HGFA inhibe la actividad proteolítica HGFA.

En un aspecto, la invención provee antagonistas que alteran la ruta de señalización HGF/c-met. Por ejemplo, la invención provee una molécula que inhibe la segmentación HGFA de proHGF (por ejemplo, segmentación en la posición R494-V495). La molécula puede ejercer su función inhibidora en cualquier número de formas, incluidas, sin limitación, la unión a HGFA en su sitio activo y/o en un sitio distinto del sitio activo (por ejemplo, un exosite) de modo que se inhiba la segmentación HGFA de proHGF. La molécula puede unirse a HGFA en forma acomplejada o no acomplejada. Asimismo la molécula puede ejercer su función inhibidora interfiriendo uno o más de los aspectos del proceso de activación HGF. Por ejemplo, en una incorporación, una molécula antagonista de la invención se une a un complejo HGFA-proHGF de modo que se inhiba la segmentación

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de proHGF. En una incorporación, la unión de la molécula a proHGF o HGFA (individualmente o en complejo) inhibe la liberación de HGF subsiguiente a la segmentación por HGFA. En una incorporación, una molécula antagonista de la invención no inhibe la unión HGF a c-met. Por ejemplo, en una incorporación, una molécula antagonista de la invención no es un anticuerpo o fragmento del mismo que tiene capacidad de unión y/o inhibidora similar como el anticuerpo producido por la línea de células de hibridoma depositada bajo el American Type Culture Collection Accession Number ATCC HB-11894 (hibridoma 1A3.3.13) o HB-11895 (hibridoma 5D5.11.6). En una incorporación, una molécula antagonista de la invención inhibe las actividades biológicas asociadas con la activación de HGF/c-met.

En un aspecto, la invención provee un anticuerpo que comprende una región CDR-H1 que incluye la secuencia de SEQ ID NO:3,6,9,12,15,18,21, 24,27,30,33,36,39 ó 42. En un aspecto, la invención provee un anticuerpo que comprende una región CDR-H2 que incluye la secuencia de SEQ ID NO:4,7,10,13,16,19,22,25,28,31,34,37,40 ó 43. En un aspecto, la invención provee un anticuerpo que comprende una región CDR-H3 que incluye la secuencia de SEQ ID NO:5,8,11,14,17,20,23,26,29,32,35,38,41 ó 44. En una incorporación, la invención provee un anticuerpo que comprende una región CDR-H1 que incluye la secuencia de SEQ ID NO:3,6,9,12,15,18,21,24,27,30,33,36,39 ó 42, y una región CDR-H2 que incluye la secuencia de SEQ ID NO:4,7,10,13,16,19,22,25,28,31,34,37,40 ó 43. En una incorporación, la invención provee un anticuerpo que comprende una región CDR-H1 que incluye la secuencia de SEQ ID NO:3,6,9,12,15,18,21,24,27,30,33,36,39 ó 42, y una región CDR-H3 que incluye la secuencia de SEQ ID NO:5,8,11,14,17,20,23,26,29,32,35,38,41 ó 44. En una incorporación, la invención provee un anticuerpo que comprende una región CDR-H2 que incluye la secuencia de SEQ ID NO:4,7,10,13,16,19,22,25,28,31,34,37,40 ó 43, y una región CDR-H3 que incluye la secuencia de SEQ ID NO:5,8,11,14,17,20,23,26,29,32,35,38,41 ó 44. En una incorporación, la invención provee un anticuerpo que comprende una región CDR-H1 que incluye la secuencia de SEQ ID

NO:3,6,9,12,15,18,21,24,27,30,33,36,39 ó 42, una región CDR-H2 que incluye la secuencia de SEQ ID NO:4,7,10,13,16,19,22,25,28,31,34,37,40 ó 43, y una región CDR-H3 que incluye la secuencia de SEQ ID NO:5,8,11,14,17,20,23,26,29,32,35,38,41 ó 44.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO:3;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO:4;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO:5.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO:6;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO:7;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO:8.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO:9;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO:10;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO:11.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO:12;

- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 13;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO: 14.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO: 15;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 16;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO: 17.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO: 18;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 19;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO:20.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO:21;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO:22;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO:23.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO: 24;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 25;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO: 26.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO: 27;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 28;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO: 29.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO: 30;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 31;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO: 32.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO: 33;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 34;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO: 35.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

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- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO: 36;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 37;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO: 38.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO: 39;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 40;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO: 41.

En un aspecto, la invención provee un anticuerpo que comprende por lo menos una, por lo menos dos, o todas las tres de las siguientes:

- (i) una secuencia CDR-H1 que incluye la secuencia de SEQ ID NO: 42;
- (ii) una secuencia CDR-H2 que incluye la secuencia de SEQ ID NO: 43;
- (iii) una secuencia CDR-H3 que incluye la secuencia de SEQ ID NO: 44.

Las secuencias de aminoácidos de SEQ ID NOs:3-44 se numeran con respecto a la CDR individual (es decir, H1, H2 o H3) como se indica en la Figura 1, siendo consistente la numeración con el sistema de numeración de Kabat como se describe a continuación.

En una incorporación, un anticuerpo de la invención comprende una secuencia(s) CDR de dominio variable de cadena pesada, que incluye la secuencia de por lo menos una, por lo menos dos, o todas las tres de las secuencias H1 (SEQ ID NO: 71-84), H2 (SEQ ID NO: 85-98) y/o H3 (SEQ ID NO: 99-112) para cada clón descrito en las Figuras 1B, 1C y 1D.

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En un aspecto, la invención provee anticuerpos que comprenden secuencias CDR de cadena pesada como se describe en las Figuras 1A, B, C y D. En una incorporación, estos anticuerpos comprenden además un dominio variable de cadena ligera del anticuerpo 4D5 humanizado (huMAb4D5-8) (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (también mencionado en la Patente de los Estados Unidos No. 6,407,213 y Lee et al., J. Mol. Biol. (2004), 340(5): 1073-93) como se describe en SEQ ID NO:45 a continuación.

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)

En una incorporación, la secuencia de dominio variable de cadena ligera huMAb4D5-8 se modifica en una o más de las posiciones 30, 66 y 91 (Asn, Arg y His como se indica en negrilla/cursiva más arriba, respectivamente). En una incorporación, la secuencia huMAb4D5-8 modificada comprende Ser en la posición 30, Gly en la posición 66 y/o Ser en la posición 91. En consecuencia, en una incorporación, un anticuerpo de la invención comprende un dominio variable de cadena ligera que incluye la secuencia descrita en SEQ ID NO: 54 a continuación:

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)

Residuos sustituidos con respecto a huMAb4D5-8 se indican en negrita/cursiva más arriba. Los anticuerpos de la invención pueden comprender además cualesquiera secuencias de dominio variable de cadena

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y/o de región de estructura alterna apropiadas, siempre que se conserve sustancialmente la actividad de unión HGFA. Por ejemplo, en algunas incorporaciones, estos anticuerpos comprenden además una secuencia de consenso de estructura del subgrupo III humano. En una incorporación de estos anticuerpos, las secuencias de consenso de región de estructura alterna comprende una sustitución en la posición 71, 73 y/o 78. En algunas incorporaciones de estos anticuerpos, la posición 71 es A, 73 es T y/o 78 es A. En una incorporación, estos anticuerpos comprenden secuencias de región de estructura alterna de dominio variable de cadena pesada del anticuerpo 4D5 humanizado (huMAb 4D5-8) (HERCEPTIN[®], Genentech, Inc., South San Francisco, CA, USA) (también mencionado en la Patente de los Estados Unidos No. 6,407,213 y Lee et al., J. Mol. Biol. (2004), 340(5): 1073-93). En una incorporación, el anticuerpo 4D5-8 humanizado es como se describe en la Patente de los Estados Unidos No. 6,407,213. En una incorporación, estos anticuerpos comprenden además una secuencia de consenso de estructura de cadena ligera kl humana. En una incorporación, estos anticuerpos comprenden secuencias de dominio variable de cadena ligera de anticuerpo 4D5 humanizado (huMAb 4D5-8) (SEQ ID NO:45) (HERCEPTIN[®], Genentech, Inc., South San Francisco, CA, USA) (también mencionado en la Patente de los Estados Unidos 6,407,213 y Lee et al., J. Mol. Biol. (2004), 340(5): 1073-93), o la variante modificada de la misma como se describe en SEQ ID NO: 54.

En una incorporación, un anticuerpo de la invención comprende un dominio variable de cadena pesada, en donde la secuencia de región de estructura alterna comprende las secuencias de SEQ ID NO: 46, 47, 48 y 49 (FR1, 2, 3, y 4, respectivamente), y las secuencias CDR H1, H2 y H3 se describen en las Figuras 1A, B, C, y/o D. En una incorporación, un anticuerpo de la invención comprende un dominio variable de cadena ligera, en donde la secuencia de región de estructura alterna comprende la secuencia de SEQ ID NO: 50, 51, 52 y 53 (FR1, 2, 3, y 4, respectivamente), y las secuencias CDR L1, L2 y L3 como se describe en SEQ ID NO: 54.

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En una incorporación, un anticuerpo de la invención comprende un dominio variable de cadena pesada, en donde la secuencia de región de estructura alterna comprende la secuencia de SEQ ID NO: 59, 60, 61 y 62 (FR1, 2, 3 y 4, respectivamente) (FIG. 1E), y las secuencias CDR H1, H2 y H3 como se describe en la Figura 1. En una incorporación, un anticuerpo de la invención comprende un dominio variable de cadena ligera, en donde la secuencia de región de estructura alterna comprende la secuencia de SEQ ID NO: 55, 56, 57, y 58 (FR1, 2, 3 y 4, respectivamente) (FIG. 1E), y las secuencias CDR L1, L2 y L3 como se describe en SEQ ID NO: 54.

En una incorporación, un anticuerpo de la invención comprende un dominio variable de cadena pesada, en donde la secuencia de región de estructura alterna comprende la secuencia de SEQ ID NO: 67, 68, 69 y 70 (FR1, 2, 3 y 4, respectivamente) (FIG. 1F), y las secuencias CDR H1, H2 y H3 como se describe en FIG. 1A, B, C y/o D. En una incorporación, un anticuerpo de la invención comprende un dominio variable de cadena ligera, en donde la secuencia de región de estructura alterna comprende la secuencia de SEQ ID NO: 63, 64, 65, y 66 (FR1, 2, 3 y 4, respectivamente) (FIG. 1F), y las secuencias CDR L1, L2 y L3 como se describe en SEQ ID NO: 54.

En un aspecto, la invención provee un anticuerpo que compite con cualquiera de los anticuerpos ya mencionados para unión a HGFA. En un aspecto, la invención provee un anticuerpo que se une al mismo epítipo en HGFA como cualquiera de los anticuerpos ya mencionados. En una incorporación, un anticuerpo de la invención es madurado por afinidad, humanizado, quimérico o humano. En una incorporación, un anticuerpo de la invención es un fragmento de anticuerpo (como se describe en esta solicitud), o un anticuerpo de longitud sustancialmente completa. En una incorporación, un anticuerpo de la invención comprende una región Fc tipo natural, o una variante de la misma. En una incorporación, un anticuerpo de la invención es una IgG (por ejemplo, IgG1, IgG2, IgG3, IgG4), IgM, IgE o IgD.

En un aspecto, una molécula antagonista de la invención se enlaza a una toxina tal como un agente citotóxico. Estas moléculas/sustancias pueden formularse o administrarse en combinación con un aditivo/agente potenciador, tal como radiación y/o agente quimioterapéutico.

La invención provee asimismo métodos y composiciones útiles para modular estados patológicos asociados con la alteración de la regulación del eje de señalización de HGF/c-met. En consecuencia, en un aspecto, la invención provee un método de modular la activación de c-met en un sujeto, comprendiendo dicho método administrar al sujeto una molécula moduladora de la invención que inhibe la segmentación HGFA de proHGF, con lo cual se modula la activación de c-met. En un aspecto, la invención provee un método de tratar una condición patológica asociada con la activación de c-met en un sujeto, comprendiendo dicho método administrar a un sujeto una molécula moduladora de la invención que inhibe la segmentación HGFA de proHGF, con lo cual se inhibe la activación de c-met. En una incorporación, la molécula moduladora de la invención es un anticuerpo que se une a HGFA.

La ruta de señalización de HGF/c-met se encuentra involucrada en múltiples funciones biológicas y fisiológicas, incluidas, por ejemplo, la estimulación del crecimiento celular (por ejemplo, proliferación celular, supervivencia celular, migración celular, morfogénesis celular) y angiogénesis. En consecuencia, en otro aspecto, la invención provee un método de inhibir el crecimiento celular activado por c-met (por ejemplo, proliferación y/o supervivencia), comprendiendo dicho método contactar una célula o tejido con un antagonista de la invención, con lo cual se inhibe la proliferación celular asociada con la activación de c-met.

En todavía otro aspecto, la invención provee un método de inhibir angiogénesis, comprendiendo dicho método administrar a una célula, tejido, y/o sujeto con una condición asociada con angiogénesis anormal un antagonista de la invención, con lo cual se inhibe la angiogénesis. En un aspecto, la invención provee el uso de una molécula moduladora de la invención en la preparación de un medicamento para el tratamiento

terapéutico y/o profiláctico de una enfermedad, tal como cáncer, un tumor, un trastorno de proliferación celular, un trastorno inmunitario (tal como autoinmunitario) y/o un trastorno relacionado con angiogénesis.

En un aspecto, la invención provee el uso de un ácido nucleico de la invención en la preparación de un medicamento para el tratamiento terapéutico y/o profiláctico de una enfermedad, tal como cáncer, un tumor, un trastorno de proliferación celular, un trastorno inmunitario (tal como autoinmunitario) y/o un trastorno relacionado con angiogénesis.

En un aspecto, la invención provee el uso de un vector de expresión de la invención en la preparación de un medicamento para el tratamiento terapéutico y/o profiláctico de una enfermedad, tal como cáncer, un tumor, un trastorno de proliferación celular, un trastorno inmunitario (tal como autoinmunitario) y/o un trastorno relacionado con angiogénesis.

En un aspecto, la invención provee el uso de una célula hospedera de la invención en la preparación de un medicamento para el tratamiento terapéutico y/o profiláctico de una enfermedad, tal como cáncer, un tumor, un trastorno de proliferación celular, un trastorno inmunitario (tal como autoinmunitario) y/o un trastorno relacionado con angiogénesis.

En un aspecto, la invención provee el uso de un artículo de fabricación de la invención en la preparación de un medicamento para el tratamiento terapéutico y/o profiláctico de una enfermedad, tal como cáncer, un tumor, un trastorno de proliferación celular, un trastorno inmunitario (tal como autoinmunitario) y/o un trastorno relacionado con angiogénesis.

En un aspecto, la invención provee el uso de un kit de la invención en la preparación de un medicamento para el tratamiento terapéutico y/o profiláctico de una enfermedad, tal como cáncer, un tumor, un trastorno de proliferación celular, un trastorno inmunitario (tal como autoinmunitario) y/o un trastorno relacionado con angiogénesis.

En un aspecto, la invención provee un método de inhibir una proliferación celular activada por c-met, comprendiendo dicho método contactar una célula o tejido con una cantidad efectiva de una molécula

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moduladora de la invención, con lo cual se inhibe la proliferación celular asociada con la activación de c-met.

En un aspecto, la invención provee un método de tratar una condición patológica asociada con alteración de la regulación de la activación de c-met en un sujeto, comprendiendo dicho método administrar al sujeto una cantidad efectiva de una molécula moduladora de la invención, con lo cual se trata dicha condición.

En un aspecto, la invención provee un método de inhibir el crecimiento de una célula que expresa c-met o factor de crecimiento hepatocítico, o ambos, comprendiendo dicho método contactar dicha célula con una molécula moduladora de la invención, ocasionando así una inhibición del crecimiento de dicha célula. En una incorporación, la célula se contacta por HGF expresado por una célula diferente (por ejemplo, a través de un efecto paracrino).

En un aspecto, la invención provee un método de tratar terapéuticamente un mamífero con un tumor canceroso que comprende una célula que expresa c-met o factor de crecimiento hepatocítico, o ambos, comprendiendo dicho método administrar a dicho mamífero una cantidad efectiva de una molécula moduladora de la invención, tratando así efectivamente a dicho mamífero. En una incorporación, la célula contactada por HGF expresado por una célula diferente (por ejemplo, a través de un efecto paracrino).

En un aspecto, la invención provee un método para tratar o prevenir un trastorno de proliferación celular asociado con una expresión o actividad creciente de HGFA, comprendiendo dicho método administrar a un sujeto necesitado de dicho tratamiento una cantidad efectiva de una molécula moduladora de la invención, tratando o previniendo así efectivamente dicho trastorno de proliferación celular. En una incorporación, dicho trastorno de proliferación es cáncer.

En un aspecto, la invención provee un método para tratar o prevenir un trastorno de proliferación celular asociado con una expresión o actividad creciente de c-met o el factor de crecimiento hepatocítico, o ambos,

comprendiendo dicho método administrar a un sujeto necesitado de dicho tratamiento una cantidad efectiva de una molécula moduladora de la invención, tratando o previniendo así efectivamente dicho trastorno de proliferación celular. En una incorporación, dicho trastorno de proliferación es cáncer.

En un aspecto, la invención provee un método de inhibir el crecimiento de una célula, en donde el crecimiento de dicha célula es por lo menos en parte dependiente de un efecto potenciador de crecimiento de HGFA, comprendiendo dicho método contactar dicha célula con una cantidad efectiva de una molécula moduladora de la invención, inhibiendo así el crecimiento de dicha célula. En una incorporación, la célula se contacta por HGF expresado por una célula diferente (por ejemplo, a través de un efecto paracrino).

En un aspecto, la invención provee un método de inhibir el crecimiento de una célula, en donde el crecimiento de dicha célula es por lo menos en parte dependiente de un efecto potenciador de crecimiento de c-met o factor de crecimiento hepatocítico, o ambos, comprendiendo dicho método contactar dicha célula con una cantidad efectiva de una molécula moduladora de la invención, inhibiendo así el crecimiento de dicha célula. En una incorporación, la célula se contacta por HGF expresado por una célula diferente (por ejemplo, a través de un efecto paracrino).

En un aspecto, la invención provee un método de tratar terapéuticamente un tumor en un mamífero, en donde el crecimiento de dicho tumor es por lo menos en parte dependiente de un efecto potenciador de crecimiento de HGFA, comprendiendo dicho método contactar dicha célula con una cantidad efectiva de una molécula moduladora de la invención, tratando así efectivamente dicho tumor. En una incorporación, la célula se contacta por HGF expresado por una célula diferente (por ejemplo, a través de un efecto paracrino).

En un aspecto, la invención provee un método de tratar terapéuticamente un tumor en un mamífero, en donde el crecimiento de dicho tumor es por lo menos en parte dependiente de un efecto potenciador

de crecimiento de c-met o factor de crecimiento hepatocítico, o ambos, comprendiendo dicho método contactar dicha célula con una cantidad efectiva de una molécula moduladora de la invención, tratando así efectivamente dicho tumor. En una incorporación, la célula se contacta por HGF expresado por una célula diferente (por ejemplo, a través de un efecto paracrino).

Los métodos de la invención pueden usarse para afectar cualquier estado patológico apropiado, por ejemplo, células y/o tejidos asociados con alteración de la regulación de la ruta de señalización de HGF/c-met, por ejemplo a través de actividad HGF creciente, asociada con la activación HGFA de HGF. En una incorporación, una célula que se toma como diana en un método de la invención es una célula cancerosa. Por ejemplo, una célula cancerosa puede ser una seleccionada del grupo conformado por una célula de cáncer de mama, una célula de cáncer colorrectal, una célula de cáncer pulmonar, una célula de carcinoma papilar (por ejemplo, de la glándula tiroides), una célula de cáncer de colon, una célula de cáncer de páncreas, una célula de cáncer de ovarios, una célula de cáncer cervical, una célula de cáncer del sistema nervioso central, una célula de sarcoma osteógeno, una célula de carcinoma renal, una célula de carcinoma hepatocelular, una célula de cáncer de vejiga, una célula de cáncer próstata, una célula de carcinoma gástrico, una célula de carcinoma escamoso de cabeza y cuello, una célula de melanoma y una célula de leucemia. En una incorporación, una célula que se toma como diana en un método de la invención es una célula hiperproliferativa y/o hiperplásica.

En una incorporación, una célula que se toma como diana en un método de la invención es una célula displásica. En todavía otra incorporación, una célula que se toma como diana en un método de la invención es una célula metastásica.

Los métodos de la invención pueden comprender asimismo pasos adicionales de tratamiento. Por ejemplo, en una incorporación, un método comprende además un paso en donde una célula tomada como diana y/o

tejido (por ejemplo, una célula cancerosa) se expone a tratamiento de radiación o a un agente quimioterapéutico.

Como se describe en esta solicitud, la activación de HGF/c-met es un proceso biológico importante cuya alteración de la regulación lleva a numerosas condiciones patológicas. En consecuencia, en una incorporación de los métodos de la invención, una célula que se toma como diana (por ejemplo, una célula cancerosa) es una en la cual se potencia la activación de HGF/c-met, comparada con una célula normal del mismo origen tisular. En una incorporación, un método de la invención causa la muerte de una célula tomada como diana. Por ejemplo, el contacto con una molécula moduladora de la invención puede resultar en la incapacidad de una célula de señalizar a través de la ruta c-met, lo cual resulta en muerte celular.

La alteración de la regulación de la activación de c-met (y, por lo tanto, la señalización) puede resultar de un número de cambios celulares, incluidos, por ejemplo, la sobreexpresión de HGF (ligando afin de c-met) y/o HGFA, y/o activación creciente de HGF por HGFA. En consecuencia, en algunas incorporaciones, un método de la invención comprende tomar como diana ("*targeting*") un tejido en donde uno o más de HGFA, c-met y factor de crecimiento hepatocítico, se expresa más abundantemente y/o está presente (por ejemplo, un cáncer), comparado con un tejido normal del mismo origen. Un HGF o una célula que expresa c-met pueden regularse por HGFA de una variedad de fuentes, es decir en una manera autocrina o paracrina. Por ejemplo, en una incorporación de métodos de la invención, una célula tomada como diana se contacta con/une un factor de crecimiento hepatocítico activado por HGFA expresado en una célula diferente (por ejemplo, mediante un efecto paracrino). Dicha célula diferente puede ser del mismo origen tisular o diferente. En una incorporación, una célula tomada como diana se contacta/une por HGF activado por HGFA expresado por la misma célula tomada como diana (por ejemplo, mediante un efecto/lazo autocrino).

En un aspecto, la invención provee composiciones que comprenden una o más moléculas moduladoras de la invención y un vehículo. En una incorporación, el vehículo es farmacéuticamente aceptable.

En un aspecto, la invención provee ácidos nucleicos que codifican una molécula moduladora de la invención. En una incorporación, un ácido nucleico de la invención codifica una molécula moduladora que es o comprende un anticuerpo o fragmento del mismo.

En un aspecto, la invención provee vectores que comprenden un ácido nucleico de la invención.

En un aspecto, la invención provee células hospederas que comprenden un ácido nucleico o un vector de la invención. Un vector puede ser de cualquier tipo, por ejemplo un vector recombinante tal como un vector de expresión. Puede usarse cualquiera de una variedad de células hospederas. En una incorporación, una célula hospedera es una célula procariota, por ejemplo, *E. coli*. En una incorporación, una célula hospedera es una célula eucariota, por ejemplo una célula de mamífero tal como una célula de Ovario de Hámster de la China (CHO).

En un aspecto, la invención provee métodos para elaborar una molécula moduladora de la invención. Por ejemplo, la invención provee un método de elaborar una molécula moduladora, que es o comprende un anticuerpo (o fragmento del mismo), comprendiendo dicho método expresar en una célula hospedera apropiada un vector recombinante de la invención, que codifica dicho anticuerpo (o fragmento del mismo), y recuperar dicho anticuerpo.

En un aspecto, la invención provee un artículo de fabricación que comprende un envase; y una composición contenida en el envase, en donde la composición incluye una o más moléculas moduladoras de la invención. En una incorporación, la composición comprende un ácido nucleico de la invención. En una incorporación, una composición que comprende una molécula moduladora incluye además un vehículo, el cual en algunas incorporaciones es farmacéuticamente aceptable. En una incorporación, un artículo de fabricación de la invención comprende instrucciones para

administrar la composición (por ejemplo, la molécula moduladora) a un sujeto.

En un aspecto, la invención provee un kit que comprende un primer envase que contiene una composición que incluye una o más moléculas moduladoras de la invención; y un segundo envase que contiene un amortiguador ("*a buffer*"). En una incorporación, el amortiguador es farmacéuticamente aceptable. En una incorporación, una composición que comprende una molécula moduladora contiene además un vehículo, el cual en algunas incorporaciones es farmacéuticamente aceptable. En una incorporación, un kit comprende además instrucciones para administrar la composición (por ejemplo, la molécula moduladora) a un sujeto.

En un aspecto la invención provee un método de diagnosticar una enfermedad, que comprende determinar el nivel de HGFA en una muestra de prueba de células tisulares contactando la muestra con un anticuerpo de la invención, con lo cual el HGFA unido por el anticuerpo indica la presencia y/o cantidad de HGFA en la muestra. En otro aspecto, la invención provee un método de determinar si un individuo está en riesgo de una enfermedad, que comprende determinar el nivel de HGFA en una muestra de prueba de célula de tejido contactando la muestra de prueba con un anticuerpo de la invención y, con ello, determinar la cantidad de HGFA presente en la muestra, en donde un mayor nivel de HGFA en la muestra de prueba, comparada con una muestra de control que comprende tejido normal del mismo origen celular de la muestra de prueba, constituye un indicio de que el individuo está en riesgo de la enfermedad. En una incorporación de métodos de la invención, el nivel de HGFA se determina basándose en la cantidad de polipéptido HGFA indicada por la cantidad de HGFA unido por el anticuerpo en la muestra de prueba. Opcionalmente, un anticuerpo empleado en el método puede marcarse en forma detectable, unido a soporte sólido, o similar.

En un aspecto, la invención provee un método de unir un anticuerpo de la invención a HGFA presente en un fluido corporal, por ejemplo, sangre.

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En todavía otro aspecto, la invención se dirige a un método de unir un anticuerpo de la invención a una célula que expresa y/o es sensible a HGFA, en donde el método comprende contactar dicha célula con dicho anticuerpo bajo condiciones que son apropiadas para la unión del anticuerpo a HGFA y permitir la unión entre ellos. En una incorporación, la unión de dicho anticuerpo a HGFA en la célula inhibe una función biológica HGFA. En una incorporación, dicho anticuerpo no inhibe la interacción de HGFA con su molécula sustrato. En una incorporación, dicho anticuerpo se une a una molécula HGFA en la célula e inhibe la unión de otra molécula (tal como proHGF) a la molécula HGFA.

En un aspecto, la invención provee un método de dirigir un agente terapéutico a un tejido asociado con HGFA en un hospedero, método que comprende administrar al hospedero dicho agente terapéutico en una forma que se enlace a un anticuerpo de la invención, con lo cual el agente se dirige al tejido asociado con HGFA en el hospedero. En una incorporación, el anticuerpo que une HGFA es capaz de unirse específicamente a HGFA localizado en una célula (*in vitro* o *in vivo*), por ejemplo donde HGFA está presente en la superficie de una célula.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

Fig. 1 (A) Secuencias de lazo CDR de cadena pesada de anticuerpos anti-HGFA. La figura muestra las secuencias CDR de cadena pesada, H1, H2, y H3. La secuencia de cadena ligera es la secuencia de 4D5 humanizado (véase Lee et al., *supra*). La numeración de secuencias es la siguiente: clón 33 (CDRH1 es SEQ ID NO:3; CDRH2 es SEQ ID NO:4; CDRH3 es SEQ ID NO:5); clón 35 (CDRH1 es SEQ ID NO:6; CDRH2 es SEQ ID NO:7; CDRH3 es SEQ ID NO:8); clón 37 (CDRH1 es SEQ ID NO:9; CDRH2 es SEQ ID NO: 10; CDRH3 es SEQ ID NO: 11); clón 39 (CDRH1 es SEQ ID NO: 12; CDRH2 es SEQ ID NO:13; CDRH3 es SEQ ID NO: 14); clón 42 (CDRH1 es SEQ ID NO: 15; CDRH2 es SEQ ID NO: 16; CDRH3 es SEQ ID NO: 17); clón 49 (CDRH1

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es SEQ ID NO:18; CDRH2 es SEQ ID NO:19; CDRH3 es SEQ ID NO:20); clón 58 (CDRH1 es SEQ ID NO:21; CDRH2 es SEQ ID NO:22; CDRH3 es SEQ ID NO:23); clón 61 (CDRH1 es SEQ ID NO:24; CDRH2 es SEQ ID NO:25; CDRH3 es SEQ ID NO:26); clón 74 (CDRH1 es SEQ ID NO:27; CDRH2 es SEQ ID NO:28; CDRH3 es SEQ ID NO:29); clón 75 (CDRH1 es SEQ ID NO:30; CDRH2 es SEQ ID NO:31; CDRH3 es SEQ ID NO:32); clón 86 (CDRH1 es SEQ ID NO:33; CDRH2 es SEQ ID NO:34; CDRH3 es SEQ ID NO:35); clón 90 (CDRH1 es SEQ ID NO:36; CDRH2 es SEQ ID NO:37; CDRH3 es SEQ ID NO:38); clón 91 (CDRH1 es SEQ ID NO:39; CDRH2 es SEQ ID NO:40; CDRH3 es SEQ ID NO:41); clón 95 (CDRH1 es SEQ ID NO:42; CDRH2 es SEQ ID NO:43; CDRH3 es SEQ ID NO:44). Las posiciones de aminoácidos se numeran de acuerdo con el sistema de numeración de Kabat como se describe a continuación. Se indican asimismo los valores IC₅₀ en la última columna (derecha).

(B), (C) y (D) Secuencias de lazo CDR de cadena pesada de anticuerpos anti-HGFA. (E) y (F) Secuencias ejemplares de región de estructura alterna del anticuerpo ("*framework region*"). (E) Secuencias de región de estructura alterna HuMAb4D5-8. (F) Secuencias de región de estructura alterna HuMAb4D5-8 que comprenden modificaciones.

Fig. 2 Inhibición de la activación proHGF, mediada por HGFA, por anticuerpos anti-HGFA. HGFA se incubó con proHGF marcado con ¹²⁵I y anticuerpos anti-HGFA durante 4 horas a 37°C. Las concentraciones de reactante fueron de 50 µg/ml proHGF, 2 nM HGFA y 0.1mg/ml (0.67µM) anticuerpos. Se analizaron alícuotas por SDS-PAGE bajo condiciones de reducción. Se utilizó HAI-1B (sHAI-1B) soluble como un inhibidor de control con una concentración final de 1 µM. A. Banda 1: (t=0) es una alícuota tomada al comienzo de la reacción, banda 2: sin inhibidor, banda

3: sHAI-1B (1 μ M), banda 4: #33, banda 5: #35, banda 6: #39, banda 7: #49, banda 8: #74, banda 9: #61. B. Banda 1: #42, banda 2: #91, banda 3: 58, banda 4: #37, banda 5: #75, banda 6: #90, banda 7: #86, banda 8: #95.

Fig. 3. Potente inhibición de la conversión de proHGF, mediada por HGFA, por el anticuerpo #58. Tres diferentes concentraciones del anticuerpo #58 y el anticuerpo no bloqueante #49 se utilizaron en experimentos de conversión de proHGF marcado con ^{125} I, llevados a cabo como se describe en la figura 1. Banda 1: (t=0) es una alícuota tomada al comienzo de la reacción, banda 2: sin inhibidor, banda 3: sHAI-1B (1 μ M), banda 4: 0.67 μ M Ab#49, banda 5: 0.13 μ M Ab#49, banda 6: 0.03 μ M Ab#49, banda 7: 0.67 μ M Ab#58, banda 8: 0.13 μ M Ab#58, banda 9: 0.03 μ M Ab#58.

Fig. 4. Inhibición, dependiente de la concentración, de la actividad amidolítica de HGFA por anticuerpos anti-HGFA 58 y 75. Varias concentraciones de anticuerpos se incubaron con HGFA (concentración final 5nM) en amortiguador HBSA durante 20 minutos a temperatura de la sala. Luego de la adición de Spectrozyme® fVIIa (concentración final 200 μ M, K_M = 200 μ M), se midieron las tasas lineales de activación del sustrato en un lector cinético de microplacas. La inhibición de la actividad enzimática se expresó como actividad fraccionaria (v_i/v_o) de la actividad no inhibida.

Fig. 5. Inhibición la actividad amidolítica de HGFA por IV-49C y un ligador/inhibidor de sitio activo de molécula pequeña. Varias concentraciones de inhibidores se incubaron con HGFA (2.5nM para IV-49C y 5nM para la molécula pequeña, respectivamente) en amortiguador HBSA durante 20 minutos a temperatura ambiente. La inhibición enzimática de la activación de Spectrozyme® fVIIa

se midió como se describe en figura 4. A. Inhibición por el inhibidor de dominio Kunitz IV-49C (círculos llenos) en comparación con el inhibidor del factor XIIa específico, inhibidor de tripsina de maíz (círculos abiertos). B. Inhibición del inhibidor de molécula pequeña (triángulos llenos).

Fig. 6 Mediciones con resonancia de plasmones superficiales de la unión de HGFA a anticuerpos anti-HGFA #58 y #75. Anticuerpos anti-HGFA (IgG1 de cadena completa) se inmovilizaron en chips BIAcore y se recolectaron los datos de unión de varias concentraciones de HGFA. Para estudios de unión de competición, HGFA (70 nM) se pre-incubó con varias concentraciones de sHAI-1B, IV-49C o ligador de sitio activo de molécula pequeña. A - D: Unión de HGFA a anticuerpo #58 (A) en la ausencia de inhibidor, o en la presencia de (B) sHAI-1B, (C) FV-49C y (D) ligador de sitio activo de molécula pequeña. E-H: Unión de HGFA a anticuerpo #75 (E) en la ausencia de inhibidor, o en la presencia de (F) sHAI-1B, (G) IV-49C y (H) ligador de sitio activo de molécula pequeña.

Fig. 7 Secuencias de proteína HGFA humana (línea superior; SEQ ID NO: 1) y murina (línea inferior; SEQ ID NO:2).

Fig. 8 Tabla que muestra los datos relativos a la inhibición de la actividad enzimática HGFA por varios anticuerpos anti-HGFA.

Fig. 9 Tabla que muestra los datos relativos a la unión de HGFA a anticuerpos anti-HGFA.

MODOS DE LLEVAR A CABO LA INVENCION

La invención provee métodos, composiciones, kits y artículos de fabricación que comprenden moduladores de la función del activador del

factor de crecimiento hepatocítico, incluidos métodos de usar tales moduladores.

Detalles de estos métodos, composiciones, kits y artículos de fabricación se consignan en esta solicitud.

Técnicas Generales

La práctica de la presente invención empleará, a menos se indique lo contrario, técnicas convencionales de biología molecular (incluidas técnicas recombinantes), microbiología, biología celular, bioquímica e inmunología, las cuales se encuentran dentro de las habilidades del arte. Tales técnicas se explican en la literatura, tales como: "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, y actualizaciones periódicas); "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).

Definiciones

El término "activador de factor de crecimiento hepatocítico" o "HGFA", tal como se emplea aquí abarca polipéptidos de secuencia nativa, variantes polipeptídicas y fragmentos de un polipéptido de secuencia nativa y variantes polipeptídicas (que se definen con más detalle en esta solicitud), que es capaz de segmentación proHGF en una manera similar al HGFA tipo natural. El polipéptido HGFA descrito en esta solicitud puede ser aquel que se aísla de una variedad de fuentes, tales como de tipo de tejido humano o de otra fuente, o se prepara por métodos recombinantes o sintéticos. Los términos "HGFA", "polipéptido HGFA", "enzima HGFA", y "proteína HGFA" incluyen asimismo variantes de un polipéptido HGFA como se divulga en esta solicitud.

Un "polipéptido HGFA de secuencia nativa" comprende un polipéptido que tiene la misma secuencia de aminoácidos como el

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polipéptido HGFA correspondiente derivado de la naturaleza (por ejemplo, las secuencias descritas en la Figura 7). En una incorporación, un polipéptido HGFA de secuencia nativa comprende la secuencia de aminoácidos de SEQ ID NO:1 (véase la Figura 7; secuencia superior). Tal polipéptido HGFA de secuencia nativa puede aislarse de la naturaleza o puede producirse por medios sintéticos o recombinantes. El término "polipéptido HGFA de secuencia nativa" engloba específicamente formas truncadas o segregadas de ocurrencia natural del polipéptido HGFA específico (por ejemplo, una secuencia de dominio extracelular), formas variantes de ocurrencia natural (por ejemplo, formas alternativamente empalmadas) y variantes alélicas de ocurrencia natural del polipéptido.

"Variante de polipéptido HGFA", o sus variaciones, significa un polipéptido HGFA, generalmente un polipéptido HGFA activo, como se define en esta solicitud, que tiene por lo menos alrededor del 80% de identidad de secuencia con cualquiera de las secuencias de polipéptido HGFA de secuencia nativa, como se divulga en esta solicitud. Tales variantes de polipéptido HGFA incluyen, por ejemplo, polipéptidos HGFA HGFA en donde uno o más residuos de aminoácidos se agregan, delecionan, en el N- o C-término de una secuencia de aminoácidos nativa. De ordinario, una variante de polipéptido HGFA tendrá por lo menos alrededor del 80% de identidad de secuencia de aminoácidos, alternativamente por lo menos alrededor de 81 %, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, o 99% de identidad de secuencia de aminoácidos, con una secuencia de polipéptido HGFA de secuencia nativa, como se divulga en esta solicitud. De ordinario, polipéptidos de variante HGFA tienen por lo menos alrededor de 10 aminoácidos de longitud, alternativamente por lo menos alrededor de 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 aminoácidos de longitud, o más. Opcionalmente, polipéptidos de variante HGFA tendrán

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no más de una sustitución de aminoácido conservadora, comparada con una secuencia de polipéptido HGFA nativa, alternativamente no más de 2, 3, 4, 5, 6, 7, 8, 9, o 10 sustituciones de aminoácido conservadoras, comparadas con la secuencia de polipéptido HGFA nativa,.

"Identidad porcentual (%) de secuencia de aminoácidos" con respecto a una secuencia de péptidos o polipéptidos se define como el porcentaje de residuos de aminoácidos en una secuencia candidata, que son idénticos con los residuos de aminoácidos en la secuencia específica de péptidos o polipéptidos, luego de alinear las secuencias e introducir espacios vacíos ("*gaps*"), si es necesario, para lograr la máxima identidad porcentual de secuencia, y sin considerar sustituciones conservadoras de ninguna clase como parte de la identidad de secuencia. La alineación para efectos de determinar la identidad porcentual de secuencia de aminoácidos puede lograrse en varias formas, que se encuentran al alcance de la habilidad en el arte, por ejemplo, usando software de computador a disposición del público, tal como software BLAST, BLAST-2, ALIGN, ALIGN-2 o Megalign (DNASTAR). Aquellas personas expertas en el arte pueden determinar parámetros apropiados para medir la alineación, incluidos todos los algoritmos necesarios para lograr la alineación máxima sobre la longitud completa de las secuencias que se están comparando. Para los propósitos contemplados en esta solicitud, sin embargo, se generan valores de identidad % de secuencia de aminoácidos usando el programa de computador de comparación de secuencias ALIGN-2, en donde el código fuente completo para el programa ALIGN-2 se provee en la siguiente Tabla A. El programa de computador de comparación de secuencia ALIGN-2 fue creado por Genentech, Inc. y el código fuente se ha depositado con documentación de usuario ante la Oficina de Derechos de Autor, Washington, D.C, 20559, en donde está registrado bajo el Registro de Derechos de Autor de los Estados Unidos No. TXU510087. El programa ALIGN-2 se encuentra a disposición del público a través de Genentech, Inc., South San Francisco, California o puede compilarse del código fuente provisto en la siguiente Figura 8. El programa ALIGN-2 debe compilarse

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para uso en un sistema operativo UNIX, preferiblemente UNIX V4.0D digital. Todos los parámetros de comparación de secuencias se fijan por el programa ALIGN-2 y no varían.

En situaciones donde ALIGN-2 se emplea para comparaciones de secuencias de aminoácidos, el % de identidad de secuencia de aminoácidos de una secuencia dada de aminoácidos A respecto a, con, o frente a una secuencia dada de aminoácidos B (lo cual puede expresarse alternativamente como una secuencia dada de aminoácidos A que tiene o comprende un cierto % de identidad de secuencia de aminoácidos respecto a, con, o frente a una secuencia dada de aminoácidos B) se calcula en la siguiente forma:

$$100 \text{ veces la fracción } X/Y$$

donde X es el número de residuos de aminoácidos obtenidos como equivalentes idénticos por el programa de alineación de secuencias ALIGN-2 en la alineación de ese programa de A y B, y donde Y es el número total de residuos de aminoácidos en B. Se observará que donde la longitud de la secuencia de aminoácidos A no es igual a la secuencia de aminoácidos B, el % de identidad de secuencia de aminoácidos de A a B no equivaldrá al % de identidad de secuencia de aminoácidos de B a A.

A menos que se indique específicamente lo contrario, todos los valores % de identidad de secuencia de aminoácidos, usados en esta solicitud, se obtienen como se describe en el párrafo inmediatamente precedente usando el programa de computador ALIGN-2.

Tabla 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, 0, _M, 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},

```

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```

/* 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,
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, 0, _M, 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, 0, _M, 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 */

```

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```

    },                                     /* limits seq to 2^16 -1 */

    struct diag {
        int      score;      /* score at last jmp */
        long     offset;     /* offset of prev block */
        short    jmp;        /* 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();

```

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```
/* 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 and 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, 0xFFFFFFFF, 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) {
```

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```
/* 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 and 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, 0xFFFFFFFF, 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) {
```


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```

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) {
```

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```

    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);

```

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```
        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
 */

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

...NW

```

    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 || (ndclx >= 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] = ndclx;
        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];
    }
}

```

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```

        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()
{
    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;

```

print

```

        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;

```

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```

while ( *p0 && *p1 ) {
    if (siz0) {
        p1++;
        n1++;
        siz0--;
    }
    else if (siz1) {
        p0++;
        n0++;
        siz1--;
    }
    else {
        if (xbrn[*p0-'A']&xbrn[*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);
if (gapx) {
    (void) sprintf(outx, " (%d %s%s)",
        gapx, (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)",
            gapy, (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 */

```

...getmat

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```

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(name[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;

```

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```

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();
}

```

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```

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

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

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

    (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);
        }
    }
}

```

dumpblock

...dumpblock

```

/*
 * 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);
}

```

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```

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

    int    i;                                ...putl
    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:
     * n1[] 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

```

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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
 * readjumps() -- get the good jumps, from tmp file if necessary
 * writejumps() -- write a filled array of jumps to a tmp file: nw()
 */
#include "nw.h"
#include <sys/file.h>

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

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

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

```

cleanup

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```

        int    i;
    {
        if (fj)
            (void) unlink(jname),
            exit(1);
    }

/*
 * read, return ptr to seq, set dna, len, maxlen
 * skip lines starting with ';', '<', or '>'
 * seq in upper or lower case
 */
char    *
getseq(file, len)
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';

    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);
}

```

...getseq

```

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

```

g_calloc

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```

    if ((px = calloc((unsigned)nx, (unsigned)sz)) == 0) {
        if (*msg) {
            fprintf(stderr, "%s: g_malloc() failed %s (n=%d, sz=%d)\n", prog, msg, nx
sz);

            exit(1);
        }
    }
    return(px);
}

```

/*

* get final jmps from dx[] or tmp file, set pp[], reset dmax. main()

*/

readjmps()

readjmps

{

```

    int          fd = -1;
    int          siz, i0, i1;
    register     i, j, xx;

```

if (fj) {

(void) fclose(fj);

if ((fd = open(jname, O_RDONLY, 0)) < 0) {

fprintf(stderr, "%s: can't open() %s\n", prog, jname);

cleanup(1);

}

}

for (i = i0 = i1 = 0, dmax0 = dmax, xx = len0; ; i++) {

while (1) {

for (j = dx[dmax].ijmp; j >= 0 && dx[dmax].jp.x[j] >= xx; j--)

;

...readjmps

if (j < 0 && dx[dmax].offset && fj) {

(void) lseek(fd, dx[dmax].offset, 0);

(void) read(fd, (char *)&dx[dmax].jp, sizeof(struct jmp));

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```

        (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()
 */
writejumps(ix)
    int    ix;
{
    char    *mktemp();

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

```

writejumps

```
    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);
```

El término "vector", tal como se emplea en esta solicitud, tiene como finalidad referirse a una molécula de ácido nucleico capaz de transportar otro ácido nucleico, al cual se ha enlazado. Un tipo de vector es un "plásmido", un lazo circular de ADN bicatenario en el cual pueden ligarse segmentos de ADN adicionales. Otro tipo de vector es un vector de fago. Otro tipo de vector es un vector vírico, en donde segmentos de ADN adicionales pueden ligarse en el genoma vírico. Ciertos vectores son capaces de replicación autónoma en una célula hospedera, en la cual se introducen (por ejemplo, vectores bacterianos que tienen un origen bacteriano de replicación y vectores mamíferos episómicos). Otros vectores (por ejemplo, vectores mamíferos no episómicos) pueden integrarse en el genoma de una célula hospedera con la introducción en la célula hospedera y, con ello, se replican junto con el genoma hospedero. Más aún, ciertos vectores son capaces de dirigir la expresión de genes a los cuales se enlazan funcionalmente. Tales vectores se designan en esta solicitud como "vectores de expresión recombinantes" (o simplemente, "vectores recombinantes"). En general, vectores de expresión de utilidad en técnicas de ADN recombinante se encuentran a menudo en forma de plásmidos. En la presente especificación, "plásmido" y "vector" pueden emplearse indistintamente, ya que el plásmido es la forma más comúnmente usada de vector.

"Polinucleótido" o "ácido nucleico", tal como se utilizan indistintamente en esta solicitud, se refieren a polímeros de nucleótidos de

cualquier longitud, e incluyen ADN y ARN. Los nucleótidos pueden ser deoxirribonucleótidos, ribonucleótidos, nucleótidos modificados o bases, y/o sus análogos, o cualquier sustrato que pueda incorporarse en un polímero por ADN o ARN polimerasa o por una reacción sintética. Un polinucleótido puede comprender nucleótidos modificados, tales como nucleótidos metilados y sus análogos. Si se halla presente, la modificación a la estructura del nucleótido puede impartirse antes o después del ensamble del polímero. La secuencia de nucleótidos puede interrumpirse por componentes no nucleótido. Un polinucleótido puede modificarse aún más luego de la síntesis, tal como mediante conjugación con una etiqueta. Otros tipos de modificaciones incluyen, por ejemplo, "caperuzas", sustitución de uno o más de los nucleótidos de ocurrencia natural con un análogo, modificaciones internucleótidos tales como, por ejemplo, aquellas con ligamientos sin carga (por ejemplo, metilfonatos, fosfotriésteres, fosfoamidatos, carbamatos, etc.) y con ligamientos con carga (por ejemplo, fosforotioatos, fosforoditioatos, etc.), aquellos que contienen fracciones pendientes, tales como, por ejemplo, proteínas (por ejemplo, nucleasas, toxinas, anticuerpos, péptidos de señal, ply-L-lisina, etc.), aquellos con intercaladores (por ejemplo, acridina, psoralen, etc.), aquellos que contienen quelantes (por ejemplo, metales, metales radioactivos, boro, metales oxidativos, etc.), aquellos que contienen alquilantes, aquellos con ligamientos modificados (por ejemplo, ácidos nucleicos alfa anoméricos, etc.), así como formas no modificadas del polinucleótido(s). Además, cualquiera de los grupos hidroxilo ordinariamente presentes en los azúcares puede reemplazarse, por ejemplo, por grupos fosfonato, grupos fosfato, protegidos por grupos protectores estándar, o activarse para preparar ligamientos adicionales a nucleótidos adicionales, o pueden conjugarse a soportes sólidos o semisólidos. El OH 5' y 3' terminal puede fosforilarse o sustituirse con aminas o fracciones de grupo caperuza orgánico ("*organic capping group moieties*") de desde 1 a 20 átomos de carbono. Además, otros hidroxilos pueden derivarse para formar grupos protectores estándar. Los polinucleótidos pueden contener asimismo formas análogas de azúcares

ribosa o desoxirribosa, que se conocen generalmente en el arte, incluidos, por ejemplo, 2'-O-metil-, 2'-O-alil, 2'-fluoro- o 2'-azido-ribosa, análogos de azúcar carbocíclicos, azúcares α -anoméricos, azúcares epiméricos tales como arabinosa, xilosas o lixosas, azúcares de piranosa, azúcares de furanosa, pseudoheptulosas, análogos acíclicos, y análogos de nucleósidos abásicos tales como ribósido de metilo. Uno o más ligamientos fosfodiéster pueden reemplazarse por grupos enlazadores alternativos. Estos grupos enlazadores alternativos incluyen, pero no se limitan a, incorporaciones en donde el fosfato se reemplaza por P(O)S("tioato"), P(S)S ("ditioato"), "(O)NR₂ ("amidato"), P(O)R, P(O)OR', CO o CH₂ ("formacetal"), en los cuales cada R o R' es independientemente H o alquilo sustituido o no sustituido (1-20 C), que contienen opcionalmente un ligamiento éter (-O-), arilo, alquenilo, cicloalquilo, cicloalquenilo, o araldilo. No todos los ligamientos en un polinucleótido necesitan ser idénticos. La descripción anterior se aplica a todos los polinucleótidos mencionados en esta solicitud, incluidos ARN y ADN.

"Oligonucleótido," tal como se emplea en esta solicitud, se refiere generalmente a polinucleótidos cortos, generalmente monocatenarios, generalmente sintéticos que por lo general, pero no necesariamente, tienen menos de alrededor de 200 nucleótidos de longitud. Los términos "oligonucleótido" y "polinucleótido" no son mutuamente excluyentes. La anterior descripción para polinucleótidos es igual y completamente aplicable a oligonucleótidos.

Un anticuerpo "aislado" es uno que se ha identificado y separado y/o recuperado de un componente de su entorno natural. Componentes contaminantes de su entorno natural son materiales que interferirían los usos diagnósticos o terapéuticos del anticuerpo, y pueden incluir enzimas, hormonas, y otros solutos proteináceos o no proteináceos. En algunas incorporaciones, el anticuerpo se purificará (1) a más del 95% en peso del anticuerpo, según se determine por el método de Lowry, y muy preferiblemente más del 99% en peso, (2) a un grado suficiente para obtener por lo menos 15 residuos de la secuencia de aminoácidos N-terminal o

interna mediante el uso de un secuenciador de vaso giratorio, o (3) a homogeneidad por SDS-PAGE bajo condiciones reductoras o no reductoras usando azul Coomassie o, preferiblemente, tintura de plata. Un anticuerpo aislado incluye el anticuerpo *in situ* en células recombinantes, puesto que por lo menos un componente del entorno natural del anticuerpo no estará presente. Ordinariamente, sin embargo, el antagonista aislado se preparará en por lo menos un paso de purificación.

El término "numeración de residuos de dominio variable como en Kabat" o "numeración de posiciones de aminoácidos como en Kabat", y sus variaciones, se refiere al sistema de numeración utilizado para dominios variables de cadena pesada o para dominios variables de cadena ligera de la compilación de anticuerpos en Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). Usando este sistema de numeración, la secuencia lineal actual de aminoácidos puede contener pocos o aminoácidos adicionales que correspondan a un acortamiento de, o una inserción en, una FR o CDR del dominio variable. Por ejemplo, un dominio variable de cadena pesada puede incluir un solo inserto de aminoácidos (residuo 52a de acuerdo con Kabat) después del residuo 52 de H2 y residuos insertados (por ejemplo, los residuos 82a, 82b, y 82c, etc., de acuerdo con Kabat) después del residuo 82 de la FR de cadena pesada. La numeración de Kabat de los residuos puede determinarse para un anticuerpo dado mediante alineación en las regiones de homología de la secuencia del anticuerpo con una secuencia numerada "estándar" de Kabat. A menos que se indique lo contrario, la numeración de todas las posiciones de aminoácidos en esta solicitud está de acuerdo con el sistema de numeración de Kabat.

Una "estructura ("*framework*") de consenso humana" es una estructura que representa el residuo de aminoácidos de ocurrencia más común en una selección de secuencias de estructura VL o VH de inmunoglobulina humana. Generalmente, la selección de secuencias VL o VH de inmunoglobulina humana es de un subgrupo de secuencias de dominio variable. Generalmente, el subgrupo de secuencias es un subgrupo

como en Kabat *et al.* En una incorporación, para el VL, el subgrupo es el subgrupo kappa I como en Kabat *et al.* En una incorporación, para el VH, el subgrupo es el subgrupo III como Kabat *et al.*

Una "estructura de consenso del subgrupo III de VH" comprende la secuencia de consenso obtenida de las secuencias de aminoácidos en el subgrupo III de cadena pesada variable de Kabat *et al.* En una incorporación, la secuencia de aminoácidos de estructura de consenso del subgrupo III VH comprende por lo menos una porción o todas de cada una de las siguientes secuencias:

EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO:46)-H1-
WVRQAPGKGLEWV (SEQ ID NO:47)-H2-
RFTISRDN SKNTLYLQMNSLRAEDTAVYYC (SEQ ID NO:48)-H3-
WGQGTL VTVSS (SEQ ID NO:49).

Una "estructura de consenso de subgrupo I de VL" comprende la secuencia de consenso obtenida de las secuencias de aminoácidos en el subgrupo I kappa ligero variable de Kabat *et al.* En una incorporación, la secuencia de aminoácidos de estructura de consenso de subgrupo I de VL comprende por lo menos una porción o todas de cada una de las siguientes secuencias:

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

El término "factor de crecimiento hepatocítico" o "HGF", tal como se emplea en esta solicitud, se refiere, a menos que específica o contextualmente se indique lo contrario, a cualquier polipéptido HGF nativo o variante (de ocurrencia natural o sintético), que es capaz de activa la ruta de señalización HGF/c-met bajo condiciones que permitan que tal proceso ocurra. El término "HGF de tipo natural" se refiere generalmente a un polipéptido que comprende la secuencia de aminoácidos de una proteína HGF de ocurrencia natural. El término "secuencia HGF de tipo natural" se

refiere generalmente a una secuencia de aminoácidos encontrada en un HGF de ocurrencia natural.

Los términos "anticuerpo" e "inmunoglobulina" se usan indistintamente en el sentido más amplio e incluyen anticuerpos monoclonales (*por ejemplo*, anticuerpos monoclonales intactos o de longitud total), anticuerpos policlonales, anticuerpos multivalentes, anticuerpos multiespecíficos (*por ejemplo*, anticuerpos biespecíficos, siempre y cuando exhiban la actividad biológica deseada) y pueden incluir asimismo ciertos fragmentos de anticuerpo (como se describe con mayor detalle en esta solicitud). Un anticuerpo puede ser humano, humanizado y/o madurado por afinidad.

"Fragmentos de anticuerpo" comprenden sólo una porción de un anticuerpo intacto, en donde la porción retiene preferiblemente como mínimo una, preferiblemente la mayoría o la totalidad de las funciones normalmente asociadas con esa porción, cuando están presentes en un anticuerpo intacto. En una incorporación, un fragmento de anticuerpo comprende un sitio de unión a antígeno del anticuerpo intacto y, por consiguiente, retiene la capacidad de fijar antígeno. En otra incorporación, un fragmento de anticuerpo, *por ejemplo* uno que comprende la región Fc, retiene como mínimo una de las funciones biológicas, asociadas normalmente con la región Fc cuando están presentes en un anticuerpo intacto, tal como unión FcRn, modulación de la semivida del anticuerpo, función ADCC, y/o unión de complemento. En una incorporación, un fragmento de anticuerpo es un anticuerpo monovalente que tiene una semivida *in vivo* sustancialmente similar a un anticuerpo intacto. *Por ejemplo*, tal fragmento de anticuerpo puede comprender un brazo de unión a antígeno enlazado a una secuencia de Fc capaz de conferir estabilidad *in vivo* al fragmento.

El término "anticuerpo monoclonal", como se emplea en esta solicitud, se refiere a un anticuerpo obtenido de una población de anticuerpos sustancialmente homogéneos, *es decir*, los anticuerpos individuales que componen la población son idénticos, excepto por posibles

mutaciones de ocurrencia natural, que puedan estar presentes en cantidades menores. Los anticuerpos monoclonales son altamente específicos, dirigiéndose contra un solo antigénico. Más aún, en contraste con preparaciones de anticuerpo policlonal, que típicamente incluyen diferentes anticuerpos dirigidos contra diferentes determinantes (epítomos), cada anticuerpo monoclonal se dirige contra un solo determinante en el antígeno.

Los anticuerpos monoclonales en esta solicitud incluyen anticuerpos "quiméricos", en los cuales una porción de la cadena pesada y/o ligera es idéntica con, u homóloga a, secuencias correspondientes en anticuerpos derivados de una especie particular, o pertenecientes a una clase o subclase de anticuerpos, mientras que el resto de la cadena(s) es idéntico con, u homólogo a, secuencias correspondientes en anticuerpos derivados de otra especie, o pertenecientes a otra clase o subclase de anticuerpos, así como fragmentos de tales anticuerpos, siempre que exhiban la actividad biológica deseada (Patente de los Estados Unidos 4,816,567; y Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

Formas "humanizadas" de anticuerpos no humanos (*por ejemplo*, murinos) son anticuerpos quiméricos que contienen una secuencia mínima derivada de inmunoglobulina no humana. En su mayor parte, los anticuerpos humanizados son inmunoglobulinas humanas (anticuerpo destinatario), en las cuales residuos de una región hipervariable del destinatario se reemplazan por residuos de una región hipervariable de una especie no humana (anticuerpo donante) tal como ratón, rata, conejo o primate no humano, que tienen la especificidad, afinidad y capacidad deseadas. En algunos casos, residuos de la región de estructura alterna del anticuerpo (FR) de la inmunoglobulina humana se reemplazan por residuos no humanos correspondientes. Más aún, los anticuerpos humanizados pueden comprender residuos, que no se encuentran en el anticuerpo destinatario ni en anticuerpo donante. Estas modificaciones se hacen para refinar aún más el desempeño del anticuerpo. En general, el anticuerpo humanizado comprenderá sustancialmente la totalidad de por lo menos uno y, típicamente dos, dominios variables, en los cuales la totalidad o

sustancialmente todos los lazos hipervariable corresponden a los de una inmunoglobulina no humana, y todas o sustancialmente todas las FR son las de una secuencia de inmunoglobulina humana. Opcionalmente, el anticuerpo humanizado comprenderá también por lo menos una porción de una región constante de inmunoglobulina (Fc), típicamente la de una inmunoglobulina humana. Para mayores detalles, véase Jones *et al.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-329 (1988); y Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). Véanse también los siguientes artículos de reseña y las referencias citadas allí: 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).

Un "anticuerpo humano" es uno que posee una secuencia de aminoácidos que corresponde a la de un anticuerpo producido por un humano y/o se ha elaborado usando cualquiera de las técnicas para producir anticuerpos humanos, como se divulga en esta solicitud. Esta definición de un anticuerpo humano excluye específicamente un anticuerpo humanizado que comprende residuos de unión a antígeno no humanos.

Un anticuerpo "madurado de afinidad" es uno con una o más alteraciones en una o más CDR del mismo, que resultan en una mejora en la afinidad del anticuerpo para el antígeno, comparada con un anticuerpo progenitor que no posee esa(s) alteración (alteraciones). Anticuerpos preferidos maduros de afinidad tendrán afinidades nanomolares o, incluso, picomolares para el antígeno diana. Anticuerpos maduros de afinidad se producen por procedimientos conocidos en el arte. Marks *et al.* *Bio/Technology*, 10:779-783 (1992) describe maduración de afinidad por entremezcla de dominio VH y VL. Mutagénesis aleatoria de CDR y/o residuos de región de estructura alterna ("*framework residues*") se describe por: 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); y Hawkins *et al.*, *J. Mol. Biol.*, 226:889-896 (1992).

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Un anticuerpo "bloqueante" o un anticuerpo "antagonista" es uno que inhibe o reduce la actividad biológica del antígeno que une. Anticuerpos bloqueantes preferidos o anticuerpos antagonistas inhiben sustancial o completamente la actividad biológica del antígeno.

Un "anticuerpo agonista", tal como se emplea aquí, es un anticuerpo que imita por lo menos una de las actividades funcionales de un polipéptido de interés.

Un "trastorno" es cualquier enfermedad que se beneficiaría del tratamiento con una sustancia/molécula o método de la invención. Esto incluye trastornos crónicos y agudos o enfermedades, incluidas aquellas afecciones patológicas que predisponen el mamífero al trastorno en cuestión. Ejemplos no limitantes de trastornos a tratarse en esta solicitud incluyen tumores malignos y benignos; neoplasias malignas distintas de leucemias y neoplasias malignas linfoides; trastornos neuronales, gliales, astrocíticos, hipotalámicos y demás trastornos glandulares, macrofágicos, epiteliales, estromales y blastocélicos; y trastornos inflamatorios, inmunitarios y otros trastornos relacionados con angiogénesis.

Los términos "trastorno de proliferación celular" y "trastorno proliferativo" se refieren a trastornos que se asocian con algún grado de proliferación celular anormal. En una incorporación, el trastorno de proliferación celular es cáncer.

"Tumor", tal como se emplea en esta solicitud, se refiere a todo crecimiento y proliferación de células neoplásicas, malignos o benignos, y a todas las células y tejidos canceroso y pre-cancerosos. Los términos "cáncer", "canceroso", "trastorno de proliferación celular", "trastorno proliferativo" y "tumor" no son mutuamente excluyentes, como se menciona en esta solicitud.

Los términos "cáncer" y "canceroso" se refieren a, o describen, la condición fisiológica en mamíferos, que se caracteriza típicamente por un crecimiento/proliferación celular descontrolado. Ejemplos de cáncer incluyen, pero no se limitan a, carcinoma, linfoma, blastoma, sarcoma y leucemia. Ejemplos más particulares de tales neoplasias malignas incluyen

carcinoma epidermoide ("*squamous cell cancer*"), cáncer de pulmón microcítico, cáncer de pulmón amicrocítico, adenocarcinoma del pulmón, carcinoma escamoso del pulmón, cáncer del peritoneo, cáncer hepatocelular, cáncer gastrointestinal, cáncer pancreático, glioblastoma, cáncer cervical, cáncer de ovarios, cáncer del hígado, cáncer de la vejiga, hepatoma, cáncer de mama, cáncer de colon, cáncer colorrectal, carcinoma del endometrio o uterino, carcinoma de glándula salival, cáncer del riñón, cáncer del hígado, cáncer de próstata, cáncer de la vulva, cáncer de tiroides, carcinoma hepático y varios tipos de cáncer de cabeza y cuello.

La alteración de la regulación de angiogénesis puede llevar a muchos trastornos que pueden tratarse por composiciones y métodos de la invención. Estos trastornos incluyen afecciones neoplásicas y no neoplásicas. Las neoplasias incluyen, sin limitación, las ya descritas. Los trastornos no neoplásicos incluyen, sin limitación, hipertrofia no deseada o aberrante, artritis, artritis reumatoide (RA), psoriasis, placas psoriáticas, sarcoidosis, aterosclerosis, placas ateroscleróticas, retinopatías diabéticas y otras retinopatías proliferativas incluidas retinopatía de la prematuridad, fibroplasia retrolental, glaucoma neovascular, degeneración macular relacionada con la edad, edema macular diabético, neovascularización de la córnea, neovascularización de injerto de córnea, rechazo de injerto de córnea, neovascularización retiniana/coroidea, neovascularización del ángulo (rubeosis), enfermedad neovascular ocular, restenosis vascular, malformaciones arteriovenosas (AVM), meningioma, hemangioma, angiofibroma, hiperplasias tiroideas (incluida la enfermedad de Grave), trasplante de córnea y otros tejidos, inflamación crónica, inflamación pulmón, lesión pulmonar aguda /síndrome disneico agudo en el adulto (ARDS), sepsis, hipertensión pulmonar primaria, efusiones pulmonares malignas, edema cerebral (*por ejemplo*, asociado con infarto cerebral agudo /traumatismo craneoencefálico / trauma), inflamación sinovial, formación de tejido de granulación sinovial en RA, miositis ossificans, formación ósea hipertrófica, osteoartritis (OA), ascitis resistente a tratamiento, enfermedad de ovario poliquístico, endometriosis, enfermedades del tercer

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espacio de fluido (pancreatitis, síndrome de compartimiento abdominal, ardores de estómago, síndrome de colon irritable), fibroides uterinos, parto prematuro, inflamación crónica tal como IBD (enfermedad de Crohn y colitis ulcerosa), rechazo de aloinjerto renal, síndrome de intestino irritable, síndrome nefrótico, crecimiento de masa tisular no deseado o aberrante (distinto de cáncer), articulaciones hemofílicas, cicatrices hipertróficas, inhibición del crecimiento del cabello, síndrome de Osler-Weber, fibroplasias retrolentales de granuloma piógeno, escleroderma, tracoma, adhesiones vasculares, sinovitis, dermatitis, preeclampsia, ascitis, efusión pericardiaca (tal como la asociada con pericarditis), y efusión pleural.

Una "enfermedad autoinmunitaria", en esta solicitud, es una enfermedad o trastorno no maligno originado en, y dirigido contra, los propios tejidos de un individuo. Las enfermedades autoinmunitarias descritas en esta solicitud excluyen específicamente enfermedades o afecciones malignas o cancerosas, excluyendo particularmente linfoma de linfocitos B, leucemia linfoblástica aguda (ALL), leucemia linfocítica crónica (CLL), tricoleucemia, y leucemia mieloblástica crónica. Ejemplos de enfermedades o trastornos autoinmunitarios incluyen, pero no se limitan a, respuestas inflamatorias tales como enfermedades cutáneas inflamatorias incluidas psoriasis y dermatitis (por ejemplo, dermatitis atópica); escleroderma sistémico y esclerosis; respuestas asociadas con síndrome de intestino inflamado (tal como enfermedad de Crohn y colitis ulcerosa); síndrome disneico (incluido síndrome disneico agudo del adulto; ARDS); dermatitis; meningitis; encefalitis; uveítis; colitis; glomerulonefritis; afecciones alérgicas tales como eczema y asma y otras enfermedades que involucran la infiltración de linfocitos T y respuestas inflamatorias crónicas; aterosclerosis; deficiencia de adhesión de leucocitos; artritis reumatoide; lupus eritematoso sistémico (SLE); diabetes sacarina (por ejemplo, diabetes sacarina tipo I o diabetes sacarina dependiente de insulina); esclerosis múltiple; síndrome de Reynaud; tiroiditis autoinmunitaria; encefalomiелitis alérgica; síndrome de Sjögren; diabetes juvenil; y respuestas inmunitarias asociadas con hipersensibilidad aguda y retardada, mediada por citocinas y

linfocitos T, hallados típicamente en tuberculosis, sarcoidosis, polimiositis, granulomatosis y vasculitis; anemia perniciosa (enfermedad de Addison); enfermedades que involucran diapédesis de leucocitos; trastorno inflamatorio del sistema nervioso central (CNS); síndrome de lesión de órganos múltiples; anemia hemolítica (incluidas, pero no limitadas a, crioglobulinemia o anemia positiva de Coomb); miastenia grave; enfermedades mediadas por complejo antígeno-anticuerpo; enfermedad de la membrana basal anti-glomerular; síndrome antifosfolípido; neuritis alérgica; enfermedad de Graves; síndrome miasténico de Lambert-Eaton; penfigoide vesicular; pénfigo; poliendocrinopatías autoinmunitarias; enfermedad de Reiter; síndrome de la persona rígida; enfermedad de Behçet; arteritis de células gigantes; nefritis de complejo inmunitario; nefropatía IgA; polineuropatías IgM; púrpura trombocitopénica inmunitaria (ITP) o trombocitopenia autoinmunitaria, etc..

Tal como se emplea en esta solicitud, "tratamiento" se refiere a una intervención clínica en un intento por alterar el ciclo natural del individuo o célula que se están tratando, y puede realizarse con fines profilácticos o durante el ciclo de la patología clínica. Los efectos deseables de tratamiento incluyen prevenir la ocurrencia o recurrencia de la enfermedad, alivio de los síntomas, disminución de cualesquiera consecuencias directas o indirectas de la enfermedad, impedir la metástasis, reducir la velocidad de evolución de la enfermedad, mejora o mitigación del estado patológico, y remisión o pronóstico mejorado. En algunas incorporaciones, los anticuerpos de la invención se utilizan para retrasar el desarrollo de una enfermedad o trastorno.

Una "cantidad efectiva" se refiere a una cantidad efectiva, en dosis y durante períodos de tiempo necesarios, para conseguir el resultado terapéutico o profiláctico deseado.

Una "cantidad terapéuticamente efectiva" de una sustancia/molécula de la invención, agonista o antagonista puede variar de acuerdo con factores tales como el estado patológico, edad, sexo, y peso del individuo, y la capacidad de la sustancia/molécula, agonista o antagonista para provocar

una respuesta deseada en el individuo. Una cantidad terapéuticamente efectiva es también una en la cual cualesquiera efectos nocivos o tóxicos de la sustancia/molécula de la invención, agonista o antagonista son superados por los efectos terapéuticamente benéficos. Una "cantidad profilácticamente efectiva" se refiere a una cantidad efectiva, en dosis y períodos de tiempo necesarios, para conseguir el resultado profiláctico deseado. Típica, pero no necesariamente, puesto que se usa una dosis profiláctica en sujetos antes de, o en una fase más temprana, la enfermedad, la cantidad profilácticamente efectiva será inferior a la cantidad terapéuticamente efectiva.

El término "agente citotóxico", tal como se usa en esta solicitud, se refiere a una sustancia que inhibe o previene la función de las células y/o causa destrucción de las células. El término tiene como finalidad incluir isótopos radioactivos (*por ejemplo*, At^{211} , I^{131} , I^{125} , Y^{90} , Re^{186} , Re^{188} , Sm^{153} , Bi^{212} , P^{32} e isótopos radioactivos de Lu), agentes quimioterapéuticos, *por ejemplo*, metotrexato, adriamicina, alcaloides vinca (vincristina, vinblastina, etopósido), doxorubicina, melfalán, mitomicina C, clorambucil, daunorubicina u otros agentes intercalantes, enzimas y sus fragmentos, tales como enzimas nucleolíticas, antibióticos, y toxinas tales como toxinas de molécula pequeña o toxinas enzimáticamente activas de origen bacteriano, fúngico, vegetal o animal, incluidos sus fragmentos y/o variantes, y los diferentes agentes antitumorales o antineoplásicos divulgados más adelante. A continuación se describen otros agentes citotóxicos. Un agente tumoricida causa la destrucción de las células tumorales.

Un "agente quimioterapéutico" es un compuesto químico útil en el tratamiento del cáncer. Ejemplos de agentes quimioterapéuticos incluyen agentes alquilantes tales como tiotepa y ciclosfosfamida CYTOXAN®; alquilosulfonatos tales como busulfán, improsulfán y piposulfán; aziridinas tales como benzodopa, carbocuna, meturedopa y uredopa; etileniminas y metilamelaminas incluidas altretamina, trietilenmelamina, trietilenfosforamida, trietilentiofosforamida y trimetilomelamina; acetogeninas (especialmente bulatacina y bulatacinona); delta-9-

tetrahidrocannabinol (dronabinol, MARINOL®); beta-lapachona; lapacol; colquicinas; ácido betulínico; una camptotecina (incluido el análogo sintético topotecán); (HYCAMTIN®), CPT-11 (irinotecán, CAMPTOSAR®), acetilcamptotecina, escopoletina, y 9-aminocamptotecina); briostatina; calistatina; CC-1065 (incluidos sus análogos sintéticos adozelesina, carzelesina y bizelesina); podofilotoxina; ácido podofilínico; tenipósido; criptoficinas (particularmente criptoficina 1 y criptoficina 8); dolastatina; duocarmicina (incluidos los análogos sintéticos, KW-2189 y CB1-TM1); eleuterobina; pancratistatina; una sarcodictina; espongistatina; mostazas nitrogenadas tales como clorambucil, clornafazina, colofosfamida, estramustina, ifosfamida, mecloretamina, clorhidrato de óxido de mecloretamina, melfalán, novembichin, phenesterine, prednimustina, trofosfamida, mostaza de uracilo; nitrosureas tales como carmustina, clorozotocina, fotemustina, lomustina, nimustina, y ranimustina; antibióticos tales como los antibióticos enediyne (*por ejemplo*, caliqueamicina, especialmente caliqueamicina gamma11 y caliqueamicina omega11 (véase, *por ejemplo*, Agnew, *Chem Intl. Ed. Engl.*, 33: 183-186 (1994))); dinemicina, incluida dinemicina A; una esperamicina; así como cromóforo de neocarzinostatina y cromóforos antibióticos relacionados de cromoproteína enediño), aclacinomisinas, actinomicina, autramicina, azaserina, bleomicinas, cactinomicina, carabicina, carminomicina, carzinofilina, cromomicinas, dactinomicina, daunorubicina, detorubicina, 6-diazo-5-oxo-L-norleucina, doxorubicina (incluida ADRIAMYCIN®, morfolino-doxorubicina, cianomorfolino-doxorubicina, 2-pirrolino-doxorubicina, inyección de liposomas de HCl de doxorubicina (DOXIL®) y deoxidoxorubicina), epirubicina, esorubicina, idarubicina, marcelomicina, mitomicinas tales como mitomicina C, ácido micofenólico, nogalamicina, olivomicinas, peplomicina, potfiromicina, puromicina, quelamicina, rodorubicina, estreptonigrina, estreptozocina, tubercidina, ubenimex, zinostatina, zorubicina; anti-metabolitos tales como metotrexato, gemcitabina (GEMZAR®), tegafur (UFTORAL®), capecitabina (XELODA®), una eptilona, y 5-fluorouracilo (5-FU); análogos de ácido

fólico tales como denopterina, metotrexato, pteropterina, trimetrexato; análogos de purina tales como fludarabina, 6-mercaptopurina, tiamiprina, tioguanina; análogos de pirimidina tales como ancitabina, azacitidina, 6-azauridina, carmofur, citarabina, dideoxiuridina, doxifluridina, enocitabina, floxuridina; andrógenos tales como calusterona, dromostanolona propionato, epitioestanol, mepitioestano, testolactona; anti-adrenales tales como aminoglutetimida, mitotano, trilostano; reabastecedor de ácido fólico tal como ácido frolinico; aceglatona; glucósido de aldofosfamida; ácido aminolevulínico; eniluracil; amsacrina; bestrabucil; bisantreno; edatraxato; defofamine; demecolcine; diaziquone; elfornithine; acetato de eliptinio; etoglucid; nitrato de galio; hidroxurea; lentinan; lonidamine; maitansinoides tales como maitansina y ansamitocinas; mitoguazona; mitoxantrona; mopidamol; nitracrine; pentostatina; phenamet; pirarubicina; losoxantrona; 2-etilhidrazida; procarbazona; complejo polisacárido PSK[®] (JHS Natural Products, Eugene, OR); razoxano; rizoxina; sizofirano; espirogermanio; ácido tenuazónico; triazicuona; 2,2',2"- triclortrietilamina; tricotecenos (especialmente T-2 toxina, verracurin A, roridin A y anguidine); uretano; vindesina (ELDISINE[®], FILDESIN[®]); dacarbazina; mannomustina; mitobronitol; mitolactol; pipobroman; gacytosine; arabinósido ("Ara-C"); tiotepa; taxoides, *por ejemplo*, paclitaxel (TAXOL[®]), formulación de nanopartículas modificadas con albúmina de paclitaxel (ABRAXANE[™]), y doxetaxel (TAXOTERE[®]); clorambucil; 6-tioguanina; mercaptopurina; metotrexato; análogos de platino tales como cisplatino y carboplatino; vinblastina (VELBAN[®]); platino; etopósido (VP-16); ifosfamida; mitoxantrona; vincristina (ONCOVIN[®]); oxaliplatino; leucovorina; vinorelbina (NAVELBINE[®]); novantrona; edatrexato; daunomicina; aminopterina; ibandronato; inhibidor de topoisomerasa RFS 2000; difluorometilornitina (DMFO); retinoides tales como ácido retinoico; sales, ácidos o derivados farmacéuticamente aceptables de cualquiera de los anteriores; así como combinaciones de dos o más de los anteriores tal como CHOP, una abreviatura para una terapia combinada de ciclofosfamida, doxorubicina, vincristina, y prednisolona, y FOLFOX, una abreviatura de

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un régimen de tratamiento con oxaliplatino (ELOXATIN™) combinado con 5-FU y leucovorina.

También incluidos en esta definición se encuentran agentes anti-hormonales que actúan para regular, reducir, bloquear, o inhibir los efectos de hormonas que pueden promover el crecimiento de cáncer, y están a menudo en la forma de tratamiento sistémico, o tratamiento de todo el cuerpo. Pueden ser hormonas en sí mismas. Los ejemplos incluyen anti-estrógenos y moduladores selectivos de receptor de estrógeno (SERMs), incluidos por ejemplo tamoxifeno (incluido NOLVADEX® tamoxifeno), raloxifeno (EVISTA®), droloxifeno, 4-hidroxitamoxifeno, trioxifeno, keoxifeno, LY117018, onapristona, y toremifeno (FARESTON®); anti-progesteronas; reguladores por disminución de receptor de estrógeno (ERDs); antagonistas de receptor de estrógeno tales como fulvestrant (FASLODEX®); agentes que funcionan para suprimir o inhabilitar los ovarios, por ejemplo, agonistas de luliberina (LHRH) tales como acetato de leuprolida (LUPRON® y ELIGARD®), acetato de goserelina, acetato de buserelina y tripterelina; otros anti-andrógenas tales como flutamida, nilutamida y bicalutamida; e inhibidores de aromatasa que inhiben la enzima aromatasa, la cual regula la producción de estrógeno en las glándulas suprarrenales, tales como, por ejemplo, 4(5)-imidazoles, aminoglutetimida, acetato de megestrol (MEGASE®), exemestano (AROMASIN®), formestanie, fadrozol, vorozol (RIVISOR®), letrozol (FEMARA®), y anastrozol (AREMIDEX®). Además, tal definición de ácidos quimioterapéuticos incluye bisfosfonatos tales como clodronato (por ejemplo, BONEFOS® u OSTAC®), etidronato (DIDROCAL®), NE-58095, ácido zoledrónico/zoledronato (ZOMETA®), alendronato (FOSAMAX®), pamidronato (AREDIA®), tiludronato (SKELID®), o risedronato (ACTONEL®); así como troxacitabina (un análogo de citosina de 1,3-dioxolán nucleósido); oligonucleótidos complementarios, particularmente aquellos que inhiben la expresión de genes en rutas de señalización implicadas en proliferación celular aberrante, tal como, por ejemplo, PKC-alpha, Raf, H-Ras, y receptor de factor de crecimiento

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epidérmico (EGF-R); vacunas tales como la vacuna THERATOPE® y vacunas de terapia génica, por ejemplo, la vacuna ALLOVECTIN®, la vacuna LEUVECTIN®, y la vacuna VAXID®; inhibidor de topoisomerasa 1 (*por ejemplo*, LURTOTECAN®); mRH (*por ejemplo*, ABARELIX®); lapatinib ditosilato (un inhibidor de molécula pequeña de tirosina cinasa dual ErbB-2 y EGFR también conocido como GW572016); inhibidores de COX-2 tales como celecoxib (CELEBREX®; 4-(5-(4-metilfenil)-3-(trifluorometil)-1H-pirazol-1-il)bencenosulfonamida; y sales, ácidos o derivados farmacéuticamente aceptables de cualquiera de los anteriores.

Un "agente inhibidor de crecimiento" se refiere a un compuesto o composición que inhibe el crecimiento de una célula, cuyo crecimiento es dependiente de la activación de HGF/c-met *in vitro* o *in vivo*. Por consiguiente, el agente inhibidor de crecimiento puede ser uno que reduzca significativamente el porcentaje de células dependientes de HGF/c-met en la fase S. Ejemplos de agentes inhibidores de crecimiento incluyen agentes que bloquean la progresión del ciclo celular (en un sitio distinto de la fase S), tales como agentes que inducen la detención G1 y la detención de fase M. Bloqueadores clásicos de fase M incluyen las vincas (vincristina y vinblastina), taxanos, e inhibidores de topoisomerasa II tales como doxorubicina, epirubicina, daunorubicina, etopósido, y bleomicina. Aquellos agentes que detienen G1 pasan también a la detención de la fase S, por ejemplo, agentes alquilantes de ADN tales como tamoxifeno, prednisona, dacarbazina, mecloretamina, cisplatino, metotrexato, 5-fluorouracil, y ara-C. Más información puede encontrarse en 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), especialmente página 13). Los taxanos (paclitaxel y docetaxel) son fármacos antineoplásicos derivados ambos del árbol del tejo ("*yew tree*"). El docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derivado del tejo europeo, es un análogo semisintético de paclitaxel (TAXOL®, Bristol-Myers Squibb). El paclitaxel y el docetaxel promueven el ensamble de microtúbulos de

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dímeros de tubulina y estabilizan los microtúbulos previniendo la despolimerización, lo cual se traduce en la inhibición de la mitosis en las células.

"Doxorubicina" es un antibiótico de antraciclina. El nombre químico completo de la doxorubicina es (8S-cis)-10-[(3-amino-2,3,6-trideoxi- α -L-lixo-hexapiranosil)oxi]-7,8,9,10-tetrahidro-6,8,11-trihidroxi-8-(hidroxiacetil)-1-metoxi-5,12-naftacenodiona.

Vectores, Células Hospederas y Métodos Recombinantes

Para la producción recombinante de un anticuerpo de la invención, el ácido nucleico que lo codifica se aísla e inserta en un vector replicable para clonación posterior (amplificación del ADN) o para expresión. ADN que codifica el anticuerpo se aísla fácilmente y se secuencia usando procedimientos convencionales (*por ejemplo*, usando sondas oligonucleotídicas que son capaces de unirse específicamente a genes que codifican las cadenas pesadas y ligeras del anticuerpo). Se dispone de muchos vectores. La elección de un vector depende en parte de la célula hospedera a utilizarse. Generalmente, células hospederas preferidas son de origen procariota o eucariota (generalmente de mamífero).

Generación de anticuepos usando células hospederas procariotas:

Contrucción de Vectores

Secuencias polinucleotídicas que codifican componentes polipeptídicos del anticuerpo de la invención pueden obtenerse usando técnicas recombinantes estándar. Secuencias polinucleotídicas deseadas pueden aislarse y secuenciarse de células productoras de anticuerpos, tales como células de hibridoma. Alternativamente, pueden sintetizarse polinucleótidos usando un sintetizador de nucleótidos o técnicas PCR. Una vez obtenidos, secuencias que codifican los polipéptidos se insertan en un vector recombinante capaz de replicar y expresar polinucleótidos heterólogos en hospederos procariotas. Vectores disponibles y conocidos en el arte pueden utilizarse para el propósito de la presente invención. La selección de un vector apropiado dependerá principalmente del tamaño de los ácidos nucleicos a insertarse en el vector y la célula hospedera particular

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a transformarse con el vector. Cada vector contiene varios componentes, dependiendo de su función (amplificación o expresión de polinucleótido heterólogo, o ambas) y su compatibilidad con la célula hospedera particular en la cual reside. Los componentes del vector incluyen generalmente, pero no se limitan a: un origen de replicación, un gen marcador de selección, un promotor, un sitio de unión a ribosoma (RBS), una secuencia señal, el inserto de ácido nucleico heterólogo y una secuencia de terminación de transcripción.

En general, vectores de plásmido que contienen secuencias de replicones y control, las cuales se derivan de especies compatibles con la célula hospedera, se utilizan en conexión con estos hospederos. El vector lleva ordinariamente un sitio de replicación, así como secuencias marcadoras que son capaces de proporcionar una selección fenotípica en células transformadas. Por ejemplo, *E. coli* se transforma típicamente usando pBR322, un plásmido derivado de una especie de *E. coli*. pBR322 contiene genes que codifican resistencia a ampicilina (Amp) y tetraciclina (Tet) y, en consecuencia, provee un medio fácil para identificar células transformadas. pBR322, sus derivados, u otros plásmidos microbianos o bacteriófagos pueden contener también, o modificarse para contener, promotores que pueden utilizarse por el organismo microbiano para expresión de proteínas endógenas. Ejemplos de derivados de pBR322 utilizados para expresión de anticuerpos particulares se describen en detalle en Carter et al., Patente de los Estados Unidos No. 5,648,237.

Además, vectores de fagos que contienen secuencias de replicones y control que son compatibles con el microorganismo hospedero pueden usarse como vectores transformantes en conexión con estos hospederos. Por ejemplo, un bacteriófago tal como λ GEM.TM.-11 puede utilizarse en producir un vector recombinante, que puede empelarse para transformar células hospederas susceptibles tales como *E. coli* LE392.

El vector de expresión de la invención puede comprender dos o más cistrón-promotor, cada uno de los cuales codifica los componentes del polipéptido. Un promotor es una secuencia reguladora no traducida

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localizada antes (5') de un cistrón que modula su expresión. Típicamente los promotores procariotas caen dentro de dos clases, inducibles y constitutivos. El promotor inducible es un promotor que inicia niveles cada vez mayores de transcripción de un cistrón bajo su control en respuesta a cambios en la condición de cultivo, *por ejemplo* la presencia o ausencia de un nutriente o un cambio en temperatura.

Se conoce un gran número de promotores reconocidos por una variedad de células hospederas potenciales. El promotor seleccionado puede enlazarse funcionalmente a un ADN de cistrón que codifica la cadena ligera o pesada retirando el promotor del ADN fuente con digestión de enzimas de restricción e insertando la secuencia promotora aislada en el vector de la invención. Tanto la secuencia promotora nativa y muchos promotores heterólogos pueden usarse para dirigir la amplificación y/o expresión de los genes diana. En algunas incorporaciones, se utilizan promotores heterólogos, ya que generalmente permiten una mayor transcripción y rendimientos más altos del gen diana expresado, comparado con el promotor polipentídico diana nativo.

Promotores apropiados para uso con hospederos procariotas incluyen el promotor PhoA, los sistemas promotores de β -galactamasa y lactosa, un sistema promotor de triptófano (*trp*) y promotores híbridos tales como los promotores *tac* o *trc*. Sin embargo otros promotores que son funcionales en bacterias (tales como otros promotores de fagos o promotores bacterianos conocidos) son igualmente apropiados. Se han publicado sus secuencias nucleotídicas, permitiéndole así al profesional experto ligarlos funcionalmente a cistrones que codifican las cadenas ligeras y pesadas diana (Siebenlist et al. (1980) Cell 20: 269) usando conectores o adaptadores para suministrar cualesquiera sitios de restricción requeridos.

En un aspecto de la invención, cada cistrón en el vector recombinante comprende un componente de secuencia señal de secreción que dirige la translocación de los polipéptidos expresados a través de una membrana. En general, la secuencia señal puede ser un componente del vector, o puede ser una parte del ADN del polipéptido diana que se inserta en el vector. La

secuencia señal seleccionada para el propósito de esta invención debe ser una que se reconoce y se procesa (es decir, se corta proteolíticamente por una peptidasa de señal) por la célula hospedera. Para células procariotas hospederas que no reconocen y procesan las secuencias señal nativas con respecto a los polipéptidos heterólogos, la secuencia señal se sustituye por una secuencia señal procariota, seleccionada, por ejemplo, del grupo conformado de fosfatasa alcalina, penicilinas, Ipp, o líderes de enterotoxina II estable al calor (STII), LamB, PhoE, PeIB, OmpA y MBP. En una incorporación de la invención, las secuencias señal usadas en ambos cistrones del sistema de expresión son secuencias señal STII o sus variantes.

En otro aspecto, la producción de las inmunoglobulinas de acuerdo con la invención puede ocurrir en el citoplasma de la célula hospedera y, en consecuencia, no requiere la presencia de secuencias señales de secreción en cada cistrón. En ese aspecto, las cadenas ligeras y pesadas de inmunoglobulina expresan, pliegan y ensamblan para formar inmunoglobulinas funcionales en el citoplasma. Ciertas cepas hospederas (*por ejemplo*, las cepas *txrB* de *E. coli*) proveen condiciones de citoplasma que son favorables para la formación de puentes disulfuro, permitiendo en esa forma el plegamiento y ensamblado apropiado de subunidades de proteínas expresadas. Proba and Plückthun *Gene*, 159:203 (1995).

La presente invención provee un sistema de expresión en el cual el índice cuantitativo de componentes polipeptídicos expresados puede modularse con el fin de maximizar el rendimiento de los anticuerpos segregados y convenientemente ensamblados de la invención. Tal modulación se lleva a cabo por lo menos en parte modulando simultáneamente intensidades translacionales para los componentes polipeptídicos. Una técnica para modular intensidad translacional se divulga en Simmons et al., Patente de los Estados Unidos No. 5,840,523. Ésta utiliza variantes de la región de iniciación translacional (TIR) en un cistrón. Para una TIR dada, puede crearse una serie de variantes de secuencia de aminoácidos o ácidos nucleicos con un rango de intensidades translacionales, proveyendo en esa forma un medio conveniente con el cual

ajustar este factor para el nivel de expresión deseada de la cadena específica. Pueden generarse variantes TIR por técnicas de mutagénesis convencional que resultan en cambios de codón, los cuales alteran la secuencia de aminoácidos, aunque se prefieren cambios silenciosos en la secuencia de nucleótidos. Las alteraciones en la TIR pueden incluir, por ejemplo, alteraciones en el número o espaciamiento de secuencias Shine-Dalgarno, junto con alteraciones en la secuencia señal. Un método de generar secuencias señal mutantes es la generación de un "banco de codones" al comienzo de la secuencia codificante, que no cambia la secuencia de aminoácidos de la secuencia señal (es decir, los cambios son silenciosos). Esto puede llevarse a cabo cambiando la tercera posición del nucleótido de cada codón; adicionalmente, algunos aminoácidos, tales como leucina, serina y arginina, tienen múltiples posiciones primeras y segundas que pueden añadir complejidad en la elaboración del banco. Este método de mutagénesis se describe en detalle en Yansura et al. (1992) METHODS: A Companion to Methods in Enzymol. 4:151-158.

Preferiblemente, se genera un conjunto de vectores con un rango de intensidades TIR para cada cistrón en ellos. Este conjunto limitado ofrece una comparación de niveles de expresión de cada cadena, así como el rendimiento de los productos de anticuerpo deseados bajo varias combinaciones de intensidades TIR. Las intensidades TIR pueden determinarse cuantificando el nivel de expresión de un gen indicador como se describe en detalle en Simmons et al., Patente de los Estados Unidos No. 5,840,523. Basándose en la comparación de la intensidad translacional, se seleccionan las TIR individuales deseadas para combinarse en los constructos del vector de expresión de la invención.

Células hospederas eucariotas apropiadas para expresar anticuerpos de la invención incluyen Archaeobacteria y Eubacteria, tales como organismos Gram-negativos o Gram-positivos. Ejemplos de bacterias útiles incluyen *Escherichia* (por ejemplo, *E. coli*), *Bacilli* (por ejemplo, *B. subtilis*), *Enterobacteria*, especies de *Pseudomonas* (por ejemplo, *P. aeruginosa*), *Salmonella typhimurium*, *Serratia marcescans*, *Klebsiella*,

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Proteus, Shigella, Rhizobia, Vitreoscilla, o Paracoccus. En una incorporación, se utilizan células gram-negativa. En una incorporación, se usan células de *E. coli* como hospederos para la invención. Ejemplos de cepas de *E. coli* incluyen la cepa W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) y sus derivados, incluida la cepa 33D3 que tiene el genotipo W3110 Δ fhuA (Δ tonA) ptr3 lac Iq lacL8 Δ ompT Δ (nmpc-fepE) degP41 kan^R (Patente de los Estados Unidos No. 5,639,635). También son apropiadas otras cepas y sus derivados, tales como *E. coli* 294 (ATCC 31, 446), *E. coli* B, *E. coli* 1776 (ATCC 31,537) y *E. coli* RV308 (ATCC 31, 608). Estos ejemplos son ilustrativos y no limitantes. Métodos para construir derivados de cualquiera de las bacterias ya mencionadas, con genotipos definidos, son conocidos en el arte y se describen en, por ejemplo, Bass et al., Proteins, 8:309-314 (1990). Por lo general, es necesario seleccionar las bacterias apropiadas tomando en consideración la replicabilidad del replicón en las células de una bacteria. Por ejemplo, las especies *E. coli*, Serratia, o Salmonella pueden usarse convenientemente como el hospedero cuando se emplean para suministrar el replicón plásmidos bien conocidos tales como pBR322, pBR325, pACYC177, o pKN410. Típicamente la célula hospedera debe segregar cantidades mínimas de enzimas proteolíticas y, deseablemente, pueden incorporarse inhibidores adicionales de proteasa en el cultivo celular.

Producción de Anticuerpos

Células hospederas se transforman con los vectores de expresión ya descritos y se cultivan en medios nutrientes convencionales, modificados según sea apropiado, para inducir promotores, seleccionar transformantes, o amplificar los genes que codifican las secuencias deseadas.

Transformación significa introducir ADN en el hospedero procariota, de manera que el ADN sea replicable, ya sea como elemento extracromosómico o ya sea por integrante cromosómico. Dependiendo de la célula hospedera utilizada, la transformación se realiza usando técnicas

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estándar apropiadas a tales células. El tratamiento de calcio empleando cloruro de calcio se utiliza generalmente para células bacterianas que contienen barreras sustanciales de pared celular. Otro método de transformación emplea polietilen glicol/DMSO. Otra técnica adicional usada es la electroporación.

Células procariotas usadas para producir los polipéptidos de la invención se cultivan en medios conocidos en el arte y apropiados para cultivo de las células hospederas seleccionadas. Ejemplos de medios adecuados incluyen Luria Broth (LB) más suplementos nutrientes necesarios. En algunas incorporaciones, el medio contiene asimismo un agente de selección, escogido basándose en la construcción del vector de expresión, para permitir selectivamente el crecimiento de células procariotas que contengan el vector de expresión. Por ejemplo, se agrega ampicilina al medio para el crecimiento de células que expresan el gen de la resistencia a la ampicilina.

Cualesquiera suplementos necesarios aparte de carbono, nitrógeno y fuentes de fosfato orgánico pueden incluirse asimismo en concentraciones apropiadas, introducidas solas o como una mezcla con otro suplemento o medio tal como una fuente compleja de nitrógeno. Opcionalmente el medio de cultivo puede contener uno o más agentes reductores, seleccionados del grupo conformado por glutatión, cisteína, cistamina, tioglicolato, ditioeritritol y ditiotreitól.

Las células hospederas procariotas se cultivan a temperaturas adecuadas. Para el crecimiento de *E. coli*, por ejemplo, la temperatura preferida varía desde alrededor de 20°C a alrededor de 39°C, más preferiblemente desde alrededor de 25°C a alrededor de 37°C, aún más preferiblemente a alrededor de 30°C. El pH del medio puede ser cualquier pH que varíe desde alrededor de 5 a alrededor de 9, dependiendo principalmente del organismo hospedero. Para *E. coli*, el pH va preferiblemente desde alrededor de 6.8 a alrededor de 7.4 y, más preferiblemente, alrededor de 7.0.

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Si un promotor inducible se utiliza en el vector de expresión de la invención, la expresión de proteína se induce bajo condiciones apropiadas para la activación del promotor. En un aspecto de la invención, se utilizan promotores PhoA para controlar la transcripción de los polipéptidos. En consecuencia, las células hospederas transformadas se cultivan en un medio limitante de fosfato para inducción. Preferiblemente, el medio limitante de fosfato es el medio C.R.A.P (véase, *por ejemplo*, Simmons et al., J. Immunol. Methods (2002), 263: 133-147). Puede usarse una variedad de otros inductores, de acuerdo con el constructo del vector empleado, como se conoce en el arte.

En una incorporación, los polipéptidos expresados de la presente invención se segregan en y se recuperan del periplasma de las células hospederas. La recuperación de proteínas involucra típicamente romper el microorganismo, generalmente por medios tales como choque osmótico, sonicación o lisis. Una vez rotas las células, los desechos celulares o células enteras pueden eliminarse por centrifugación o filtración. Las proteínas pueden purificarse posteriormente, por ejemplo, por cromatografía de afinidad en resina. Alternativamente, las proteínas pueden transportarse en el medio de cultivo y aislarse allí. Las células pueden retirarse del cultivo y filtrarse y concentrarse el sobrenadante del cultivo para purificación posterior de las proteínas producidas. Los polipéptidos expresados pueden aislarse e identificarse posteriormente usando métodos comúnmente conocidos tales como electroforesis en gel de poliacrilamida (PAGE) y análisis Western blot.

En un aspecto de la invención, la producción de anticuerpos se conduce en gran cantidad por un proceso de fermentación. Para la producción de proteínas recombinantes se encuentran disponibles diferentes procesos de fermentación a gran escala con adición progresiva de sustrato ("*fed-batch*"). Las fermentaciones a gran escala tienen por lo menos 1.000 litros de capacidad, preferiblemente alrededor de 1.000 a 100.000 litros de capacidad. Estos fermentadores utilizan impulsores de agitación para distribuir oxígeno y nutrientes, especialmente glucosa (la fuente preferida

de carbono/energía). La fermentación a pequeña escala se refiere generalmente a la fermentación en un fermentador que no tiene más de aproximadamente 100 litros de capacidad volumétrica, y puede variar desde alrededor de 1 litro a alrededor de 100 litros.

En un proceso de fermentación, la inducción de la expresión de proteína se inicia típicamente una vez que las células se han cultivado bajo condiciones apropiadas a la densidad deseada, *por ejemplo*, una OD₅₅₀ de alrededor de 180-220, fase en la cual las células se encuentran en fase estacionaria temprana. Puede utilizarse una variedad de inductores, de acuerdo con el constructo de vector empleado, como se conoce en el arte y se describe más arriba. Las células pueden cultivarse por períodos más cortos antes de la inducción. Usualmente las células se inducen durante alrededor de 12-50 horas, aunque puede emplearse un tiempo de inducción más corto o más prolongado.

Para mejorar el rendimiento de la producción y la calidad de los polipéptidos de la invención, pueden modificarse varias condiciones de fermentación. Por ejemplo, para mejorar el ensamblaje y plegamiento apropiados de los polipéptidos de anticuerpo segregados, pueden usarse vectores adicionales que sobreexpresan proteínas chaperonas, tales como proteínas Dsb (DsbA, DsbB, DsbC, DsbD y/o DsbG) o FkpA (una peptidilprolil cis,trans-isomerasa con actividad chaperona) para co-transformar las células procariotas hospederas. Se ha demostrado que las proteínas chaperonas facilitan el plegamiento apropiado y la solubilidad de proteínas heterólogas producidas en células hospederas bacterianas. Chen et al. (1999) J. Bio. Chem 274:19601- 19605; Georgiou et al., Patente de los Estados Unidos No. 6,083,715; Georgiou et al., Patente de los Estados Unidos 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.

Para minimizar la proteólisis de proteínas heterólogas expresadas (especialmente aquellas que son proteolíticamente sensibles), pueden usarse para la presente invención ciertas cepas hospederas deficientes en enzimas

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proteolíticas. Por ejemplo, cepas de células hospederas pueden modificarse para efectuar mutación (mutaciones) genética(s) en los genes que codifican proteasas bacterianas conocidas tales como Proteasa III, OmpT, DegP, Tsp, Proteasa I, Proteasa Mi, Proteasa V, Proteasa VI y sus combinaciones. Algunas cepas deficientes en proteasa de *E. coli* se encuentran disponibles y se describen en, por ejemplo, Joly et al. (1998), *supra*; Georgiou et al., Patente de los Estados Unidos No. 5,264,365; Georgiou et al., Patente de los Estados Unidos No. 5,508,192; Hara et al., Microbial Drug Resistance, 2:63-72 (1996).

En una incorporación, cepas de *E. coli* deficientes en enzimas proteolíticas y transformadas con plásmidos, que sobreexpresan una o más proteínas chaperonas, se utilizan como células hospederas en el sistema de expresión de la invención.

Purificación de Anticuerpos

En una incorporación, la proteína de anticuerpo producida en esta solicitud se purifica posteriormente para obtener preparaciones que son sustancialmente homogéneas para análisis y usos adicionales. Pueden emplearse métodos estándar de purificación de proteínas conocidos en el arte. Los siguientes procedimientos son ejemplares de procedimientos apropiados de purificación: fraccionamiento con respecto a inmunoafinidad o columnas de intercambio iónico, precipitación en etanol, HPLC de fase inversa, cromatografía en sílice o en una resina de intercambio catiónico tal como DEAE, cromatofocado, SDS-PAGE, precipitación en sulfato de amonio, y filtración en gel usando, por ejemplo, Sephadex G-75.

En un aspecto, Proteína A inmovilizada en una fase sólida se usa para purificación por inmunoafinidad de los productos de anticuerpo de longitud total de la invención. Proteína A es una proteína de pared celular de 41kD de *Staphylococcus aureus* que se une con una alta afinidad a la región Fc de anticuerpos. Lindmark et al (1983) J. Immunol. Meth. 62:1-13. La fase sólida en la cual se inmoviliza la Proteína A es preferiblemente una columna que comprende una superficie de vidrio o sílice, más preferiblemente una columna de vidrio de poros controlados o una columna

de ácido silícico. En algunas aplicaciones, la columna puede recubrirse con un reactivo, tal como glicerina, en un intento por prevenir la adherencia no específica de contaminantes.

Como el primer paso de purificación, la preparación derivada del cultivo celular, como ya se describió, se aplica en la fase inmovilizada de la Proteína A para permitir la unión específica del anticuerpo de interés a la Proteína A. A continuación se lava la fase sólida para eliminar los contaminantes no ligados específicamente a la fase sólida. Finalmente, se recupera el anticuerpo de interés de la fase sólida mediante elución.

Generación de anticuerpos utilizando células hospederas eucariotas:

Los componentes del vector incluyen generalmente, pero no se limitan a, uno o más de los siguientes: una secuencia señal, un origen de replicación, uno o más genes marcadores, un elemento potenciador, un promotor, y una secuencia de terminación de transcripción,

(i) Componente de secuencia señal

Un vector para uso en una célula hospedera eucariota puede contener asimismo una secuencia señal u otro polipéptido con un sitio de corte proteolítico específico en el N-término de la proteína madura o polipéptido de interés. La secuencia señal heteróloga seleccionada es preferiblemente una que es reconocida y procesada (es decir, segmentada por una peptidasa de señal) por la célula anfitriona. En expresión de células de mamífero, se dispone de las secuencias señal de mamífero, así como líderes secretorios víricos, por ejemplo, la señal gD del herpes simplex.

El ADN para tal región de precursor se liga en el de región de estructura alterna de lectura a ADN que codifica el anticuerpo.

(ii) Origen de replicación

Generalmente, no se necesita un componente de origen de replicación para vectores de expresión mamíferos. Por ejemplo, el origen SV40 puede usarse típicamente sólo porque contiene el promotor precoz.

(iii) Componente del gen de selección

Vectores de expresión y clonación pueden contener un gen de selección, conocido también como un marcador seleccionable. Genes de

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selección típicos codifican proteínas que (a) confieren resistencia a antibióticos u otras toxinas, *por ejemplo*, ampicilina, neomicina, metotrexato, o tetraciclina, (b) complementan deficiencias auxotróficas, donde sea relevante, o (c) aportan nutrientes críticos no disponibles del medio complejo.

Un ejemplo de un esquema de selección utiliza un medicamento para detener el crecimiento de una célula hospedera. Aquellas células, que se transforman exitosamente con un gen heterólogo, producen una proteína que confiere resistencia a medicamento y, por consiguiente, sobreviven al régimen de selección. Ejemplos de tal selección dominante utilizan los medicamentos neomicina, ácido micofenólico e higromicina.

Otro ejemplo de marcadores seleccionables apropiados para células de mamífero son aquellos que permiten la identificación de células competentes para incorporar el ácido nucleico del anticuerpo, tales como DHFR, timidina cinasa, metalotioneína-I y -II, preferiblemente genes metalotioneína de primate, adenosina deaminasa, ornitina decarboxilasa, etc.

Por ejemplo, células transformadas con el gen de selección DHFR se identifican primeramente cultivando la totalidad de los transformantes en un medio de cultivo que contiene metotrexato (Mtx), un antagonista competitivo de DHFR. Una célula hospedera apropiada, cuando se emplea DHFR natural, es la línea celular de ovario de hámster chino (CHO), deficiente en actividad DHFR (*por ejemplo*, ATCC CRL-9096).

Alternativamente, células hospederas (particularmente hospederas naturales que contienen DHFR endógeno) transformadas o co-transformadas con secuencias de ADN que codifica un anticuerpo, proteína DHFR natural, y otro marcador seleccionable tal como aminoglucósido 3'-fosfotransferasa (APH) pueden seleccionarse por crecimiento celular en un medio que contiene un agente de selección para el marcador seleccionable tal como un antibiótico aminoglucosídico, *por ejemplo*, canamicina, neomicina, o G418. Véase Patente de los Estados Unidos No. 4,965,199.

(iv) Componente de promotor

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Usualmente los vectores de expresión y clonación contienen un promotor que es reconocido por el organismo hospedero y se enlaza operacionalmente al ácido nucleico del polipéptido del anticuerpo. Secuencias promotoras son conocidas para eucariotas. Virtualmente todos los genes eucariotas que tienen una región rica en AT localizada aproximadamente 25 a 30 bases antes del sitio donde se inicia la transcripción. Otra secuencia encontrada 70 a 80 bases antes del inicio de transcripción de muchos genes es una región CNCAAT donde N puede ser cualquier nucleótido. En el extremo 3' de la mayoría de los genes eucariotas está una secuencia AATAAA que puede ser la señal para adición de la cola poly A al extremo 3' de la secuencia codificante. Todas estas secuencias se insertan apropiadamente en vectores de expresión eucariotas.

La transcripción de polipéptidos de anticuerpo de vectores en células huéspedes mamíferas puede controlarse, por ejemplo, por promotores obtenidos de los genomas de virus tales como el virus como el virus polioma, el virus de la viruela aviar, adenovirus (tales como Adenovirus 2), virus del papiloma bovino, virus del sarcoma aviar, citomegalovirus, un retrovirus, virus de la hepatitis-B y el virus de los simios 40 (SV40), de promotores heterólogos mamíferos, *por ejemplo*, el promotor de actina o un promotor de inmunoglobulina, y de promotores de choque térmico, a condición de que tales promotores sean compatibles con los sistemas de células huéspedes.

Los promotores precoces y tardíos del virus SV40 se obtienen convenientemente de un fragmento de restricción de SV40 que contiene asimismo el origen de replicación vírica SV40. El promotor precoz inmediato del citomegalovirus humano se obtiene convenientemente como fragmento de restricción HindIII E. Un sistema para expresar ADN, en hospederos mamíferos usando el virus de papiloma bovino como vector, se divulga en la Patente de los Estados Unidos No. 4,419,446. Una modificación de este sistema se describe en la Patente de los Estados Unidos No. 4,601,978. Véase también Reyes *et al.*, *Nature* 297:598-601 (1982) respecto a la expresión de ADNc de interferón β humano en células

de ratón bajo el control de un promotor de timidina cinasa del virus herpes simplex. Alternativamente, la repetición de terminal larga del virus de sarcoma de Rous puede usarse como el promotor.

(v) Componente del elemento intensificador

La transcripción de un ADN que codifica el polipéptido de anticuerpo de esta invención por eucariotas superiores se incrementa a menudo insertando una secuencia intensificadora en el vector. Actualmente se conocen muchas secuencias de intensificadores de genes de mamífero (globina, elastasa, albúmina, α -fetoproteína e insulina). Típicamente, sin embargo, se usará un intensificador de un virus de célula eucariótica. Ejemplos incluyen el intensificador SV40 en el lado tardío del origen de replicación (bp 100-270), el intensificador de promotor precoz de citomegalovirus, el intensificador de poliovirus en el lado tardío del origen de replicación e intensificadores de adenovirus. Véase también Yaniv, *Nature* 297:17-18 (1982) con respecto a elementos intensificadores para activación de promotores eucariotas. El intensificador puede empalmarse en el vector en una posición 5' ó 3' a la secuencia codificante del anticuerpo, pero se localiza preferiblemente en el sitio 5' del promotor.

(vi) Componente de terminación de transcripción

Típicamente, los vectores de expresión usados en células hospederas eucariotas contendrán asimismo secuencias necesarias para la terminación de transcripción y para estabilizar el ARNm. Tales secuencias se encuentran comúnmente disponibles de las regiones 5' y, ocasionalmente 3', no traducidas, de ADN víricos o eucariotas o ADNc. Estas regiones contienen segmentos de nucleótido transcritos como fragmentos poliadenilados en la porción no traducida del ARNm que codifica un anticuerpo. Un componente útil de terminación de transcripción es la región de poliadenilación de somatropina bovina. Véase WO94/11026 y el vector de expresión divulgado en esta solicitud.

(vii) Selección y transformación de células hospederas

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Células hospederas apropiadas para clonar o expresar el ADN en los vectores en esta solicitud incluyen células eucariotas más especializadas descritas aquí, incluidas células hospederas de vertebrados. Propagación de células de vertebrado en cultivo (cultivo de tejido) se ha vuelto un procedimiento de rutina. Ejemplos de líneas celulares de hospederos mamíferos útiles son la línea CV1 de riñón de mono transformada por SV40 (COS-7, ATCC CRL 1651); la línea de riñón embrionario humano (células 293 ó 293 subclonadas para crecimiento en cultivo en suspensión, Graham et al., J. Gen Virol. 36:59 (1977)); células de riñón de crías de hámster (BHK, ATCC CCL 10); células de ovario de hámster chino/- DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)); células Sertoli de ratón (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); células de riñón de mono (CV1 ATCC CCL 70); células de riñón de mono African green (VERO-76, ATCC CRL-1587); células de carcinoma cervical humano (HELA, ATCC CCL 2); células de riñón canino (MDCK, ATCC CCL 34); células hepáticas de rata buffalo (BRL 3A, ATCC CRL 1442); células de pulmón humanas (W138, ATCC CCL 75); células hepáticas humanas (Hep G2, HB 8065); tumor mamario de ratón (MMT 060562, ATCC CCL51), células TRI (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 (1982)), células MRC 5; células FS4; una línea de hepatoma humano (Hep G2).

Células hospederas se transforman con los vectores de expresión y clonación ya descritos para producción de anticuerpos y se cultivan en medio nutriente convencional, modificado según sea apropiado para inducir promotores, seleccionar transformantes, o amplificar los genes que codifican las secuencias deseadas.

(viii) Cultivo de las células hospederas

Las células hospederas usadas para producir el anticuerpo de esta invención pueden cultivarse en una variedad de medios. Medios disponibles en el comercio tales como Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), y Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) son apropiados para cultivar las células hospederas. Adicionalmente, cualquiera de los medios descritos

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en Ham et al., Meth. Enz. 58:44 (1979), Barnes et al., Anal. Biochem. 102:255 (1980), Patentes de los Estados Unidos Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; o 5,122,469; WO 90/03430; WO 87/00195; o Patente de los Estados Unidos Re. 30,985 puede utilizarse como medio de cultivo para las células hospederas. Cualquiera de estos medios pueden suplementarse, según sea necesario, con hormonas y/u otros factores de crecimiento (tales como insulina, transferrina, o factor de crecimiento epidérmico), sales (tales como cloruro de sodio, calcio, magnesio, y fosfato), tampones (tales como HEPES), nucleótidos (tales como adenosina y timidina), antibióticos (tales como el medicamento GENTAMYCIN™), oligoelementos (definidos como compuestos inorgánicos usualmente presentes en concentraciones finales en el rango micromolar), y glucosa o una fuente de energía equivalente. Cualesquiera otros suplementos necesarios pueden incluirse asimismo en concentraciones apropiadas serían conocidos a los expertos en el arte. Las condiciones de cultivo, tales como temperatura, pH, y similares, son las empleadas previamente con la célula hospedera seleccionada para expresión, y serán evidentes para el profesional de experiencia ordinaria.

(ix) Purificación de anticuerpo

Cuando se utilizan técnicas recombinantes, el anticuerpo puede producirse intracelularmente, o secretarse directamente en el medio. Si el anticuerpo se produce intracelularmente, como un primer paso, los desechos de particulado, ya sea células hospederas o fragmentos lisados, se retiran, por ejemplo, por centrifugación o ultrafiltración. Donde el anticuerpo se secreta en el medio, generalmente los sobrenadantes de tales sistemas de expresión se concentran primero usando un filtro de concentración de proteína disponible en el comercio, por ejemplo, una unidad de ultrafiltración Amicon o Millipore Pellicon. Un inhibidor de proteasa tal como PMSF puede incluirse en cualquiera de los pasos anteriores para inhibir proteólisis, y antibióticos pueden incluirse para prevenir el crecimiento de contaminantes adventicios.

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La composición de anticuerpo, preparada de las células, puede purificarse usando, por ejemplo, cromatografía de hidroxilapatita, electroforesis en gel, diálisis, y cromatografía de afinidad, siendo la cromatografía de afinidad la técnica de purificación preferida. La idoneidad de la proteína A como ligando de afinidad depende de la especie e isotipo de cualquier región Fc de inmunoglobulina que está presente en el anticuerpo. La proteína A puede usarse para purificar anticuerpos que se basan en cadenas pesadas humanas $\gamma 1$, $\gamma 2$, ó $\gamma 4$ (Lindmark *et al.*, *J. Immunol. Meth.* 62:1-13 (1983)). La proteína G se recomienda para todos los isotipos de ratón y para $\gamma 3$ humana (Guss *et al.*, *EMBO J.* 5:15671575 (1986)). La matriz a la cual se une el ligando de afinidad es muy a menudo agarosa, pero se encuentran disponibles otras matrices. Matrices mecánicamente estables, tales como cristal poroso controlado o poli(estirendivinil)benceno, permiten velocidades de flujo más rápidas y tiempos más cortos de procesamiento de los que pueden lograrse con agarosa. Donde el anticuerpo comprende un dominio C_H3 , la resina Bakerbond ABX™ (J. T. Baker, Phillipsburg, NJ) es útil para purificación. Dependiendo del anticuerpo a recuperarse, se encuentran disponibles también otras técnicas para purificación de proteínas tales como fraccionamiento en columna de intercambio iónico, precipitación en etanol, HPLC de fase reversa, cromatografía en sílice, cromatografía en heparina, cromatografía en SEPHAROSE™ o una resina de intercambio aniónico o catiónico (tal como una columna de ácido poliaspártico), cromatofocoso ("chromatofocusing"), SDS-PAGE, y precipitación en sulfato de amonio.

Luego de cualquier paso(s) de purificación preliminar, la mezcla que comprende el anticuerpo de interés y contaminantes puede someterse a cromatografía de interacción hidrófoba de pH bajo usando un amortiguador de elución con un pH entre alrededor de 2.5-4.5, preferiblemente se realiza con concentraciones bajas de sal (*por ejemplo*, desde alrededor de 0-0.25M de sal).

Análisis de Actividad

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Los anticuerpos de la presente invención pueden caracterizarse con respecto a sus propiedades físicas/químicas y funciones biológicas mediante varios análisis conocidos en el arte. Las inmunoglobulinas purificadas pueden caracterizarse además por una serie de análisis incluidos, pero no limitados a, secuenciación N-terminal, análisis de aminoácidos, cromatografía líquida de alto rendimiento con exclusión de tamaño no desnaturizadora (HPLC), espectrometría de masas, cromatografía de intercambio iónico, y digestión con papaína.

En algunas incorporaciones, las inmunoglobulinas de la presente invención se prueban en cuanto a su actividad de unión de antígeno. Los análisis de unión de antígeno son conocidos en el arte y los que pueden emplearse en esta solicitud incluyen sin limitación cualesquiera análisis de unión competitiva que usan técnicas tales como western blots, radioinmunoensayos, ELISA (enzimoinmunoanálisis absorbente), inmunoensayos tipo "sandwich", análisis de inmunoprecipitación, inmunoensayos fluorescentes, e inmunoensayos de proteína A. Análisis ilustrativos de unión de antígeno se proveen más adelante en la sección de Ejemplos.

En una incorporación, la presente invención contempla un anticuerpo alterado que posee algunas, pero no todas las funciones efectoras, que lo hacen un candidato deseado para muchas aplicaciones en las cuales es importante la semivida del anticuerpo *in vivo*, pero algunas funciones efectoras (tales como complemento y ADCC) son innecesarias o nocivas. En ciertas incorporaciones, las actividades Fc de la inmunoglobulina producida se miden para garantizar que únicamente se mantengan las propiedades deseadas. Análisis de citotoxicidad *in vitro* y/o *in vivo* pueden conducirse para confirmar la reducción/depleción de actividades CDC y/o ADCC. Por ejemplo, pueden conducirse análisis de unión de receptor Fc (FcR) para garantizar que el anticuerpo carezca de unión FcγR (careciendo, por lo tanto, igualmente de actividad ADCC), pero retiene la capacidad de unión FcRn. Las células primarias para mediar ADCC, linfocitos citolíticos naturales ("*NK cells*"), expresan FcγRIII únicamente, mientras que los

monocitos expresan FcγRL FcγRII y FcγRIII. La expresión de FcR en células hematopoyéticas se resume en la Tabla 3 en la página 464 de Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). En ejemplo de un análisis *in vitro* para evaluar la actividad ADCC de una molécula de interés se describe en la Patente de los Estados Unidos No. 5,500,362 o 5,821,337. Células efectoras útiles para tales ensayos incluyen células mononucleares de sangre periférica (PBMC) y linfocitos citolíticos naturales (NK). Alternativa, o adicionalmente, la actividad ADCC de la molécula de interés puede evaluarse *in vivo*, por ejemplo, en un modelo animal tal como el divulgado en Clynes *et al.*, *PNAS (USA)* 95:652-656 (1998). Asimismo pueden llevarse a cabo análisis de unión de C1q para confirmar que el anticuerpo es incapaz de unir C1q y, por lo tanto, carece de actividad CDC. Para evaluar la activación de complemento, puede realizarse un análisis CDC, *por ejemplo*, como se describe en Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996). Las determinaciones de unión FcRn y depuración/semivida *in vivo* pueden realizarse igualmente usando métodos conocidos en el arte.

Anticuerpos Humanizados

La presente invención abarca anticuerpos humanizados. Varios métodos para humanizar anticuerpos no humanos son conocidos en el arte. Por ejemplo, un anticuerpo humanizado puede tener uno o más residuos de aminoácidos introducidos en él de una fuente que es no humana. Estos residuos de aminoácidos no humanos se denominan a menudo residuos de "importación", los cuales se toman típicamente de un dominio variable de "importación". La humanización puede realizarse esencialmente siguiendo el método de Winter *et al.*, Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.* (1988) *Nature* 332:323-327; Verhoeyen *et al.* (1988) *Science* 239: 1534-1536), sustituyendo secuencias de región hipervariable por las secuencias correspondientes de un anticuerpo humano. En consecuencia, tales anticuerpos "humanizados" son anticuerpos quiméricos (Patente de los Estados Unidos No. 4,816,567), en donde sustancialmente menos de un dominio variable humano intacto se ha sustituido por la

secuencia correspondiente de una especie no humana. En la práctica, anticuerpos humanizados son típicamente anticuerpos humanos en los cuales algunos residuos de región hipervariable y, posiblemente, algunos residuos FR se sustituyen con residuos de sitios análogos en anticuerpos de roedor.

La elección de dominios variables humanos, livianos y pesados, para usarlos en elaborar los anticuerpos humanizados es muy importante para reducir la antigenicidad. De acuerdo con el denominado método de "ajuste óptimo", se criba la secuencia del dominio variable de un anticuerpo de roedor contra toda la biblioteca de secuencias de dominio variable conocidas. La secuencia humana que es más próxima a la del roedor se acepta entonces como la región de estructura alterna humana para el anticuerpo humanizado (Sims et al. (1993) J. Immunol. 151:2296; Chothia et al. (1987) J. Mol. Biol. 196:901. Otro método usa una región de estructura alterna particular derivada de la secuencia de consenso de todos los anticuerpos humanos de un subgrupo particular de cadenas ligeras y pesadas. La misma estructura puede utilizarse para varios anticuerpos humanizados diferentes (Carter et al. (1992) Proc. Natl. Acad. Sci. USA, 89:4285; Presta et al. (1993) J. Immunol., 151:2623.

Es además importante que los anticuerpos se humanicen conservando una alta afinidad para el antígeno y otras propiedades biológicas favorables. Para lograr este objetivo, de acuerdo con un método, anticuerpos humanizados se preparan por un proceso de análisis de las secuencias progenitoras y varios productos humanizados conceptuales usando modelos tridimensionales de las secuencias progenitoras y humanizadas. Comúnmente se dispone de modelos tridimensionales de inmunoglobulina y son familiares a los expertos en el arte. Se dispone de programas de computador, los cuales ilustran y visualizan estructuras probables de conformación tridimensional de secuencias seleccionadas de inmunoglobulina candidata. La inspección de estas visualizaciones permite el análisis del papel probable de los residuos en el funcionamiento de la secuencia de inmunoglobulina candidata, es decir, el análisis de residuos

que influyen en la capacidad de la inmunoglobulina candidata de fijar su antígeno. De esta forma, pueden seleccionarse residuos FR y combinarse de las secuencias receptoras y de importación, de modo que se logre la característica del anticuerpo deseado, tal como creciente afinidad para el antígeno(s) diana. En general, los residuos de región hipervariable se hallan directa y, la mayoría de las veces, sustancialmente implicados en influir en la unión del antígeno.

Variantes de Anticuerpo

En un aspecto, la invención provee un anticuerpo que comprende modificaciones en la interface de polipéptidos Fc, que incluyen la región Fc, en donde las modificaciones facilitan y/o promueven la heterodimerización. Estas modificaciones comprenden la introducción de una protuberancia en un primer polipéptido Fc y una cavidad en un segundo polipéptido Fc, en donde la protuberancia es posicionable en la cavidad de tal modo que promueva el acomplejamiento de los polipéptidos FC primeros y segundos. Métodos de generar anticuerpos con estas modificaciones son conocidas en el arte, *por ejemplo*, como se describe en la Patente de los Estados Unidos No. 5,731,168.

En algunas incorporaciones, se contempla(n) modificación (modificaciones) de la secuencia de aminoácidos de los anticuerpos descritos en esta solicitud. Por ejemplo, puede ser deseable mejorar la afinidad de unión y/u otras propiedades biológicas del anticuerpo. Variantes de la secuencia de aminoácidos del anticuerpo se preparan introduciendo cambios de nucleótidos apropiados en el ácido nucleico del anticuerpo, o mediante síntesis peptídica. Tales modificaciones incluyen, por ejemplo, deleciones de, y/o inserciones en y/o sustituciones de, residuos en las secuencias de aminoácidos del anticuerpo. Cualquier combinación de deleción, inserción y sustitución se hace para llegar al constructo final, siempre que el constructo final posea las características deseadas. Las alteraciones de aminoácidos pueden introducirse en la secuencia de aminoácidos del anticuerpo en cuestión en el momento de hacer la secuencia.

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Un método útil para la identificación de ciertos residuos o regiones del anticuerpo que son sitios preferidos para la mutagénesis se denomina "mutagénesis dirigida de rastreo de alanina" [*"alanine-scanning mutagenesis"*] como se describe por Cunningham y Wells (1989) Science, 244: 1081-1085. Aquí, se identifica un residuo o grupo de residuos diana (*por ejemplo*, residuos cargados tales como arg, asp, his, lys, y glu) y se reemplazan por un aminoácido neutro o cargado negativamente (muy preferiblemente alanina o polialanina) para afectar la interacción de los aminoácidos con antígeno. Aquellas localizaciones de aminoácidos, que demuestren sensibilidad funcional a las sustituciones, se refinan entonces introduciendo variantes adicionales u otras en, o para, los sitios de sustitución. Por lo tanto, aunque se predetermina el sitio para introducir una variación de secuencia de aminoácidos, la naturaleza de la mutación *per se* no necesita determinarse. Por ejemplo, para analizar el desempeño de una mutación en un sitio dado, la mutagénesis de rastreo *ala* o aleatoria se conduce en el codón o región diana y las inmunoglobulinas expresadas se criban con respecto a la actividad deseada.

Inserciones de secuencia de aminoácidos incluyen fusiones de terminal amino- y/o carboxilo, que varían en longitud desde un residuo hasta polipéptidos que contienen cien o más residuos, así como inserciones intrasecuencia de residuos de aminoácidos únicos o múltiples. Ejemplos de inserciones de terminal incluyen un anticuerpo con un residuo metionilo N-terminal o el anticuerpo fusionado a un polipéptido citotóxico. Otras variantes de inserción de la molécula del anticuerpo incluyen la fusión al término N o C del anticuerpo de una enzima (*por ejemplo*, para ADEPT) o un polipéptido que incrementa la semivida del anticuerpo.

Otro tipo de variante es una variante de sustitución de aminoácidos. Estas variantes tienen por lo menos un residuo de aminoácidos en la molécula del anticuerpo reemplazada por un residuo diferente. Los sitios de máximo interés para la mutagénesis de sustitución incluyen las regiones hipervariables, aunque se contemplan también alteraciones FR. Sustituciones conservadoras se muestran en la Tabla 1 bajo el

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encabezamiento de "sustituciones preferidas". Si tales sustituciones resultan en un cambio en la actividad biológica, entonces cambios más sustanciales, denominados "sustituciones ejemplares" en Tabla 1, o como se describe con mayor detalle más adelante con referencia a clases de aminoácidos, pueden introducirse y evaluarse selectivamente los productos.

Tabla 1

Residuo Original	Sustituciones Ejemplares	Sustituciones Preferidas
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; Norleucina	Leu
Leu (L)	Norleucina; 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; Norleucina	Leu

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Modificaciones sustanciales en las propiedades biológicas del anticuerpo se llevan a cabo seleccionando sustituciones que difieren significativamente en su efecto sobre el mantenimiento de (a) la estructura del esqueleto polipeptídico en el área de la sustitución, por ejemplo, como una hoja o conformación helicoidal, (b) la carga de hidrofobicidad de la molécula en el sitio diana, o (c) la parte principal de la cadena lateral. Los aminoácidos pueden agruparse de acuerdo con similitudes en las propiedades de sus cadenas laterales (en: A.L. Lehninger, in *Biochemistry*, second ed., pp 73-75, Worth Publishers, New York (1975))

(1) no polares Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) polares sin carga: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) ácidos: Asp (D), Glu (E)

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

Alternativamente, residuos de ocurrencia natural se dividen en grupos basados en propiedades comunes de cadena lateral:

(1) hidrófobo: Norleucina, Met, Ala, Val, Leu, Ile;

(2) hidrófilo neutro: Cys, Ser, Thr, Asn, Gln;

(3) ácido: Asp, Glu;

(4) básico: His, Lys, Arg;

(5) residuos que influyen en la orientación de la cadena: Gly, Pro;

y

(6) aromático: Trp, Tyr, Phe.

Sustituciones no conservadoras incluirán intercambiar un miembro de una de estas clases por otra clase. Tales residuos sustituidos pueden introducirse asimismo en los sitios de sustitución conservadores o, más preferiblemente, en los sitios restantes (no conservados).

Un tipo de variante de sustitución involucra sustituir uno o más residuos de región hipervariable de un anticuerpo progenitor (*por ejemplo*,

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un anticuerpo humano o humanizado). Generalmente, la variante(s) resultante para el desarrollo posterior tendrá propiedades biológicas mejoradas relativas al anticuerpo progenitor, del cual se generan. Un modo conveniente para generar tales variantes de sustitución es maduración de afinidad usando la técnica exposición en fago. En síntesis, varios sitios de región hipervariable (*por ejemplo*, 6-7 sitios) se mutan para generar todas las sustituciones posibles de aminoácidos en cada sitio. Los anticuerpos así generados se exponen de partículas de bacteriófagos filamentosos como fusiones al producto del gen III de M13 empaquetado con cada partícula. Las variantes expuestas en bacteriófagos se evalúan entonces selectivamente con respecto a su actividad biológica (*por ejemplo*, afinidad de unión), tal como se divulga en esta solicitud. Con el fin de identificar sitios candidatos de región hipervariable para modificación, puede realizarse mutagénesis dirigida de rastreo de alanina para identificar residuos de región hipervariable que contribuyen significativamente a la unión de antígeno. Alternativa, o adicionalmente, puede ser benéfico analizar una estructura cristalina del complejo antígeno-anticuerpo para identificar puntos de contacto entre el anticuerpo y el antígeno. Tales residuos de contacto y residuos contiguos son candidatos para sustitución de acuerdo con las técnicas elaboradas en esta solicitud. Una vez se generan tales variantes, el panel de variantes se somete a cribado ("*screening*"), como se describe en esta solicitud, y pueden seleccionarse para desarrollo posterior anticuerpos con propiedades superiores en uno o más análisis relevantes.

Moléculas de ácido nucleico que codifican variantes de secuencia de aminoácidos del anticuerpo se preparan por una variedad de métodos conocidos en el arte. Estos métodos incluyen, pero no se limitan a, aislamiento de una fuente natural (en el caso de variantes de secuencia de aminoácidos de ocurrencia natural) o preparación por mutagénesis mediada por oligonucleótido (o dirigida al sitio), mutagénesis de PCR, y mutagénesis de casete de una variante previamente preparada o una versión no-variante del anticuerpo.

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Puede ser deseable introducir una o más modificaciones de aminoácidos en una región Fc de los polipéptidos de inmunoglobulinas de la invención, generando así una variante de la región Fc. La variante de la región Fc puede comprender una secuencia de región Fc humana (*por ejemplo*, una región Fc de IgG1, IgG2, IgG3 o IgG4 Fc humana) que incluye una modificación de aminoácido (*por ejemplo*, una sustitución) en una o más posiciones de aminoácidos incluyendo la de una cisteína de unión flexible.

De acuerdo con esta descripción y las enseñanzas del arte, se contempla que en algunas incorporaciones, un anticuerpo utilizado en métodos de la invención puede comprender una o más alteraciones, comparado con el anticuerpo contraparte natural, *por ejemplo*, en la región Fc. No obstante, estos anticuerpos conservarían sustancialmente las mismas características requeridas para la utilidad terapéutica, comparados con su contraparte natural. *Por ejemplo*, se cree que ciertas alteraciones pueden introducirse en la región Fc, que resultarían en unión C1q alterada (es decir, mejorada o disminuída) y/o Citotoxicidad Dependiente de Complemento (CDC), *por ejemplo*, como se describe en WO99/51642. Véase también Duncan & Winter Nature 322:738-40 (1988); Patente de los Estados Unidos No. 5,648,260; Patente de los Estados Unidos No. 5,624,821; y WO94/29351 en lo concerniente a otros ejemplos de variantes de región Fc.

Inmunoconjugados

La invención concierne además a inmunoconjugados, o conjugados anticuerpo-medicamento (ADC), que comprenden un anticuerpo conjugado a un agente citotóxico tal como un agente quimioterapéutico, un medicamento, un agente inhibidor de crecimiento, una toxina (*por ejemplo*, una toxina enzimáticamente activa de origen bacteriano, fúngico, vegetal, o animal, o fragmentos de la misma), o un isótopo radioactivo (*es decir*, un radioconjugado).

El uso de conjugados anticuerpo-medicamento para la administración local de agentes citotóxicos o citostáticos, es decir fármacos para eliminar o inhibir células tumorales en el tratamiento de cáncer (Syrigos and Epenetos (1999) *Anticancer Research* 19:605-614; Niculescu-Duvaz and Springer (1997) *Adv. Drug Del. Rev.* 26:151-172; Non civium ardor prava jubentium, 4975278) permite, en teoría, la administración selectiva ("*targeted delivery*") de la fracción de medicamento a tumores, y la acumulación intracelular en ellos, donde la administración sistémica de estos agentes de medicamento no conjugado puede resultar en niveles inaceptables de toxicidad a las células normales, así como las células tumorales que se busca eliminar (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. (eds), pp.475-506). Con ello se busca la máxima eficacia con una toxicidad mínima. Se ha divulgado que los anticuerpos policlonales y los anticuerpos monoclonales son útiles en estas estrategias (Rowland et al (1986) *Cancer Immunol. Immunother.* 21:183-87). Los medicamentos empleados en estos métodos incluyen daunomicina, doxorubicina, metotrexato, y vindesina (Rowland et al., (1986) *supra*). Las toxinas usadas en conjugados anticuerpo-toxina incluyen toxinas bacterianas tales como toxina de la difteria, toxinas vegetales tales como ricina, toxinas de molécula pequeña tales como geldanamicina (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), maitansinoides (EP 1391213; Liu et al (1996) *Proc. Natl. Acad. Sci. USA* 93:8618-8623), y caliqueamicina (Lode et al (1998) *Cancer Res.* 58:2928; Hinman et al (1993) *Cancer Res.* 53:3336-3342). Las toxinas pueden producir sus efectos citotóxicos y citostáticos mediante mecanismos tales como inhibición de unión de tubulina, unión de ADN, o topoisomerasa. Algunos medicamentos citotóxicos tienden a ser inactivos, o menos activos, cuando se conjugan con grandes anticuerpos o ligandos de receptor de proteína.

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ZEVALIN® (ibritumomab tiuxetan, Biogen/Idec) es un conjugado anticuerpo-radioisótopo compuesto de un anticuerpo monoclonal de IgG1 kappa murina, dirigido contra el antígeno CD20 y ^{111}In o ^{90}Y radioisótopo unido por un quelante-enlazador tiourea (Wiseman et al (2000) Eur. J. 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). Aunque el ZEVALIN posee actividad contra Linfoma No Hodgkiniano de Linfocitos B (NHL), la administración resulta en citopenias graves y prolongadas en la mayoría de los pacientes. MYLOTARG™ (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), un conjugado anticuerpo-medicamento compuesto de un anticuerpo huCD33 enlazado a caliqueamicina, fue aprobado en 2000 para el tratamiento de leucemia mieloide mediante inyección (Drugs of the Future (2000) 25(7):686; US 4970198; US 5079233; US 5585089; US 5606040; US 5693762; US 5739116; US 5767285; US 5773001). Cantuzumab mertansina (Immunogen, Inc.), un conjugado anticuerpo-medicamento compuesto del anticuerpo huC242, enlazado mediante el enlazador disulfuro SPP a la fracción de medicamento maitansinoide, DM1, ha pasado a estudios clínicos de Fase II para el tratamiento de neoplasias malignas que expresan CanAg, tales como cáncer de colon, páncreas, gástrico, y otros. MLN-2704 (Millennium Pharm., BZL Biologies, Immunogen Inc.), un conjugado anticuerpo-medicamento compuesto del anticuerpo monoclonal del antígeno de membrana específico anti-próstata (PSMA) enlazado a la fracción de fármaco maitansinoide, DM1, se encuentra bajo desarrollo para el tratamiento potencial de tumores de la próstata. Los péptidos de auristatina, auristatina E (AE) y monometilauristatina (MMAE), análogos sintéticos de dolastatina, se conjugaron a anticuerpos monoclonales quiméricos cBR96 (específico a Lewis Y en carcinomas) y cAC10 (específico a CD30 en neoplasias malignas hematológicas) (Doronina et al., (2003) Nature Biotechnology 21(7):778-784) y se encuentran bajo desarrollo terapéutico.

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Agentes quimioterapéuticos útiles en la generación de tales inmunoconjugados se han descrito anteriormente. Toxinas enzimáticamente activas y sus fragmentos que pueden usarse incluyen cadena A de la difteria, fragmentos activos no fijadores de toxina de la difteria, cadena A de exotoxina (de *Pseudomonas aeruginosa*), cadena A de ricina, cadena A de abrina, cadena A de modeccina, alfa-sarcina, proteínas de *Aleurites fordii*, proteínas de diantina, proteínas de *Phytolaca americana* (PAPI, PAPII, y PAP-S), inhibidor de momordica charantia, curcina, crotina, inhibidor de sapaonaria officinalis, gelonina, mitogelina, restrictocina, fenomicina, enomicina y los tricotecenos. Se encuentra disponible una variedad de radionúclidos para la producción de anticuerpos radioconjugado. Los ejemplos incluyen ^{212}Bi , ^{131}I , ^{131}In , ^{90}Y , y ^{186}Re . Conjugados del anticuerpo y agente citotóxico se elaboran usando una variedad de agentes de acoplamiento de proteínas bifuncionales tales como N-succinimidil-3-(2-piridilditiol) propionato (SPDP), iminotiolano (IT), derivados bifuncionales de imidoésteres (tales como HCl de adipimidato dimetilico), ésteres activos (tales como disuccinimidil suberato), aldehídos (tales como glutareldehído), compuestos bis-azido (tales como bis (p-azidobenzoyl)hexanodiamina), derivados de bis-diazonio (tales como bis-(p-diazoniobenzoyl)-etilendiamina), diisocianatos (tales como tolien 2,6-diisocianato), y compuestos de flúor bis-activo (tales como 1,5-difluoro-2,4-dinitrobenceo). Por ejemplo, puede prepararse una inmunotoxina de ricina como se describe en Vitetta y colaboradores, *Science*, 238: 1098 (1987). Ácido 1-isotiocianatobencil-3-metildietilen triaminapentaacético marcado con carbono 14 (MX-DTPA) es un agente quelante ejemplar para conjugación de radionucleótido al anticuerpo. Véase WO94/11026.

Conjugados de un anticuerpo y una o más toxinas de molécula pequeña, tales como una caliqueamicina, maitansinoides, un tricoteceno, y CC1065, y los derivados de estas toxinas que tienen actividad de toxina, también contemplados en esta solicitud.

Maitansina y maitansinoides

En una incorporación, un anticuerpo (longitud total o fragmentos) de la invención se conjuga con una o más de las moléculas maitansinoides.

Los maitansinoides son inhibidores mitóticos que actúan inhibiendo la polimerización de tubulina. La maitansina se aisló por primera vez del arbusto este-africano *Maytenus serrata* (Patente de los Estados Unidos No. 3,896,111). Posteriormente, se descubrió que ciertos microbios también producen maitansinoides, tales como ésteres de maitansinol y C-3 maitansinol (US 4151042). Se han divulgado maitansinol sintético y análogos de maitansinol, por ejemplo, en la Patente de los Estados Unidos 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; y 4,371,533, cuyas divulgaciones se incorporan expresamente por la presente solicitud por referencia.

Conjugados maitansinoide-anticuerpo

En un intento por mejorar su índice terapéutico, maitansina y maitansinoides se han conjugado con anticuerpos que específicamente se unen a antígenos de células tumorales. Inmunoconjugados que contienen maitansinoides y su uso terapéutico se divulgan, por ejemplo, en la Patente de los Estados Unidos Nos. 5,208,020, 5,416,064 y la Patente Europea EP 0 425 235 B1, cuyas divulgaciones se incorporan aquí expresamente por referencia. Liu et al., Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996) describen inmunoconjugados que comprenden un maitansinoide designado DM1 enlazado a un anticuerpo monoclonal C242 dirigido contra cáncer colorrectal humano. Se encontró que el conjugado era altamente citotóxico contra células de cáncer de colon cultivadas, y mostró actividad antitumoral en un análisis de crecimiento de tumor *in vivo*. Chari et al., Cancer Research 52: 127-131 (1992) describen inmunoconjugados en los cuales un maitansinoide se conjugó mediante un conector disulfuro al anticuerpo A7 murino que se une a un antígeno en líneas de células humanas de cáncer de colon, o a otro anticuerpo TA.1 monoclonal murino que une el oncogén HER-2/neu. La citotoxicidad del conjugado TA.1-maitansinoide se ensayó

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in vitro en la línea de células humanas de cáncer de mama SK-BR-3, que expresan 3×10^5 antígenos de superficie HER-2 por célula. El conjugado del medicamento alcanzó un grado de citotoxicidad similar al fármaco maitansinoide libre, que podría incrementarse aumentando el número de moléculas maitansinoides por molécula de anticuerpo. El conjugado A7-maitansinoide mostró una baja citotoxicidad sistémica en ratones.

Conjugados anticuerpo-maitansinoide (inmunoconjugados)

Los conjugados anticuerpo-maitansinoide se preparan enlazando químicamente un anticuerpo a una molécula maitansinoide sin disminuir significativamente la actividad biológica del anticuerpo o la molécula maitansinoide. Un promedio de 3-4 moléculas maitansinoides, conjugadas por molécula de anticuerpo, ha mostrado eficacia en aumentar la citotoxicidad de células diana sin afectar negativamente la función o la solubilidad del anticuerpo, aunque se esperaría que incluso una molécula de toxina/anticuerpo incremente la citotoxicidad en relación con el uso de un anticuerpo desnudo. Los maitansinoides son bien conocidos en el arte y pueden sintetizarse por técnicas conocidas o aislarse de fuentes naturales. Maitansinoides apropiados se divulgan, por ejemplo, en la Patente de los Estados Unidos No. 5,208,020 y en otras patentes y publicaciones distintas de patentes mencionadas más arriba en el texto. Maitansinoides preferidas son maitansinol y análogos de maitansinol, modificados en el anillo aromático o en otras posiciones de la molécula de maitansinol, tales como varios ésteres de maitansinol.

Existen muchos grupos enlazadores conocidos en el arte para fabricar conjugados anticuerpo-maitansinoide, incluidos, por ejemplo, los divulgados en la Patente de los Estados Unidos No. 5,208,020 o la Patente EP 0 425 235 B1, y Chari et al., Cancer Research 52:127-131 (1992). Los grupos enlazadores incluyen grupos disulfuro, grupos tioéter, grupos lábiles a los ácidos, grupos fotolábiles, grupos lábiles a la peptidasa, o grupos lábiles a la esteras, como se divulga en las patentes ya identificadas, prefiriéndose los grupos disulfuro y tioéter.

Conjugados del anticuerpo y maitansinoide pueden prepararse usando una variedad de agentes de acoplamiento de proteína bifuncionales tales como N-succinimidil-3-(2-piridilditiol)propionato (SPDP), succinimidil-4-(N-maleimidometil)ciclohexano-1-carboxilato, iminotiolano (IT), derivados bifuncionales de imidoésteres (tales como dimetil adipimidato HCl), ésteres activos (tales como disuccinimidil suberato), aldehídos (tales como glutareldehído), compuestos bis-azido (tales como bis (p-azidobenzoil)hexanidiamina), derivados de bis-diazonio (tales como bis-(p-diazoniobenzoil)-etilendiamina), diisocianatos (tales como tolueno 2,6-diisocianato), y compuestos de flúor bis-activo (tales como 1,5-difluoro-2,4-dinitrobenceno).

El conector puede unirse a la molécula maitansinoide en varias posiciones, dependiendo del tipo de enlace. Por ejemplo, puede formarse un enlazamiento éster por reacción con un grupo hidroxilo usando técnicas de acoplamiento convencionales. La reacción puede ocurrir en la posición C-3 con un grupo hidroxilo, la posición C-14 modificada con hidroximetilo, la posición C-15 modificada con un grupo hidroxilo, y la posición C-20 con un grupo hidroxilo. En una incorporación preferida, el enlazamiento se forma en la posición C-3 de maitansinol o un análogo de maitansinol.

Caliqueamicina

Otro inmunoconjugado de interés comprende un anticuerpo conjugado con una o más moléculas de caliqueamicina. La familia caliqueamicina de antibióticos son capaces de producir cortes de ADN bicatenarios ("*double-stranded DNA breaks*") en concentraciones picomolares. Para la preparación de conjugados de la familia caliqueamicina, véase Patentes de los Estados Unidos 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296 (todas a American Cyanamid Company). Análogos estructurales de caliceamicina que pueden usarse incluyen, pero no se limitan a, $\gamma 1^I$, $\alpha 2^I$, $\alpha 3^I$, N-acetil- $\gamma 1^I$ PSAG y θ^I , (Hinman et al., Cancer Research 53:3336-3342 (1993), Lode et al., Cancer Research 58:2925-2928 (1998) y las Patentes de los Estados

Unidos ya mencionados American Cyanamid). Otro fármaco anti-tumoral al cual puede conjugarse el anticuerpo es QFA, que es un antifolato. Tanto la caliqueamicina como el QFA tienen sitios intracelulares de acción y no cruzan fácilmente la membrana plasmática. Por lo tanto, la captación celular de estos agentes a través de la internalización mediada por anticuerpo mejora enormemente sus efectos citotóxicos.

Otros agentes citotóxicos

Otros agentes citotóxicos que pueden conjugarse con los anticuerpos de la invención incluyen BCNU, estreptozaicina, vincristina y 5-fluorouracil, la familia de agentes conocidos colectivamente LL-E33288 complejo descrito en las Patentes de los Estados Unidos 5,053,394, 5,770,710, así como esperamicinas (Patente de los Estados Unidos 5,877,296).

Toxinas enzimáticamente activas y sus fragmentos que pueden usarse incluyen cadena A de la difteria, fragmentos activos no fijadores de toxina de la difteria, cadena A de exotoxina (de *Pseudomonas aeruginosa*), cadena A de ricina, cadena A de abrina, cadena A de modeccina, alfa-sarcina, proteínas de *Aleurites fordii*, proteínas de diantina, proteínas de *Phytolaca americana* (PAPI, PAPII, y PAP-S), inhibidor de momordica charantia, curcina, crotina, inhibidor de sapaonaria officinalis, gelonina, mitogelina, restrictocina, fenomicina, enomicina y los tricotecenos. Véase, por ejemplo, WO 93/21232 publicada el 28 de octubre de 1993.

La presente invención contempla además inmunoconjugados formados entre un anticuerpo y un compuesto con actividad nucleolítica (por ejemplo, una ribonucleasa o ADNendonucleasa tal como deoxiribonucleasa, ADNasa).

Para destrucción selectiva del tumor, el anticuerpo puede comprender un átomo altamente radioactivo. Para la producción de anticuerpos radioconjugados se dispone de una variedad de isótopos radioactivos. Ejemplos incluyen At^{211} , I^{131} , I^{125} , Y^{90} , Re^{186} , Re^{188} , Sm^{153} , Bi^{212} , P^{32} y Pb^{212} e isótopos radioactivos de Lu. Cuando el conjugado se usa para detección, puede comprender un átomo radioactivo para estudios escintigráficos, por

ejemplo Tc^{99} ó I^{123} , o una marca de espín para generación de imágenes de resonancia magnética nuclear (NMR) (también conocida como generación de imágenes de resonancia magnética, mri), tal como yodo-123, yodo-131, indio-111, flúor-19, carbono-13, nitrógeno-15, oxígeno-17, gadolinio, manganeso o hierro.

Las radiomarcas u otras marcas pueden incorporarse en el conjugado en formas conocidas. Por ejemplo, el peptide puede biosintetizarse o puede sintetizarse por síntesis química de aminoácidos usando precursores apropiados de aminoácidos que incluyan, por ejemplo, flúor-19 en lugar de hidrógeno. Marcas tales como Tc^{99} ó I^{123} , Re^{186} , Re^{188} e In^{111} pueden unirse con un residuo de cisteína en el péptido. Itrio-90 puede unirse mediante un residuo de lisina. El método IODOGEN® (Fraker et al (1978) Biochem. Biophys. Res. Commun. 80: 49-57 puede emplearse para incorporar yodo-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal, CRC Press 1989) describe otros métodos en detalle.

Conjugados del anticuerpo y agente citotóxico se elaboran usando una variedad de agentes de acoplamiento de proteínas bifuncionales tales como N-succinimidil-3-(2-piridilditiol) propionato (SPDP), succinimidil-4-(N-maleimidometil)ciclohexano-1-carboxilato, iminotiolano (IT), derivados bifuncionales de imidoésteres (tales como HCl de adipimidato dimetilico), ésteres activos (tales como disuccinimidil suberato), aldehídos (tales como glutareldérido), compuestos bis-azido (tales como bis (p-azidobenzoyl)hexanodiamina), derivados de bis-diazonio (tales como bis-(p-diazoniobenzoyl)-etilendiamina), diisocianatos (tales como tolien 2,6-diisocianato), y compuestos de flúor bis-activo (tales como 1,5-difluoro-2,4-dinitrobenceo). Por ejemplo, puede prepararse una inmunotoxina de ricina como se describe en Vitetta y colaboradores, *Science*, 238: 1098 (1987). Ácido 1-isotiocianatobencil-3-metildietilen triaminapentaacético marcado con carbono 14 (MX-DTPA) es un agente quelante ejemplar para conjugación de radionucleótido al anticuerpo. Véase WO94/11026. El enlazador puede ser un "enlazador segmentable" que facilite la liberación del medicamento citotóxico en la célula. Por ejemplo, pueden usarse un

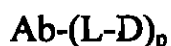
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enlazador lábil al ácido, un enlazador sensible a peptidasa, enlazador fotolábil, un enlazador dimetílico o enlazador que contenga disulfuro (Chari et al., Cancer Research 52:127-131 (1992); Patente de los Estados Unidos No. 5,208,020).

Los compuestos de la invención expresamente contemplan, pero no se limitan a, ADC preparado con reactivos de enlazamiento cruzado: 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, sulfo-SMPB, y SVSB (succinimidil-(4-vinilsulfone)benzoato), que se encuentran disponibles en el comercio (*por ejemplo*, de Pierce Biotechnology, Inc., Rockford, IL., U.S.A). Véase páginas 467-498, 2003-2004 Manual de Aplicaciones y Catálogo.

Preparación de conjugados anticuerpo-medicamento

En los conjugados medicamento-anticuerpo (ADC) de la invención, un anticuerpo (Ab) se conjuga con una o más fracciones de una o más fracciones (D), *por ejemplo* alrededor de 1 a alrededor de 20 fracciones de medicamento por anticuerpo, a través de un enlazador (L). El ADC de Fórmula I puede prepararse por varias rutas, empleando reacciones de química orgánica, condiciones, y reactivos conocidos por los expertos en el arte, incluidas: (1) reacción de un grupo nucleófilo de un anticuerpo con un reactivo enlazador bivalente, para formar Ab-L, mediante un enlace covalente, seguido por reacción con una fracción de medicamento D; y (2) reacción de un grupo nucleófilo o un grupo electrófilo de una fracción de medicamento con un reactivo enlazador, para formar el intermedio medicamento-enlazador D-L, mediante un enlace covalente, seguida por reacción con el grupo nucleófilo de un anticuerpo.



Grupos nucleófilos en anticuerpos incluyen, pero no se limitan a: (i) grupos amina N-terminal, (ii) grupos amina de cadena lateral, *por ejemplo*, lisina, (iii) grupos tiol de cadena lateral, *por ejemplo*, cisteína, y (iv) grupos

azúcar hidroxilo o amino donde el anticuerpo es glucosilado. Grupos amina, tiol, e hidroxilo son nucleófilos y capaz de reaccionar para formar enlaces covalentes con grupo electrófilos en fracciones de enlazador y reactivos de enlazador incluidos: (i) ésteres activos tales como ésteres NHS, ésteres HOBt, haloformiatos, y haluros ácidos; (ii) haluros alquílicos y bencílicos tales como haloacetamidas; (iii) aldehídos, acetonas, carboxilo, y grupos maleimida. Ciertos anticuerpos tienen disulfuros de intercadena reducibles, *es decir*, puentes cisteína. Anticuerpos pueden hacerse reactivos para conjugación con reactivos enlazadores por tratamiento con un agente reductor tal como DTT (ditiotreitól). Por consiguiente, cada puente disulfuro cisteína formará, teóricamente, dos nucleófilos tiol reactivos. Grupos nucleófilos adicionales pueden introducirse en anticuerpos mediante la reacción de lisinas con 2-iminotiolano (reactivo de Traut) resultante en la conversión de una amina en un tiol.

Conjugados anticuerpo-medicamento de la invención pueden producirse asimismo por modificación del anticuerpo para introducir fracciones electrófilas, que pueden reaccionar con sustituyentes nucleófilos en el reactivo enlazador o medicamento. Los azúcares de anticuerpos glucosilados pueden oxidarse, *por ejemplo* con reactivos de oxidación con periodato, para formar aldehído o grupos acetona, los cuales pueden reaccionar con el grupo amina de reactivos de enlazador o fracciones de medicamento. Los grupos básicos de imina Schiff resultantes pueden formar un enlazamiento estable, o pueden reducirse, *por ejemplo* por reactivos borohidruro para formar enlazamientos de amina estables. En una incorporación, la reacción de la porción carbohidrato de un anticuerpo glucosilado con galactosa oxidasa o meta-periodato de sodio puede proporcionar grupos carbonilo (aldehído y acetona) en la proteína que puede reaccionar con grupos apropiados en el medicamento (Hermanson, Bioconjugate Techniques). En otra incorporación, proteínas que contienen residuos de serina o treonina N-terminal pueden reaccionar con meta-periodato de sodio, resultante en la producción de un aldehído en lugar del primer aminoácido (Geoghegan & Stroh, (1992) Bioconjugate Chem.

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3:138-146; US 5362852). Tal aldehído puede hacerse reaccionar con una fracción de medicamento o nucleófilo enlazador.

Análogamente, grupos nucleófilos en una fracción de medicamento incluyen, pero no se limitan a: amina, tiol, hidroxilo, hidrazida, oxime, hidrazina, tiosemicarbazona, hidrazina carboxilato, y grupos arilhidrazida capaces de reaccionar para formar enlaces covalentes con grupos electrófilos en fracciones de enlazador y reactivos de enlazador incluidos: (i) ésteres activos tales como ésteres NHS, ésteres HOBt, haloformatos, y haluros ácidos; (ii) haluros alquílicos y bencílicos tales como haloacetamidas; (iii) aldehídos, acetonas, carboxilo, y grupos maleimida.

Alternativamente, puede fabricarse una proteína de fusión que comprende el anticuerpo y el agente citotóxico, *por ejemplo*, mediante técnicas recombinantes o síntesis de péptidos. La longitud del ADN puede comprender regiones respectivas que codifican dos porciones del conjugado, adjuntas unas a otras o separadas por una región que codifica un péptido enlazador, que no destruye las propiedades deseadas del conjugado.

En todavía otra incorporación, el anticuerpo puede conjugarse a un "receptor" (tal como estreptavidina) para utilización en pre-direccionamiento tumoral ("*tumor pre-targeting*"), en donde el conjugado anticuerpo-receptor se administra al paciente, seguido por retiro del conjugado no unido de la circulación, usando un agente de depuración y luego la administración de un "ligando" (*por ejemplo*, avidina), el cual se conjuga con un agente citotóxico (*por ejemplo*, un radionucleótido).

Derivados de Anticuerpo

Los anticuerpos de la presente invención pueden modificarse aún más para contener fracciones adicionales no proteináceas, que son conocidas en el arte y fácilmente disponibles. Preferiblemente, las fracciones apropiadas para derivación del anticuerpo son polímeros solubles en agua. Ejemplos no limitantes de polímeros solubles en agua incluyen, pero no se limitan a, polietilen glicol (PEG), copolímeros de etilen glicol/propilen glicol, carboximetilcelulosa, dextrina, alcohol polivinílico, pirrolidona polivinílico, poli-1,3-dioxo, poli-1,3,6-trioxano, copolímero etileno/anhídrido maleico,

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poliaminoácidos (homopolímeros o copolímeros aleatorios), y dextrina o poli(n-vinil pirrolidona)polietilen glicol, homopolímeros de propipilen glicol, copolímeros óxido de propilpropileno/óxido de etileno, polioles polioxietilados (*por ejemplo*, glicerina), alcohol polivinílico, y mezclas de los mismos. Propionaldehído de polietilen glicol puede tener ventajas en la fabricación debido a su estabilidad en agua. El polímero puede ser de cualquier peso molecular, y puede ser ramificado o no ramificado. El número de polímeros unidos al anticuerpo pueden variar y, si se adhieren más de un polímero, éstos pueden ser las mismas o moléculas diferentes. En general, el número y/o tipo de polímeros usados para derivación pueden determinarse basándose en consideraciones, incluidas, pero no limitadas a, las propiedades particulares o funciones del anticuerpo a mejorarse, si el derivado de anticuerpo se usará en una terapia bajo condiciones definidas, etc.

Formulaciones Farmacéuticas

Formulaciones terapéuticas que comprenden un anticuerpo de la invención se preparan para almacenamiento mezclando un anticuerpo que tenga el grado deseado de pureza, con vehículos, excipientes o estabilizantes opcionales, farmacéuticamente aceptables (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), en la forma de soluciones acuosas, formulaciones liofilizadas u otras formulaciones secas. Vehículos, excipientes, o estabilizantes aceptables son no tóxicos a los destinatarios en las posologías y concentraciones empleadas, e incluyen amortiguadores tales como fosfato, citrato, histidina y otros ácidos orgánicos; antioxidantes incluidos ácido ascórbico y metionina; preservantes (tales como cloruro de octadecildimetilbencil amonio; cloruro de hexametonio; cloruro de benzalconio, cloruro de benzetonio; fenol, alcohol butílico o bencílico; alquil parabenos tales como metil o propil parabeno; catecol; resorcinol; ciclohexanol; 3-pentanol; y m-cresol); polipéptidos de bajo peso molecular (menos de alrededor de 10 residuos); proteínas, tales como albúmina sérica, gelatina, o inmunoglobulinas; polímeros hidrófilos tales como polivinilpirrolidona; aminoácidos tales

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como glicina, glutamina, asparagina, histidina, arginina, o lisina; monosacáridos, disacáridos, y otros carbohidratos incluídas glucosa, mannososa, o dextrinas; agentes quelantes tales como EDTA; azúcares tales como sacarosa, mannitol, trehalosa o sorbitol; iones contrarios formadores de sal tales como sodio; complejos de metal (*por ejemplo*, complejos zinc-proteína); y/o surfactantes no iónicos tales como TWEEN™, PLURONICS™, o polietilen glicol (PEG).

La formulación en esta solicitud puede contener asimismo más de un compuesto activo según sea necesario para la afección particular que se esté tratando, preferiblemente aquellos con actividades complementarias que no se afectan negativamente entre sí. Tales moléculas están convenientemente presentes en combinación en cantidades que son efectivas para los propósitos previstos.

En igual forma los ingredientes activos pueden atraparse en microcápsulas preparadas, por ejemplo, por técnicas de aglomeración o por polimerización interfacial, por ejemplo, hidroximetilcelulosa o microcápsulas de gelatina y microcápsulas de poli-(metilmetacilato), respectivamente, en sistemas coloidales de liberación de medicamentos (por ejemplo, liposomas, microesferas de albúmina, microemulsiones, nanopartículas y nanocápsulas) o en macroemulsiones. Tales técnicas se divulgan en *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Las formulaciones a utilizarse para administración *in vivo* tienen que ser estériles. Esto se logra fácilmente por filtración a través de membranas de filtración estériles.

Pueden prepararse preparaciones de liberación sostenida. Ejemplos apropiados de preparaciones de liberación sostenida incluyen matrices semipermeables de polímeros hidrófobos sólidos que contienen la inmunoglobulina de la invención, matrices que están en la forma de artículos moldeados, *por ejemplo*, películas, o microcápsulas. Ejemplos de matrices de liberación sostenida incluyen poliésteres, hidrogeles (por ejemplo, poli(2-hidroxietil-metacrilato), o poli(vinilalcohol)), poliláctidos

(Patente de los Estados Unidos No. 3,773,919), copolímeros de ácido L-glutámico y γ -etil-L-glutamato, etilen vinil acetato, no degradable, copolímeros degradables de ácido láctico-ácido glicólico tales como los LUPRONDEPOT™ (microesferas inyectables compuestas de copolímero de ácido láctico-ácido glicólico y acetato de leuprolida), y ácido poli-D-(-)-3-hidroxibutírico. Aunque polímeros tales como etilenvinilacetato y ácido láctico-ácido glicólico permiten la liberación de moléculas durante más de 100 días, ciertos hidrogeles liberan proteínas durante períodos de tiempo más cortos. Cuando inmunoglobulinas encapsuladas permanecen en el cuerpo durante un tiempo prolongado, pueden desnaturalizarse o agregarse como resultado de la exposición a humedad a 37°C, resultando en una pérdida de actividad biológica y posibles cambios en inmunogenicidad. Pueden diseñarse estrategias racionales para estabilización dependiendo del mecanismo involucrado. Por ejemplo, si se descubre que el mecanismo de agregación es la formación del enlace S-S intermolecular mediante intercambio tio-disulfuro, la estabilización puede lograrse modificando los residuos sulfhidrilo, liofilizando a partir de soluciones ácidas, controlar el contenido de humedad, usando aditivos apropiados, y desarrollando composiciones de matriz polimérica específica.

En ciertas incorporaciones, un inmunoconjugado, que comprende un anticuerpo conjugado con un agente citotóxico, se administra al paciente. En algunas incorporaciones, el inmunoconjugado y/o antígeno al cual se une se internaliza/internalizan por la célula, resultando en una eficacia terapéutica incrementada del inmunoconjugado en eliminar la célula diana a la cual se une. En una incorporación, el agente citotóxico toma como objetivo o interfiere el ácido nucleico en la célula diana. Ejemplos de tales agentes citotóxicos incluyen cualquiera de los agentes quimioterapéuticos anotados en esta solicitud (tales como un maitansinoide o una caliqueamicina), un isótopo radioactivo, o una ribonucleasa o una ADN endonucleasa.

Anticuerpos de la invención pueden usarse solos o en combinación con otras composiciones en una terapia. Por ejemplo, un anticuerpo de la

invención puede co-administrarse con otro anticuerpo, agente(s) quimioterapéutico (incluidas mezclas de agentes quimioterapéuticos), otro(s) agente(s) citotóxicos, agente(s) anti-angiogénico(s), citocinas, y/o agente(s) inhibidor(es) de crecimiento. Donde un anticuerpo de la invención inhibe el crecimiento tumoral, puede ser particularmente deseable combinarlo con uno o más agente(s) terapéutico(s), que inhibe además el crecimiento tumoral. Por ejemplo, un anticuerpo de la invención puede combinarse con anticuerpos anti-VEGF que bloquean las actividades VEGF y/o anticuerpos anti-ErbB (*por ejemplo* el anticuerpo anti-HER2 HERCEPTIN®) en un tratamiento de cáncer de mama metastásico. Alternativa, o adicionalmente, el paciente puede recibir terapia de irradiación combinada (*por ejemplo* irradiación externa o terapia con un agente radioactivo marcado, tal como un anticuerpo). Tales terapias combinadas, ya anotadas, incluyen administración combinada (donde los dos o más agentes se incluyen en las mismas formulaciones o separadas), y administración separada, caso en el cual la administración del anticuerpo de la invención puede ocurrir antes, y/o luego, de la administración de la terapia o terapias complementarias.

El anticuerpo de la invención (y el agente terapéutico complementario) se administra/administran por cualquier medio apropiado, incluida la administración parenteral, subcutáneo, intraperitoneal, intrapulmonar, e intranasal, y, si se desea para tratamiento local, administración intralesional. Las infusiones parenterales incluyen administración intramuscular, intravenosa, intraarterial, intraperitoneal, o subcutánea. Además, el anticuerpo se administra convenientemente por infusión por pulsación, particularmente con dosis decrecientes del anticuerpo. La posología puede ser por cualquier ruta apropiada, por ejemplo, mediante inyecciones, tales como inyecciones intravenosas o subcutáneas, dependiendo en parte de si la administración es corta o crónica.

La composición de anticuerpo de la invención se formulará, dosificará y administrará en una forma consistente con la buena práctica

médica. Factores a considerarse en este contexto incluyen el trastorno particular que se está tratando, el mamífero particular que se está tratando, la condición clínica del paciente individual, la causa del trastorno, el sitio de aplicación del agente, el método de administración, la pauta de administración y otros factores conocidos por los profesionales médicos. El anticuerpo no necesita formularse, pero opcionalmente se formula con uno o más agentes usados actualmente para prevenir o tratar el trastorno en cuestión. La cantidad efectiva de tales otros agentes depende de la cantidad de anticuerpos de la invención presentes en la formulación, el tipo de trastorno o tratamiento, y otros factores ya discutidos. Estos agentes se usan generalmente en las mismas posologías y con rutas de administración como las utilizadas hasta la fecha o alrededor de 1 a 99% de las posologías empleadas hasta ahora.

Para la prevención o tratamiento de enfermedad, la posología apropiada de un anticuerpo de la invención (cuando se usa solo o en combinación con otros agentes tales como agentes quimioterapéuticos) dependerá del tipo de enfermedad a tratarse, el tipo de anticuerpo, la gravedad y evolución de la enfermedad, si el anticuerpo se administra para propósitos preventivos o terapéuticos, la terapia previa, la historia clínica del paciente y la respuesta al anticuerpo, y la descripción del médico a cargo del paciente. El anticuerpo se administra convenientemente al paciente de una sola vez o durante una serie de tratamientos. Dependiendo del tipo y gravedad de la enfermedad, alrededor de 1 $\mu\text{g/kg}$ a 15 mg/kg (*por ejemplo* 0.1 mg/kg -10 mg/kg) de anticuerpo usa una posología candidata inicial para administración al paciente, ya sea, por ejemplo, por una o más administraciones separadas, o por infusión continua. Una posología diaria típica podría variar desde alrededor de 1 $\mu\text{g/kg}$ a 100 mg/kg o más, dependiendo de los factores ya mencionados. Para administraciones repetidas durante varios días o más, dependiendo de la enfermedad, el tratamiento se mantiene hasta que ocurra una supresión deseada de los síntomas de la enfermedad. Una posología ejemplar del anticuerpo estaría en el rango desde alrededor de 0.05 mg/kg a alrededor de 10 mg/kg . En

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consecuencia, una o más dosis de alrededor de 0.5mg/kg, 2.0mg/kg, 4.0mg/kg o 10mg/kg (o cualquier combinación de las mismas) puede administrarse al paciente. Tales dosis pueden administrarse de manera intermitente, *por ejemplo* cada semana o cada tres semanas (*por ejemplo*, de manera que el paciente reciba desde alrededor de dos a alrededor de veinte, *por ejemplo* alrededor de seis dosis del anticuerpo). Puede administrarse una dosis de carga inicial más alta, seguida por una o más dosis. Un régimen posológico ejemplar comprende administrar una dosis de carga inicial de alrededor de 4 mg/kg, seguida por una dosis de mantenimiento semanal de alrededor de 2 mg/kg del anticuerpo. No obstante, pueden utilizarse otros regímenes posológicos. El progreso de esta terapia se monitorea fácilmente por técnicas y análisis convencionales.

Artículos de Fabricación

En otro aspecto de la invención, se provee un artículo de fabricación que contiene materiales útiles para el tratamiento, prevención y/ diagnóstico de los desórdenes descritos anteriormente. El artículo de fabricación comprende un recipiente y una etiqueta o prospecto de envase sobre o asociado con el recipiente. Recipientes apropiados incluyen, por ejemplo, frascos, viales, jeringas, etc. Los recipientes pueden formarse de una variedad de materiales tales como vidrio o plástico. El recipiente comprende o contiene una composición que es por sí misma, o cuando se combina con otra composición, efectiva para tratar, prevenir y/o diagnosticar la afección, y puede tener un puerto de acceso estéril (por ejemplo, el recipiente puede ser una bolsa con solución intravenosa o un vial con un tapón perforable por una aguja de inyección hipodérmica). Por lo menos un agente activo en la composición es un anticuerpo de la invención. La etiqueta o prospecto de envase indica que la composición se utiliza para tratar la afección de elección, tal como cáncer. Más aún el artículo de fabricación puede comprender (a) un primer recipiente con una composición contenida en él, en donde la composición comprende un anticuerpo de la invención; y (b) un segundo recipiente con una composición contenida en él, en donde la composición comprende un

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agente citotóxico adicional. El artículo de fabricación en esta incorporación de la invención puede comprender además un prospecto de envase indicando que las composiciones de anticuerpo primera y segunda pueden usarse para tratar una afección particular, por ejemplo cáncer. Alternativa o adicionalmente, el artículo de fabricación puede comprender asimismo un segundo (o tercer) recipiente que contenga un amortiguador farmacéuticamente aceptable, tal como agua bacteriostática para inyección (BWFI), solución salina amortiguada con fosfato, solución salina de Ringer y solución de dextrosa. Puede incluir asimismo otros materiales deseables desde un punto de vista comercial y del usuario, incluidos otros amortiguadores, diluyentes, filtros, agujas y jeringas.

Los siguientes son ejemplos de los métodos y combinaciones de la invención. Se entiende que pueden ponerse en práctica varias otras incorporaciones, dada la descripción general dada anteriormente.

EJEMPLOS

Materiales y Métodos

Reactivos

El inhibidor de tripsina de maíz era de Haematologic Technologies (Essex Junction, VT) y el sustrato cromógeno para HGFA, Spectrozyme® fVIIa, era de American Diagnostica (Stamford, CT). HAI-1B soluble (sHAI-1B) se expresó en células de Ovario de Hámster de la China y se purificó como se describe previamente (1). El inhibidor de dominio de Kunitz IV-49C ya se describió (2) (Genentech, Inc., South San Francisco). HGFA recombinante humano (HGFA) se expresó en un sistema de expresión con baculovirus como ya se describió (1).

Análisis de activación proHGF

Análisis de activación ProHGF y marcado de proHGF con Iodogen se llevaron a cabo como se describe previamente (1,3). En pocas palabras, HGFA se preincubó con anticuerpos anti-HGFA o sHAI-1B en amortiguador HNC (20 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM CaCl₂) durante 15 minutos a temperatura de la sala, tras lo cual se agregó proHGF

marcado con ^{125}I en amortiguador HNC y se incubó durante 4 horas a 37°C . Las concentraciones del reactivo en la mezcla final fueron las siguientes: 2 nM HGFA, 0.05mg/ml proHGF marcado con ^{125}I , 0.1 mg/ml anticuerpos anti-HGFA, 1 μM sHAI-1B. Transcurridas 4 horas se retiraron alícuotas y se agregaron a amortiguador de muestra (Bio-Rad Laboratories, Hercules, CA) con agente reductor ditiotreitól (BIO-Rad). Luego de calentar brevemente, se cargaron muestras (aprox. 10^6 cpm/) en una gradiente 4-20% de gel de poliacrilamida (Invitrogen Corp., Carlsbad, CA). Después de la electroforesis, los geles secos se expusieron en películas de rayos x (X-OMAT AR, Eastman Kodak Company, Rochester, NY) durante 10-20 min. Se revelaron las películas (Kodak M35A X-OMAT Processor), se escanearon (Umax S-12, Umax Data Systems, Inc., Fremont, CA) y se procesaron con software Adobe V.6.0 Photoshop (Adobe Systems Inc., San Jose, CA).

Experimentos BIAcore

Se determinaron afinidades de unión de los anticuerpos anti-HGFA a HGFA por mediciones de resonancia de plasmones superficiales en un instrumento BIAcore 3000 (Biacore, Inc.). La IgG1 anti-HGFA reformateada de longitud total se inmovilizó con una densidad de 300 unidades de resonancia (RU) en las células de flujo de un chip sensor Pioneer CM5. La inmovilización se logró por acoplamiento aleatorio a través de grupos amino usando un protocolo provisto por el fabricante. Se registraron sensogramas de la unión de HGFA a estas superficies mediante inyección de una serie de soluciones que variaban de 1 μM a 8 nM en incrementos de a 2. La señal de la célula de referencia se restó del sensograma observado. Se calcularon constantes cinéticas por análisis de regresión no lineal de los datos de acuerdo con un modelo de unión de Langmuir 1:1 usando software suministrado por el fabricante. En experimentos de competición, se pre-incubó HGFA (70 nM) con diferentes concentraciones de sHAI-1B (4nM-300nM) o IV-49C (11nM-300nM) o un ligador de sitio activo HGFA de molécula pequeña (220nM-10uM). Luego

de incubar durante 60 minutos a temperatura de la sala, la mezcla inhibidor-enzima se inyectó en las células de flujo y se registraron los sensogramas.

Análisis de inhibición de enzima HGFA

Los anticuerpos o sHAI-1B se incubaron con HGFA (concentración final 5nM) en amortiguador HBSA (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.5 mg/ml BSA, 5 mM CaCl₂) durante 20 minutos a temperatura de la sala. Se agregó Spectrozyme® fVIIa (200 µM concentración final, K_M = 200 µM) y se cuantificaron las tasas lineales del aumento en absorbencia a 405 nm en un lector cinético de microplacas (Molecular Devices, Sunnyvale, CA). La inhibición de la actividad enzimática se expresó como actividad fraccionaria (v_i/v_o) de la actividad no inhibida.

Resultados y Discusión

Identificación de anticuerpos anti-HGFA mediante exposición en bacteriófago

Un método de identificar anticuerpos es mediante el uso de una biblioteca de anticuerpos en bacteriófagos. Véase, por ejemplo, Lee et al. (4). Para identificar anticuerpos contra HGFA, se llevaron a cabo cuatro rondas de rastreo ("*rounds of panning*") usando una biblioteca de anticuerpos de fagémido sintético humano previamente divulgado (una biblioteca F(ab')₂). Las placas se recubrieron con 5 µg/pocillo de HGFA. Aumentamos la rigurosidad del lavado después de cada ronda, de 10-40 veces de lavados. Observamos enriquecimiento después de tres rondas de rastreo. Después de cuatro rondas de rastreo, se seleccionaron 95 clones para análisis ELISA. Luego del secuenciamiento, se encontró que 67 clones únicos se unían específicamente a HGFA. Luego de un ELISA de competición ("*spot competition ELISA*"), se caracterizaron 24 clones más usando bacteriófago purificado para medir los valores IC₅₀, los cuales se determinaron empleando un ELISA de competición de bacteriófagos estándar. Se subclonaron 14 clones únicos con valores IC₅₀ < 100 nM en un vector IgG1 humana PRK. Las secuencias CDR para estos clones se enumeran en Fig. 1. Cadenas pesadas y ligeras (del anticuerpo 4D5 humanizado como se describe en Lee et al. (4)) de clones anti-HGFA se co-

transfectaron en células 293 de mamífero. Luego de una semana, los sobrenadantes libres de suero se recolectaron y los anticuerpos se purificaron usando cromatografía de afinidad de proteína A.

Inhibición de la actividad enzimática HGFA por anticuerpos anti-HGFA de longitud total

Los anticuerpos seleccionados se reformatearon como anticuerpos de longitud total (IgG) por técnicas recombinantes estándar. Estos anticuerpos de longitud total se examinaron en un análisis de activación de sustrato macromolecular usando proHGF marcado con ^{125}I . Durante el experimento de 4 horas, el HGFA convirtió completamente proHGF en HGF de cadena 2, y esta reacción pudo inhibirse por 1 μM sHAI-1B (Fig. 2A) consistente con informes previos (1). Con la excepción de anticuerpo #49 (Fig. 1), todos los anticuerpos anti-HGFA analizados en la concentración probada de 0.67 μM inhibieron significativamente la conversión de proHGF (Fig. 2). Experimentos adicionales mostraron que #58 inhibió la conversión de proHGF en concentraciones tan baja como 0.03 μM (Fig. 3). Consistente con estos resultados, el anticuerpo #58 inhibió muy potentemente la actividad enzimática HGFA frente al pequeño sustrato sintético Spectrozyme® fVIIa, con un IC_{50} de 1.3 nM, mientras que el anticuerpo #49 no inhibió a 500nM (Fig. 8). Más aún, de acuerdo con sus actividades inhibitoras relativamente más débiles en análisis de activación de proHGF, los anticuerpos #39, #86, #90 y #95 tuvieron actividades comparativamente más débiles en el análisis de sustrato cromógeno, con $\text{IC}_{50} > 500\text{nM}$ (Fig. 8). Los 3 anticuerpos #42, #61, y #74 también mostraron una inhibición relativamente débil ($\text{IC}_{50} > 500\text{nM}$) a pesar de la inhibición casi completa de la conversión de proHGF a 0.67 μM (Fig. 8). De manera interesante, el anticuerpo #75 desplegó una cinética de inhibición inusual pues su actividad inhibitora alcanzó un nivel de alrededor de 70% de inhibición, comparada con la inhibición completa lograda por el anticuerpo #58 (Fig. 4).

Mecanismos inhibidores de anticuerpos #75 y #58

A la luz de la inhibición completa del procesamiento del sustrato macromolecular por el anticuerpo #75, su incapacidad de neutralizar completamente la actividad enzimática de HGFA frente al sustrato sintético pequeño sugirió que el anticuerpo #75 se une a una región HGFA funcionalmente importante localizada por fuera de, o en proximidad a, el sitio activo. En contraste, el anticuerpo #58 inhibió fuertemente tanto el procesamiento macromolecular como el de sustrato pequeño por HGFA. Para obtener una visión más detallada de los mecanismos inhibidores de los anticuerpos, se llevaron a cabo estudios de unión de competición con varios inhibidores de sitio activo conocidos. Los tres inhibidores del sitio activo de HGFA utilizados fueron el inhibidor de dominio sHAI-1B bi-Kunitz previamente descrito (1), el inhibidor de dominio IV-49C Kunitz único (2) y el ligador de sitio activo HGFA de molécula pequeña. IV-49C es un dominio Kunitz de 62 aminoácidos, derivado del inhibidor del precursor de β -proteína de Alzheimer (APPI) y es un inhibidor específico del complejo factor de tejido/factor VIIa (2). Encontramos que IV-49C es también un potente inhibidor de la actividad enzimática de HGFA, con un IC_{50} de 0.079 μ M, mientras que el ligador de sitio activo HGFA de molécula pequeña inhibió con un IC_{50} de 0.8 μ M ($K_i = 0.4 \mu$ M) como se ilustra en Fig. 5.

El KD de HGFA a anticuerpo inmovilizado #58 fue 1.3 nM (Fig. 9), similar a la afinidad determinada por análisis amidolíticos (Fig. 8). Las mediciones BIAcore mostraron que sHAI-1B, IV-49C y el ligador de sitio activo HGFA de molécula pequeña inhibió la unión de HGFA a #58. Esto sugería que #58 se une directamente al sitio activo de HGFA o ejerce influencias alostéricas sobre el sitio activo.

Anticuerpo #75 tuvo una unión más débil a HGFA (Fig. 6E; Fig. 9) que #58. Más aún, el ligador de sitio activo HGFA de molécula pequeña no tuvo efecto en la unión de HGFA al anticuerpo #75, indicando que el anticuerpo #75 no se une a la región 'núcleo' del sitio activo. En forma interesante, el anticuerpo #75 inhibió parcialmente la actividad amidolítica de HGFA, sugiriendo que incluso aunque el epítopo #75 se sitúa fuera del sitio activo, tiene que haber una conexión molecular entre estos dos sitios.

Esto explicaría los efectos parciales de sHAI-1B y IV-49C sobre la unión del anticuerpo #75 (Fig. 9; Fig. 6F, G).

Similar al anticuerpo #75, los dos anticuerpos #74 y #61 se unieron también a HGFA en la presencia del ligador de sitio activo de molécula pequeña (Fig. 9), mientras que los inhibidores del dominio Kunitz interfirieron la unión de HGFA. Estos resultados sugirieron que los epítomos de #74 y #61 están por fuera del sitio activo de HGFA. Es concebible que los anticuerpos #61, #74 y #75 se unen a una región exosite de HGFA que es importante para la interacción de sustrato macromolecular o que influyen alostéricamente la conformación de la región de sitio activo. En el factor VIIa de serina proteasa estructuralmente relacionado se encuentra localizado un exosite importante entre el sitio activo y el lazo de unión de calcio (5). Anticuerpos así como péptidos que se unen al exosite del factor VIIa son potentes inhibidores del procesamiento de sustrato macromolecular (6,7). Por ejemplo, la unión del inhibidor peptídico E76 efectúa cambios de conformación en una de los lazos del 'dominio de activación', alterando en esa forma un sitio de interacción de sustrato (7). Además, estos cambios inducen efectos alostéricos en el sitio activo, lo cual explica la observación de que el péptido E-76 inhibe la actividad amidolítica a pesar de la unión fuera de la región del sitio activo (7).

Experimentos adicionales de unión competitiva con anticuerpos biotinilados #75 y #58 indicaron que #75 y #58 tienen epítomos superpuestos en HGFA (datos no mostrados). Estudios de cinética de enzimas demostraron además que #58 es un inhibidor competitivo y que #75 es un inhibidor competitivo parcial (es decir, inhibidor competitivo hiperbólico intersector simple) (datos no mostrados). En conjunto, estos resultados sugieren que ambos anticuerpos se unen fuera del sitio activo de HGFA y que son inhibidores alostéricos de la actividad enzimática de HGFA.

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REIVINDICACIONES

1. Un anticuerpo aislado que une el activador del factor de crecimiento hepatocítico humano (HGFA).
2. El anticuerpo de la reivindicación 1, en donde el anticuerpo se une a un sitio activo de HGFA.
3. El anticuerpo de la reivindicación 1, en donde el anticuerpo se une a HGFA en una posición distinta de un sitio activo de HGFA.
4. El anticuerpo de la reivindicación 1, que es un anticuerpo bloqueante.
5. El anticuerpo de la reivindicación 4, que bloquea la actividad proteolítica de HGFA.
6. El anticuerpo de la reivindicación 5, que bloquea la proteólisis HGFA del HGF de cadena simple.
7. El anticuerpo de la reivindicación 1, que inhibe la señalización de HGF/c-met.
8. El anticuerpo de la reivindicación 7, que inhibe la proliferación celular.
9. El anticuerpo de la reivindicación 7, que inhibe la angiogénesis.
10. El anticuerpo de la reivindicación 1, en donde el anticuerpo es un inhibidor alostérico de la actividad enzimática de HGFA.
11. El anticuerpo de la reivindicación 1, en donde es un inhibidor competitivo parcial o completo de la actividad enzimática de HGFA.

12. Un método de tratar una enfermedad asociada con la alteración de la regulación de la señalización de HGF/c-met en un sujeto, que comprende administrar al sujeto una cantidad efectiva de un anticuerpo que une HGFA, de acuerdo con cualquiera de las reivindicaciones precedentes.
13. El método de la reivindicación 10, en donde la enfermedad es cáncer.
14. El método de la reivindicación 10, en donde la enfermedad se asocia con la alteración de la regulación de angiogénesis.
15. El método de la reivindicación 10, en donde la enfermedad es inmuno-relacionada.
16. Un anticuerpo aislado que específicamente une el activador del factor de crecimiento hepatocítico humano, en donde el anticuerpo comprende una secuencia CDR de cadena pesada, que comprende las secuencias respectivas H1, H2 y/o H3 descritas en la Figura 1A.
17. El anticuerpo de la reivindicación 16, en donde las secuencias H1, H2 y/o H3 son como se describe en las Figuras 1B, 1C y/o 1D.

Clón #	Sib#	H1				H2												H3														Afinidad
		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		

FIG. 1A

200

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Clón #	H1										
	26	27	28	29	30	31	32	33	34	35	SEQ ID NO:
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

Clón #	H2																		
	49	50	51	52	52a	53	54	55	56	57	58	59	60	61	62	63	64	65	SEQ ID NO:
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

Clón #	H3																
	93	94	95	96	97	98	99	100	a	b	c	d	e	f	101	102	SEQ ID NO:
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

Secuencias Framework de Cadena Ligera

LC-FR1 1 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val
 23
 Thr Ile Thr 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
88 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys (SEQ ID NO: 57)

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

Secuencias Framework de Cadena Pesada

HC-FR1 1
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg
 25
Leu Ser Cys Ala Ala 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 Asn 83
83a 83b 83c 92
Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys (SEQ ID NO: 61)

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

FIG. 1E

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Secuencias Framework de Cadena Ligera

[illegible]

Secuencias Framework de Cadena Pesada

```

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

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

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

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

```

FIG. 1F

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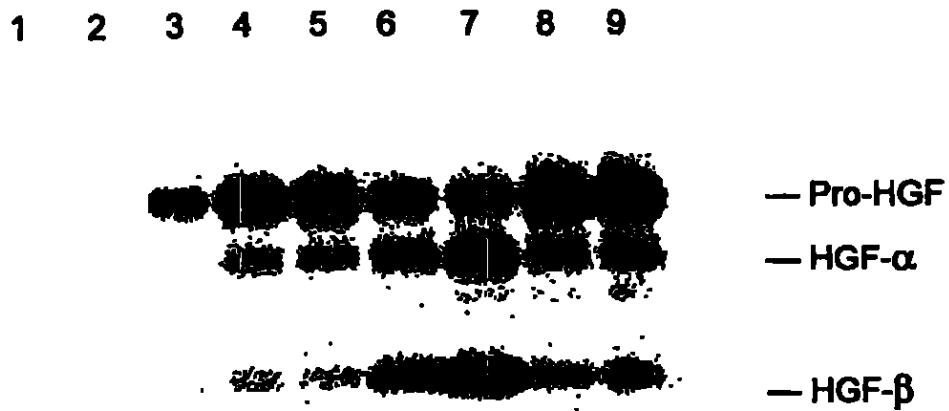


FIG. 2A

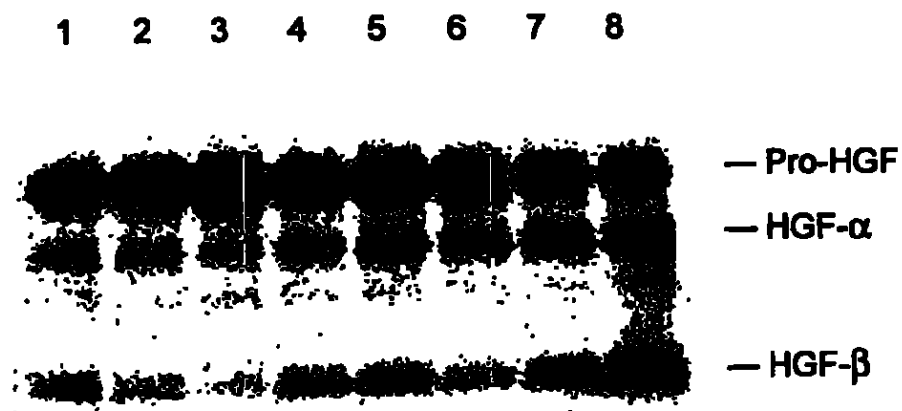


FIG. 2B

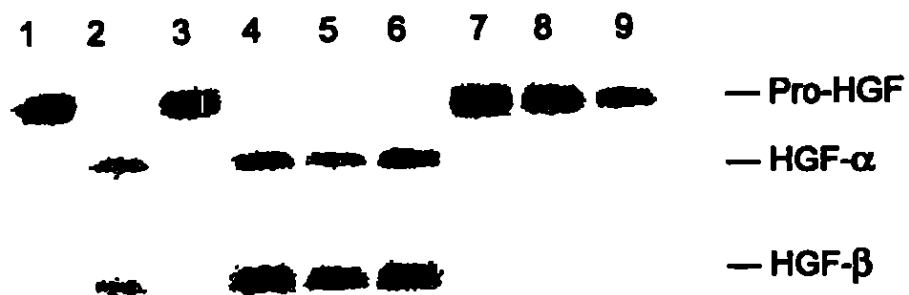
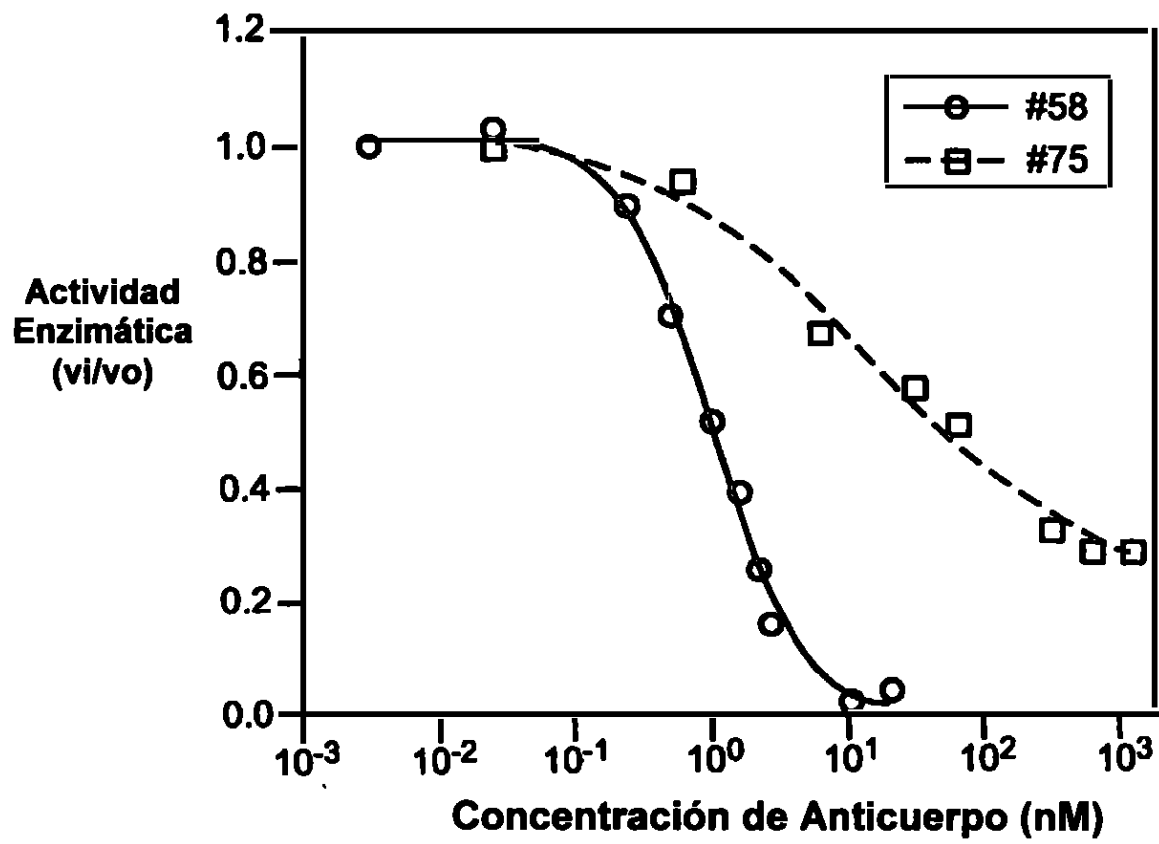


FIG. 3

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**FIG. 4**

347
247

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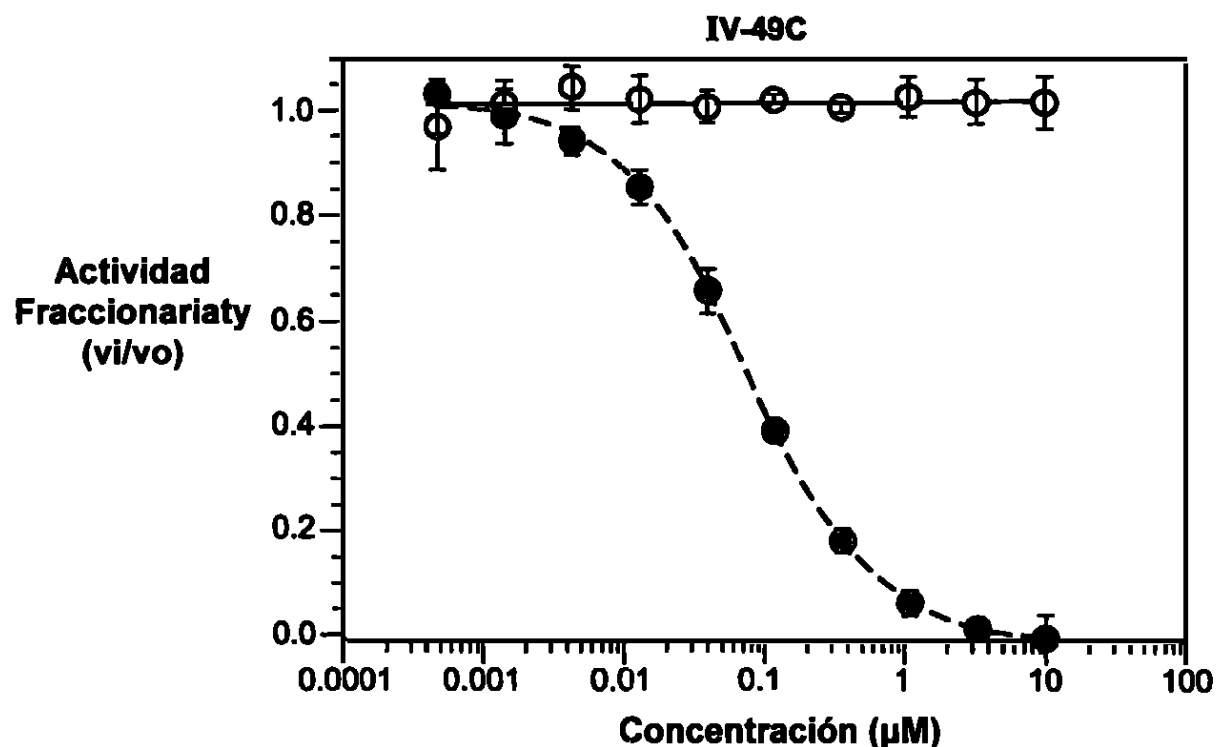


FIG. 5A

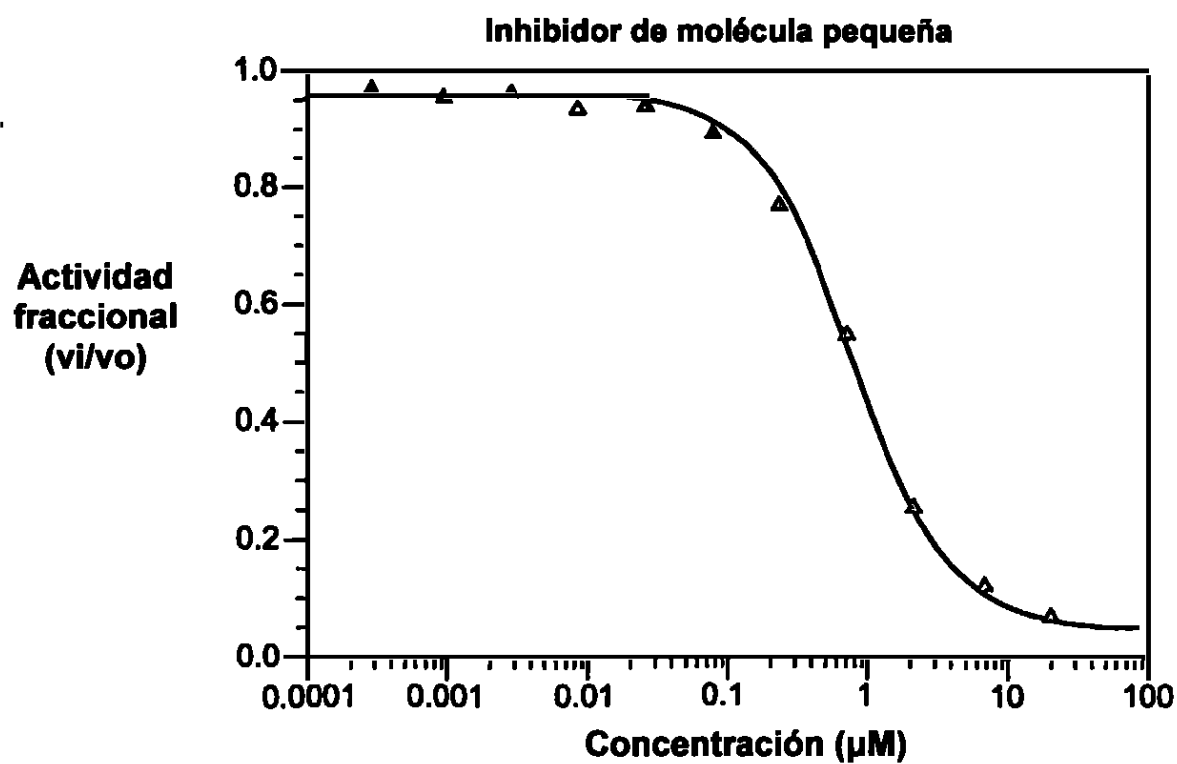


FIG. 5B

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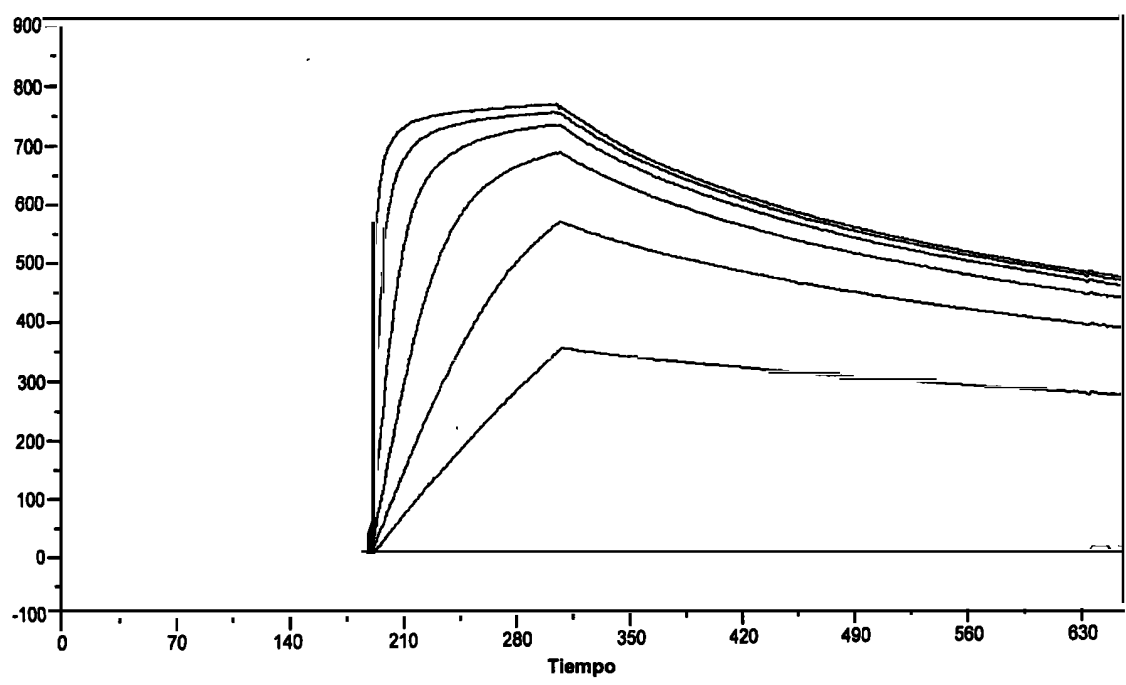


FIG. 6A

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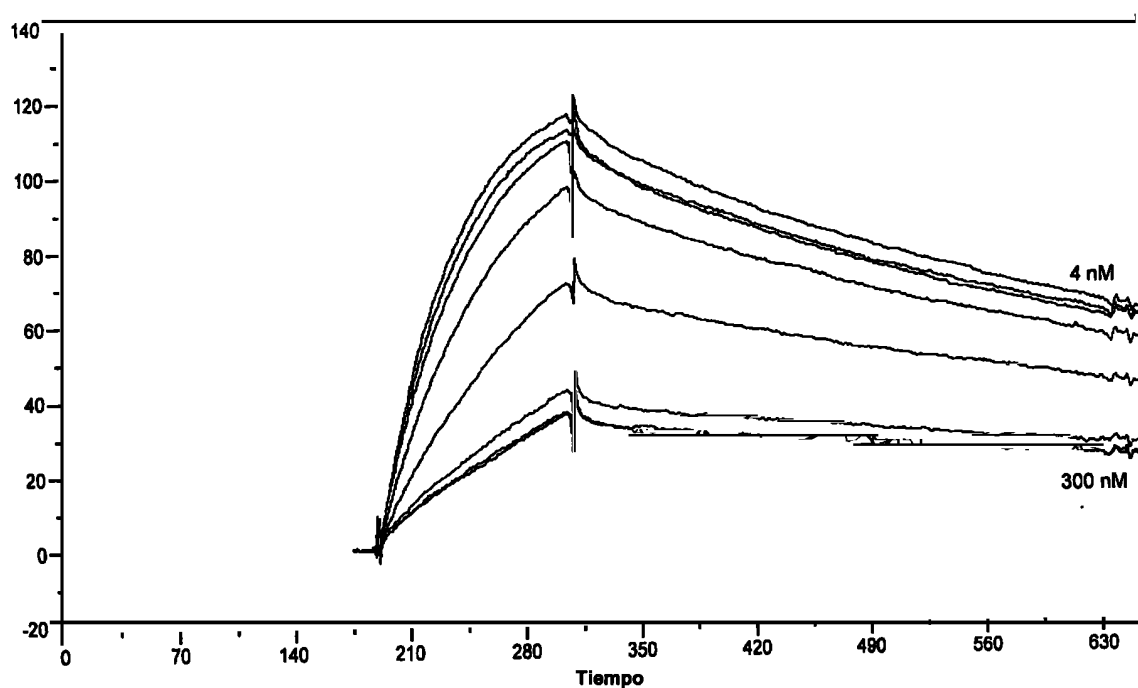


FIG. 6B

250

285

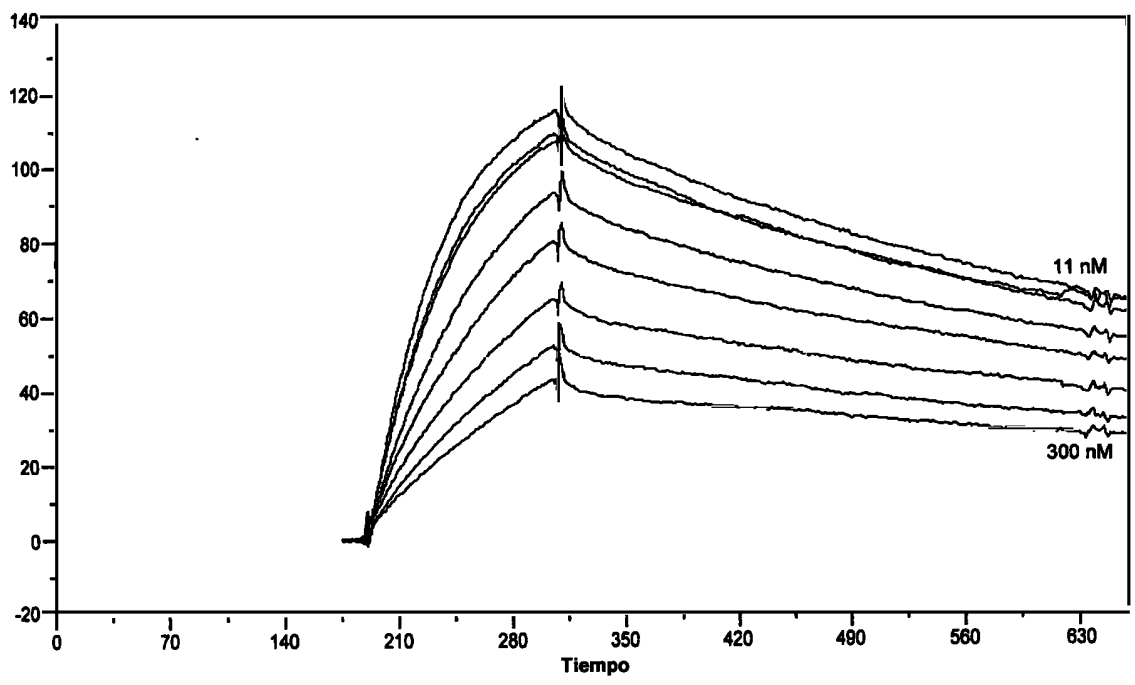
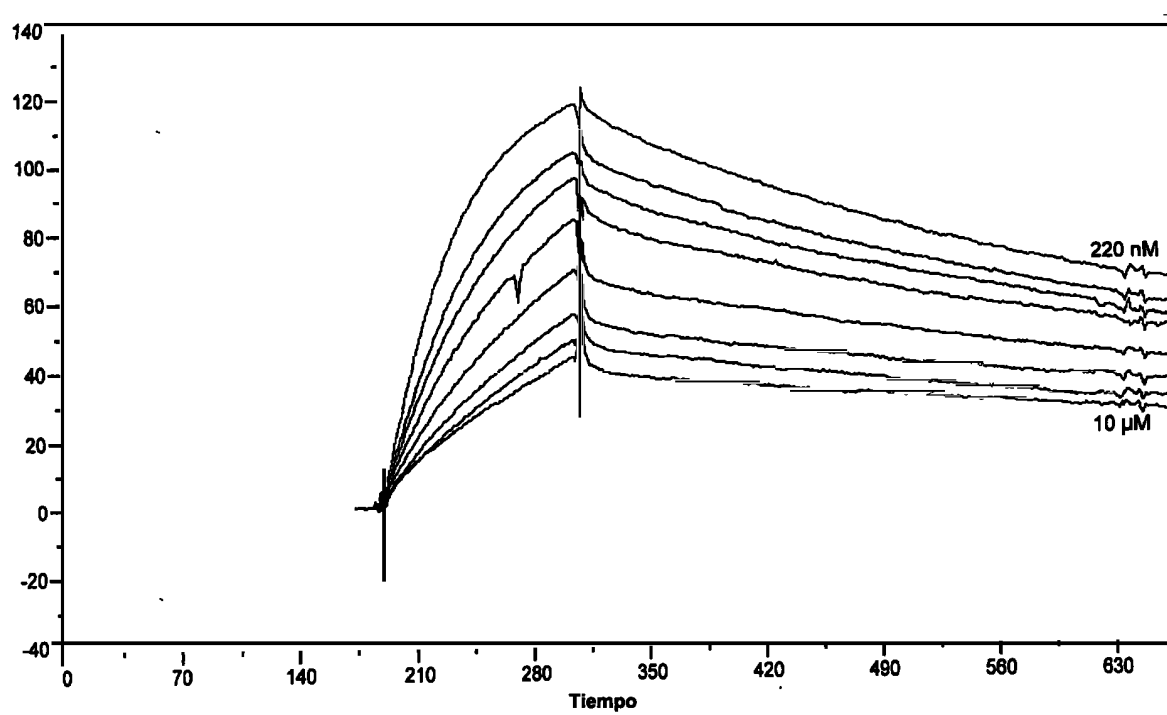


FIG. 6C

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**FIG. 6D**

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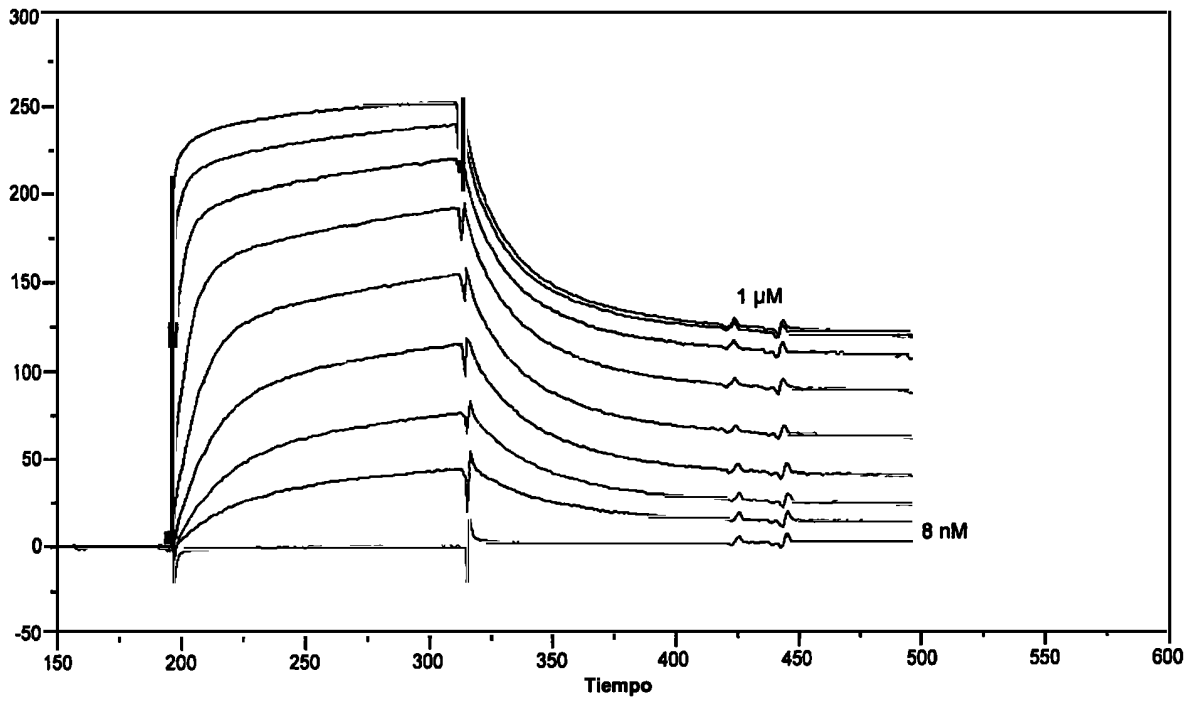


FIG. 6E

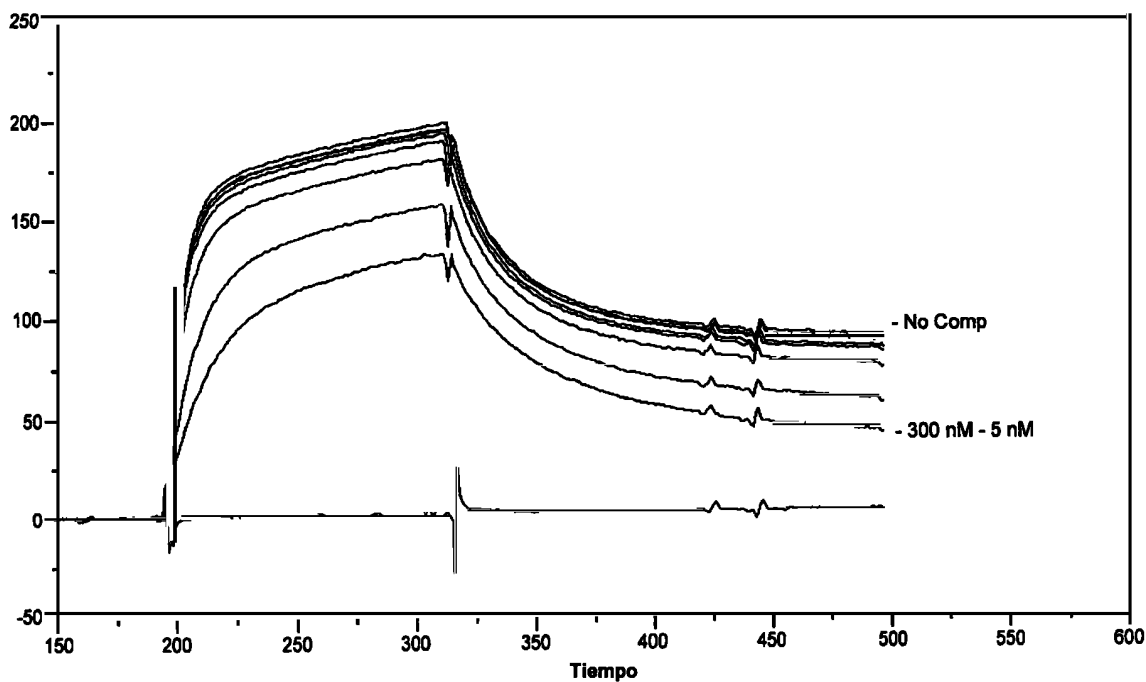


FIG. 6F

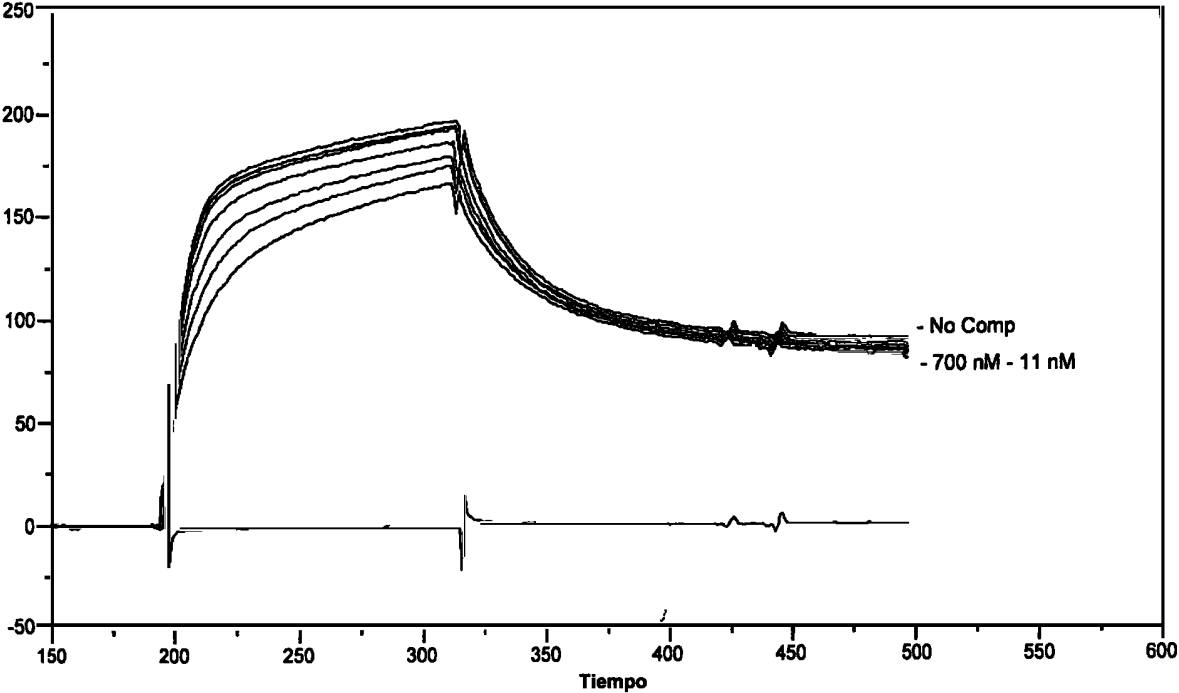


FIG. 6G

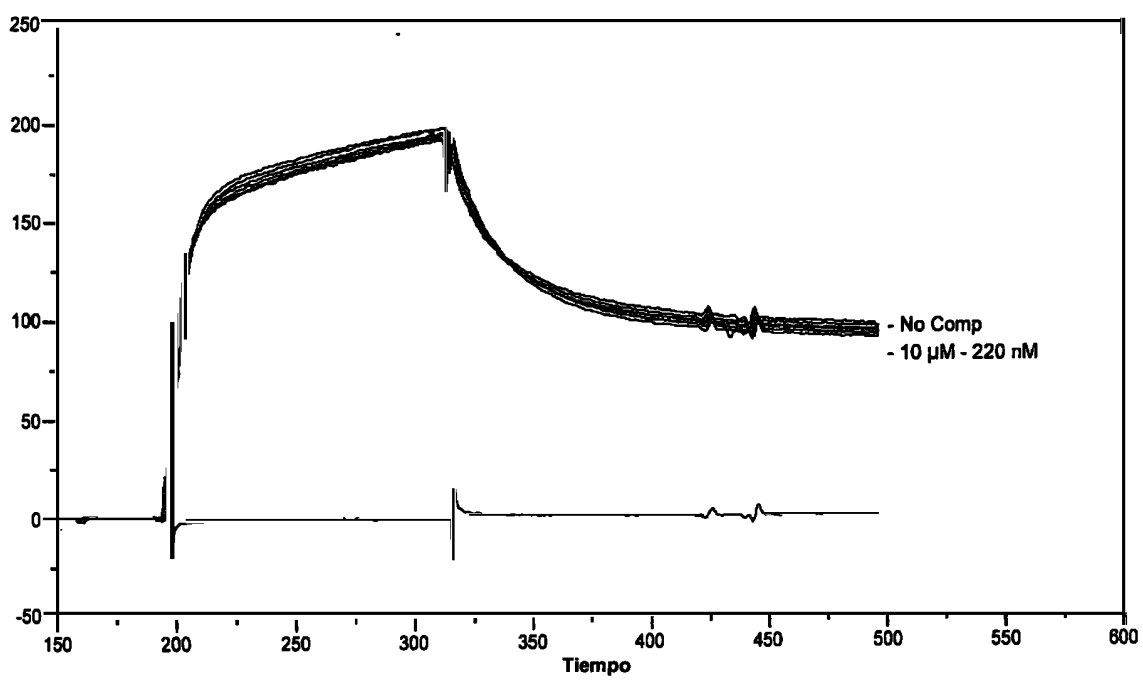


FIG. 6H

huHGFA	1	MGRWAWVPSPWPPLPGLGPFLLLLLLLLL	50
muHGFA	1	MGRQAWISSLCPLPRPCPF-LLLLLLL	49
huHGFA	51	ATPAIPTILVTS--VTSETPATSAPEAEGPQSGGLPPFPRAVPSSSSSPQA	98
muHGFA	50	ATPVTPITPVISGNVSTSTESAFAAETEGPQSERYPSPSSSSPPGG	95
huHGFA	99	QALTEDEGRPCRFPPFRYGGRMLHACTSEGSASHRKWCATTHNYDRDRAWGYC	148
muHGFA	96	QVLTESGQPCRFPPFRYGGRMLHSCTEGSAYRKWCATTHNYDRDRAWGYC	145
huHGFA	149	VETATPPPGGPAALDPCASGPCLNGGSCSNTPQDPQSYHCSCPRAFGTGKDCG	198
muHGFA	146	AETVTLPLVEGPAILDPCASWPCCLNGGTCSSTHHDHGSYHCSCPLAFGTGKDCG	195
huHGFA	199	TEKCFDETRYEYLEGGDRWARVRQGHVEQCECFPGGRTWCEGTTRHTACLS	248
muHGFA	196	TEKCFDETRYEYFEVGDHWARVSEGHVEQCGCMEGQARCEDTHHTACLS	245
huHGFA	249	PCLNGGTCHLIVATGTTTVCACPFGFAGRLCNIEPDERCFLGNGTGYRGVA	298
muHGFA	246	PCLNGGTCHLIVGTGTSVCTCPGLGYAGRFCNIVETEHCFLGNGTEYRGVA	295
huHGFA	299	STSAAGLSCLAWNSDLLYQELHVDVGAALLLGLGPHAYCRNPDNDERPW	348
muHGFA	296	STAAAGLSCLAWNSDLLYQELHVDVAAAVLLGLGPHAYCRNPDKDERPW	345
huHGFA	349	CYVVKDSALSWEYCRLTACESLTRVQ-LSPDLLATLPEPASPGRQACGR	397
muHGFA	346	CYVVKDNALSWEYCRLTACESLARVHSQTPEILAAALPESAPAVRPTCGKR	395
huHGFA	398	HKKRTFLRPRIIGGSSSLPGSHPWLAAYIYIGDSFCAGSLVHTCWVVSAAH	447
muHGFA	396	HKKRTFLRPRIIGGSSSLPGSHPWLAAYIYIGNSFCAGSLVHTCWVVSAAH	445
huHGFA	448	CFSSHSPPRDSIVSVVLGQHFFNRTTDTVQTFGIEKYIIPYTLYSVFNPSDHD	497
muHGFA	446	CFANSPPRDSITVVLGQHFFNRTTDTVQTFGIEKYVIPYTLYSVFNPNNDHD	495
huHGFA	498	LVLIRLKKKGDRCATRSQFVQPICLPEPGSTFFAGHKCQIAGWGHLDENV	547
muHGFA	496	LVLIRLKKKGERRCAVRSQFVQPICLPEAGSSFFTGHKCQIAGWGHMDENV	545
huHGFA	548	SGYSSSLRREALVPLVADHKCSSPEVYGADISPNMLCAGYFDCKSDACQGD	597
muHGFA	546	SSYSNSLLREALVPLVADHKCSSPEVYGADISPNMLCAGYFDCKSDACQGD	595
huHGFA	598	SGGPLACEKNGVAYLYGIISWGDGCGRLHKPGVYTRVANYVDWINDRIRP	647
muHGFA	596	SGGPLVCEKNGVAYLYGIISWGDGCGRLNKPVGVTYRVANYVDWINDRIRP	645
huHGFA	648	PRRLVATPS	655
muHGFA	646	PKRPVATPS	653

FIG. 7

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Inhibición de la Actividad Enzimática de HGFA por Anticuerpos Anti-HGFA

Anticuerpo #	Análisis de Sustrato Cromógeno ^a IC ₅₀ (nM)	Inhibición proHGF de Sustrato Macromolecular a 670nM Anticuerpo ^b
33	153	**
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	*

^aSustrato era Spectrozyme® fVIIa a 0.2mM (~K_M)

^bEvaluación cualitativa de la inhibición basada en la desaparición de la banda HGF de cadena única

* inhibición débil, ** inhibición fuerte, *** inhibición muy fuerte; N I , sin inhibición

FIG. 8

Unión de HGFA a Anticuerpos Anti-HGFA

Anticuerpo en Bacteriófago Anti-HGFA	Afinidad por ELISA de Bacteriófagos IC ₅₀ (nM)	Afinidad por BIAcore K _D (nM)	Inhibición de la Unión de HGFA por IV49	Inhibición de la Unión de HGFA por Molécula Pequeña	Inhibición de la Unión de HGFA por 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% Inhibición

+/-, 20-50% Inhibición

- <20% Inhibición

[HGFA]: 70 nM

[Competidor] inicio a 10 veces por encima de la KD reportada, y disminución por dilución doble

^aND, No Determinada

FIG. 9

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P2179R1
Sequence Listing

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<150> US 60/615,657
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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

P2179R1

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
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

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260

P2179R1

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
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650 655

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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

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261

P2179R1

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
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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

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262

P2179R1

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
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Asn Gly Thr Tyr

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P2179R1

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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

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

291

P2179R1

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
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95 100 105
Ile Lys Arg Thr

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 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
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 Ser Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
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Tyr Cys

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20 25 30

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Tyr

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Listado de Secuencias

<110> Genentech, Inc.
<120> MODULADORES DE ACTIVADOR DE FACTOR DE CRECIMIENTO HEPATOCÍTICO
<130> P2179R1 PCT
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<141> 2005-10-03
<150> US 60/615,657
<151> 2004-10-04
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Glu Pro Asn Ala Thr Ala Thr Pro Ala Ile Pro Thr Ile Leu Val
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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
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
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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
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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
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Ala Thr Arg Ser Gln Phe Val Gln Pro Ile Cys Leu Pro Glu Pro
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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
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Glu Val Tyr Gly Ala Asp Ile Ser Pro Asn Met Leu Cys Ala Gly
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Tyr Phe Asp Cys Lys Ser Asp Ala Cys Gln Gly Asp Ser Gly Gly
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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
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Tyr Thr Arg Val Ala Asn Tyr Val Asp Trp Ile Asn Asp Arg Ile
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Página 2

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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	
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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	
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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	
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Pro His Ala Tyr Cys	Arg Asn Pro Asp Lys Asp Glu Arg Pro Trp	
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Cys Tyr Val Val Lys	Asp Asn Ala Leu Ser Trp Glu Tyr Cys Arg	
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Leu Thr Ala Cys Glu	Ser Leu Ala Arg Val His Ser Gln Thr Pro	
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Glu Ile Leu Ala Ala	Leu Pro Glu Ser Ala Pro Ala Val Arg Pro	
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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	
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Ala Ala Ile Tyr Ile	Gly Asn Ser Phe Cys Ala Gly Ser Leu Val	
425	430	435

289 299

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His Thr Cys Trp Val Val Ser Ala Ala His Cys Phe Ala Asn Ser
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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
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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

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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
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PCT

From the INTERNATIONAL SEARCHING AUTHORITY

To:

GENENTECH, INC.

MS 49

Attn. Naik, Paul

1 DNA Way

South San Francisco CA-94080

UNITED STATES OF AMERICA

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

Calendared/CLK

30 Day US IDS - P2179R1

13 JUL 06

Due Date

(PCT Rule 44.1)

Date of mailing
(day/month/year)

13/06/2006

Applicant's or agent's file reference

P2179R1

FOR FURTHER ACTION

See paragraphs 1 and 4 below

International application No

PCT/US2005/036300

International filing date
(day/month/year)

03/10/2005

Applicant

GENENTECH, INC

Ch II Deadline/Resp. to WO - 13 SEP 06

Due Date

1. ☒ The applicant is hereby notified that the International search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 18:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46).

When? The time limit for filing such amendments is normally two months from the date of transmittal of the International Search Report.

Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes
1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338 8270

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that

- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices
- ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminders

Shortly after the expiration of 18 months from the priority date, the International application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the International application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis 1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

Within 18 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later), otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the International Searching Authority

 European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx 31 651 epo'nl,
Fax: (+31-70) 340-3018

Authorized officer

Henriëtte Huysing-Solles

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report and the written opinion of the International Searching Authority, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only (see *PCT Applicant's Guide*, Volume I/A, Annexes B1 and B2).

The attention of the applicant is drawn to the fact that amendments to the claims under Article 19 are not allowed where the International Searching Authority has declared, under Article 17(2), that no international search report would be established (see *PCT Applicant's Guide*, Volume I/A, paragraph 296).

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by canceling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference P2179R1	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, Item 5 below	
International application No. PCT/US2005/036300	International filing date (day/month/year) 03/10/2005	(Earliest) Priority Date (day/month/year) 04/10/2004
Applicant GENENTECH, INC		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 6 sheets

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a With regard to the language, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed
☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b ☒ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2 ☒ Certain claims were found unsearchable (See Box No. II)

3 ☐ Unity of invention is lacking (see Box No. III)

4. With regard to the title,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the drawings,

- a the figure of the drawings to be published with the abstract is Figure No. 1
☒ as suggested by the applicant
☐ as selected by this Authority, because the applicant failed to suggest a figure
☐ as selected by this Authority, because this figure better characterizes the invention
- b ☐ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/036300

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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a type of material
 - ☒ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b format of material
 - ☒ on paper
 - ☒ in electronic form
 - c time of filing/furnishing
 - ☐ contained in the international application as filed
 - ☐ filed together with the international application in electronic form
 - ☒ furnished subsequently to this Authority for the purpose of search
- 2 ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished
3. Additional comments:

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/40 A61P35/00 A61K39/395
ADD. C12N9/64

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	KATAOKA HIROAKI ET AL: "Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma" CANCER RESEARCH, vol. 60, no. 21, 1 November 2000 (2000-11-01), pages 6148-6159, XP002380896 ISSN: 0008-5472 the whole document ----- -/-	1-17



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "A" document member of the same patent family

Date of the actual completion of the international search

22 May 2006

Date of mailing of the international search report

13/06/2006

Name and mailing address of the ISA/

European Patent Office, P B 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Tx 31 851 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Bumb, P

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	MIYAZAWA K ET AL: "ACTIVATION OF HEPATOCYTE GROWTH FACTOR IN THE INJURED TISSUES IS MEDIATED BY HEPATOCYTE GROWTH FACTOR ACTIVATOR" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOCHEMICAL BIOLOGISTS, BIRMINGHAM,, US, vol. 271, no. 7, 16 February 1996 (1996-02-16), pages 3615-3618, XP002039579 ISSN: 0021-9258 the whole document	1-17
X	EP 1 213 302 A (MITSUBISHI CHEMICAL CORPORATION) 12 June 2002 (2002-06-12) examples 3-8	1-3,16, 17
A	CAO B ET AL: "Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON,,DC, US, vol. 98, no. 13, 19 June 2001 (2001-06-19), pages 7443-7448, XP002982804 ISSN: 0027-8424 page 7445 - page 7446	12-15
A	US 6 407 213 B1 (CARTER PAUL J ET AL) 18 June 2002 (2002-06-18) cited in the application the whole document	16,17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/036300

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Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons.

1. ☒ Claims Nos : 12-15 (with respect to industrial applicability)
because they relate to subject matter not required to be searched by this Authority, namely
Although claims 12-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos :
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically
3. ☐ Claims Nos :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos :

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 1213302	A	12-06-2002	CN 1384120 A	11-12-2002
			US 2002165362 A1	07-11-2002
			US 2006073520 A1	06-04-2006
US 6407213	B1	18-06-2002	AT 255131 T	15-12-2003
			AU 675916 B2	27-02-1997
			AU 2250992 A	12-01-1993
			CA 2103059 A1	15-12-1992
			CY 2500 A	02-09-2005
			DE 69233254 D1	08-01-2004
			DE 69233254 T2	16-09-2004
			DE 122004000008 I1	09-06-2005
			DK 590058 T3	29-03-2004
			EP 0590058 A1	06-04-1994
			ES 2206447 T3	16-05-2004
			JP 6508267 T	22-09-1994
			JP 2005000169 A	06-01-2005
			JP 2006083180 A	30-03-2006
			LU 91067 A9	02-04-2004
			NL 300145 I1	01-06-2004
			WO 9222653 A1	23-12-1992
			US 6719971 B1	13-04-2004
			US 6639055 B1	28-10-2003
			US 5821337 A	13-10-1998

From the
INTERNATIONAL SEARCHING AUTHORITY

PCT

see form PCT/ISA/220

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43bis.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No
PCT/US2005/036300

International filing date (day/month/year)
03.10.2005

Priority date (day/month/year)
04.10.2004

International Patent Classification (IPC) or both national classification and IPC
INV C07K16/40 A61P35/00 A61K39/395
ADD. C12N9/64

Applicant
GENENTECH, INC

1. This opinion contains indications relating to the following items

- ☒ Box No. I Basis of the opinion
☒ Box No. II Priority
☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐ Box No. IV Lack of unity of invention
☒ Box No. V Reasoned statement under Rule 43bis 1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐ Box No. VI Certain documents cited
☒ Box No. VII Certain defects in the international application
☒ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for International preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66 1bis(b) that written opinions of this International Searching Authority will not be so considered

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later

For further options, see Form PCT/ISA/220

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA



European Patent Office - P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk - Pays Bas
Tel +31 70 340 - 2040 Tx 31 651 epo nl
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Date of completion of
this opinion

see form
PCT/ISA/210

Authorized Officer

Bumb, P

Telephone No +31 70 340-8838



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

317 217
International application No.
PCT/US2005/036300

Box No. I Basis of the opinion

1. With regard to the language, this opinion has been established on the basis of
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).
2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of.
 - a. type of material:
 - ☒ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material.
 - ☒ on paper
 - ☒ in electronic form
 - c. time of filing/furnishing
 - ☐ contained in the international application as filed
 - ☐ filed together with the international application in electronic form
 - ☒ furnished subsequently to this Authority for the purposes of search
3. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
4. Additional comments:

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43bis.1 and 64.1) is the claimed priority date.
2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43bis.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.
3. Additional observations, if necessary:

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of

- ☐ the entire international application
- ☒ claims Nos. 12-15 (with respect to industrial applicability), 1-15 (in part)

because:

- ☒ the said international application, or the said claims Nos. 12-15 (with respect to industrial applicability) relate to the following subject matter which does not require an international search (*specify*):

see separate sheet

- ☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 1-15 (in part) are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed (*specify*):
- ☐ no international search report has been established for the whole application or for said claims Nos.
- ☐ a meaningful opinion could not be formed without the sequence listing, the applicant did not, within the prescribed time limit:
- ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it
- ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rules 13ter.1(a) or (b).
- ☐ a meaningful opinion could not be formed without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-bis of the Administrative Instructions, and such tables were not available to the International Searching Authority in a form and manner acceptable to it
- ☐ the tables related to the nucleotide and/or amino acid sequence listing, if in electronic form only, do not comply with the technical requirements provided for in Annex C-bis of the Administrative Instructions.
- ☐ See Supplemental Box for further details

Box No. V Reasoned statement under Rule 43b/s.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes	Claims	2-3,7-17
	No	Claims	1,4-6
inventive step (IS)	Yes	Claims	
	No	Claims	1-17
Industrial applicability (IA)	Yes	Claims	1-11,16,17
	No	Claims	

2 Citations and explanations

see separate sheet

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made

see separate sheet

Re Item III.

- 1.1 Claims 12-15 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(I) PCT).
- 1.2 Claims 1-15 relate to an extremely large number of possible antibodies, as any antibody may be expected to aspecifically bind HGFA, at least to some degree. Support and disclosure in the sense of Articles 5 and 6 PCT, however, are only to be found for a very limited number of the products claimed. The non-compliance with the substantive provisions is to such an extent that the search was performed taking into consideration this non-compliance in determining the extent of the search of said claims 1-15.

The search of claims 1-15 was restricted to those antibodies which **specifically** bind HGFA, as exemplified by present example 1 (see page 81, line 32 to page 82, line 3).

- 1.3 Claims 12, 14 and 15 relate to an extremely large number of possible methods of treatment. These methods relate to a vast number of possible diseases due to the fundamental unclarity of expressions such as "disease associated with" and "immune related". No support and disclosure in the sense of Articles 5 and 6 PCT is to be found for any of the *in vivo* treatment methods claimed. The non-compliance with the substantive provisions is to such an extent that the search was performed taking into consideration the non-compliance in determining the extent of the search of said claims 1-15.

The search of claims 12, 14 and 15 was restricted to the disease of cancer (see claim 13), diseases related to angiogenesis and autoimmune diseases as defined in the description (see pages 48 & 49).

Re Item V.

1. State of the Art

1.1 Reference is made to the following documents:

- D1: KATAOKA H ET AL: "Activation of Hepatocyte Growth Factor/Scatter Factor in Colorectal Carcinoma", *CANCER RESEARCH*, vol. 60, 1 November 2000 (2000-11-01), pages 6148-6159, XP002380896
- D2: MIYAZAWA K ET AL: "Activation of Hepatocyte Growth Factor in the Injured Tissues Is Mediated by Hepatocyte Growth Factor Activator", *JOURNAL OF BIOLOGICAL CHEMISTRY*, vol. 271, no. 7, 16 February 1996 (1996-02-16), pages 3615-3618, XP002039579
- D3: EP 1213302 A1 (MITSUBISHI CHEMICAL CORPORATION) (12 June 2002) (2002-06-12)
- D4: CAO B ET AL: "Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models", *PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA*, vol. 98, no. 13, 19 June 2001 (2001-06-19), pages 7443-7448, XP002982804

2. Novelty

- 2.1 D1-D3 disclose monoclonal antibodies which specifically bind human HGFA (see D1, pages 6149-6151) (see D1, Fig. 2C) (see D2, pages 3616-3617) (see D2, Fig. 2) (see D3, examples 3-7). The antibody P1-4 of D1 & D2 is shown to inhibit the proteolytic processing of single chain HGF by HGFA *in vitro* (see D1, Fig. 2C) (see D2, Fig. 2). The subject-matter of claims 1 & 4-6 is thus not new (Article 33(2) PCT).
- 2.2 Neither the binding site of anti-HGFA antibodies, nor their mode of inhibiting HGF-conversion has been previously reported. The subject-matter of claims 2-3 and 10-11 is thus novel (Article 33(2) PCT).
- 2.3 The direct inhibition of (a) cell proliferation, (b) angiogenesis or (c) HGF/c-met signalling by means of anti-HGFA antibodies has not been previously reported. The subject-matter of claims 7-9 is thus new (Article 33(2) PCT).
- 2.4 The *in vivo* application of anti-HGFA antibodies has not been previously reported. The subject-matter of claims 12-15 is thus new (Article 33(2) PCT).

- 2.5 Anti-HGFA antibodies which comprise two or more of the CDRs as put forth by Figure 1A or SEQ ID Nos: 71-112 have not been previously disclosed. The subject-matter of claims 16-17 is thus new (Article 33(2) PCT).

3. Inventive Step

- 3.1 D1 is considered to represent the closest prior art with respect to the subject-matter of claim 2 and discloses the neutralizing anti-HGFA mAb P1-4 (see D1, page 6149).

The subject-matter of (a) claim 2 differs from that of (b) mAb P1-4 in that the former recognizes an epitope within the active site of HGFA. The technical effect achieved by said difference may be expected to lie in a complete inhibition of the targeted HGFA-molecule's biological activity. The technical problem to be solved with respect to the state of the art may therefore be regarded as the provision of an antibody which blocks HGFA's active site.

Claim 2 is attempting to define the product for which protection is sought in terms of the result to be achieved ("antibody ... [which] binds to an active site") without providing a solution to the technical problem of attaining said desired properties. It does therefore not constitute an inventive technical solution in the sense of Article 33(3) PCT.

Furthermore, the aforementioned technical problem has not been solved by the present application. The present application does not provide conclusive technical support (Article 5 PCT) for an antibody which binds the **active site** of HGFA (see application, page 83, line 4). The subject-matter of claim 2 therefore does not solve the aforementioned technical problem and does not comply to Article 33(3) PCT.

- 3.2 D1 is considered to represent the closest prior art with respect to the subject-matter of claim 3 and discloses the neutralizing anti-HGFA mAb P1-4 (see D1, page 6149).

The subject-matter of (a) claim 3 differs from that of (b) mAb P1-4 in that the former recognizes an epitope outside the active site of HGFA. The technical effect achieved by said difference is not apparent, as not every such binding is expected to result in

an appreciable inhibitory effect. The technical problem to be solved with respect to the state of the art may therefore be regarded as the provision of an alternative antibody which specifically binds HGFA.

Claim 3 is attempting to define the product for which protection is sought in terms of the result to be achieved ("antibody .. [which] binds to [...] HGFA") without providing a solution to the technical problem of attaining said desired properties. It does therefore not constitute an inventive technical solution in the sense of Article 33(3) PCT.

Furthermore, the aforementioned technical problem has not been solved by the present application. The present application does not provide conclusive technical support (Article 6 PCT) for an antibody which binds HGFA outside of the **active site** of HGFA (see application, page 83, line 4). The subject-matter of claim 2 therefore does not solve the aforementioned technical problem and does not comply to Article 33(3) PCT.

3.3 Claims 7-11 are formulated in terms of the result to be achieved ("antibody which inhibits"/"antibody [which] is an [...] inhibitor") without providing a technical solution to the technical problem of achieving said desired properties. The subject-matter of said claims in itself therefore does not provide an inventive technical solution in the sense of Article 33(3) PCT).

3.4 D1 is considered to represent the closest prior art with respect to the subject-matter of claim 12 and discloses the neutralizing anti-HGFA mAb P1-4 (see D1, page 6149) which is used *in vitro* to downregulate the proteolytic processing of single chain HGF.

The subject-matter of (a) claim 12 differs from that of (b) D1 in that the former uses said known anti-HGFA antibody in an *in vivo* treatment of a disease. The technical effect achieved by said difference may be expected to lie in an *in vivo* therapeutic effect. The technical problem to be solved may therefore be regarded as the provision of an effective medicament.

The person skilled in the art is aware of the fact that interfering with HGF's biological

activity results in an antitumour activity *in vivo* (see D4, pages 7445-7446). He would therefore contemplate using the HGFA-neutralizing antibody P1-4 of D1 in a method of treatment, especially since D1 already suggests that the proteolytic activation step of HGF may serve as a target of therapeutic intervention in the treatment of tumours, i.e. colon cancer (see D1, page 8158).

The subject-matter of claims 12-13 therefore does not involve an inventive step (Article 33(3) PCT).

- 3.5 In the state of the art angiogenesis is generally implicated in the formation of tumours and the HGF/c-Met signalling pathway is well known to be implicated in angiogenesis (see i.e. D4, page 7445). The unclarity of the expression "immune related" notwithstanding, tumorigenesis may be regarded as influencing and thus relating to an immune response.

The subject-matter of claims 14-15 therefore does not introduce any inventive subject-matter over claim 12 and thus does not involve an inventive step (Article 33(3) PCT).

- 3.6 D1 is considered to represent the closest prior art with respect to the subject-matter of claim 16 and discloses the neutralizing anti-HGFA mAb P1-4 (see D1, page 6149).

The subject-matter of (a) claim 16 differs from that of (b) D1 in that the former comprises one specific CDR, wherein said CDR comprises one of the amino acid sequences depicted in Fig. 1A

As the antigen-specificity and affinity of an antibody is only sufficiently well defined when the complete antigen-binding region is defined, in other words, when the complete variable heavy and variable light chain regions are provided, the technical effect achieved by the partial definition of a single CDR is not apparent. The technical problem to be solved is therefore regarded to lie in the provision of an alternative antibody which specifically binds HGFA.

As mentioned above, the provision of a partial CDR is not sufficient to guarantee the

antigen-specificity of a given antibody. The subject-matter of claim 16 is therefore not considered to have solved the aforementioned technical problem (lack of technical support, Article 6 PCT) and a lack of inventive step arises over said claim (Article 33(3) PCT).

- 3.7 Similarly, the subject-matter of claim 17 does not solve the aforementioned technical problem of providing an antibody which specifically binds HGFA (lack of technical support, Article 6 PCT), resulting in lack of inventive step for claim 17 (Article 33(3) PCT).

4. Industrial Applicability

- 4.1 For the assessment of the present claims 12-15 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VII.

- 5.1 The present **method claims** 13-15 are formulated as direct dependent claims of **product claim** 10, thereby rendering the subject-matter of said claims unclear (Article 6 PCT).

Re Item VIII.

- 6.1 Claims 1-11, and consequently claims 12-15 which refer thereto, attempt to define the subject-matter for which protection is sought in terms of the desired properties ("antibody which binds") of the claimed products without providing the technical features or indeed any technical solution to the technical problem of achieving said desiderata. The reader is left in doubt as to the technical features referred to, thereby rendering the subject-matter of said claims unclear (Article 6 PCT).

- 6.2 Claims 1, 2, 3, 7, 8, 9, 11, 12, 14 and 15 do not meet the requirements of Article 6 PCT in that they fail to clearly define the subject-matter for which protection is sought. Said claims make use of certain unclear, vague or relative terminology which leaves the reader in doubt as to the features referred to. Said unclear terminology comprises: "antibody that binds" (claims 1-3, 12), "a position other than" (claim 3), "inhibits" (claims 7-9), "partial competitive inhibitor" (claim 11), "disease associated with", "dysregulation" (claims 12 & 14), "effective amount" (claim 12), "immune related" (claim 15).
- 6.3 Claims 16 & 17 do not meet the requirements of Article 6 PCT in that they do not clearly define the subject-matter for which protection is sought. Said claims refer to a CDR, wherein said ~~one~~ CDR may comprise multiple sequences of Fig. 1A-1D, i.e. H1, H2 and H3, thereby introducing ambiguity about the meaning of said claims.
- 6.4 Claims 16 & 17 contain references to the drawings. According to Rule 6.2(a) PCT, claims should not contain such references except where absolutely necessary, which is not the case here.

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From the INTERNATIONAL BUREAU

PCTNOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To

NAIK, Paul
Genentech, Inc
MS 49
1 DNA Way
South San Francisco, California 94080
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 16 January 2006 (16 01 2006)	
Applicant's or agent's file reference P2179R1	IMPORTANT NOTIFICATION
International application No PCT/US2005/036300	International filing date (day/month/year) 03 October 2005 (03 10 2005)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 04 October 2004 (04 10 2004)
Applicant GENENTECH, INC et al	

1 By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17 1(a) or (b)

2 (If applicable) The letters "NR" appearing in the right-hand column denote a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau under Rule 17 1(a) or (b). Where, under Rule 17 1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17 1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

3 (If applicable) An asterisk (*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b) (the priority document was received after the time limit prescribed in Rule 17 1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17 1(b)). Even though the priority document was not furnished in compliance with Rule 17 1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17 1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority datePriority application No.Country or regional Office
or PCT receiving OfficeDate of receipt
of priority document

04 October 2004 (04 10 2004)

80/815,657

US

05 December 2005 (05 12 2005)

The International Bureau of WIPO
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Form PCT/IB/304 (January 2004)

CPWU33CA



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UNITED STATES PATENT AND TRADEMARK OFFICE

UNDER SECRETARY OF COMMERCE FOR INTELLECTUAL PROPERTY AND
DIRECTOR OF THE UNITED STATES PATENT AND TRADEMARK OFFICE

OCTOBER 17, 2005

PTAS

700203905A***700203905A***

GENENTECH, INC.
PAUL NAIK, PH.D.
1 DNA WAY
SOUTH SAN FRANCISCO, CA 94080

**UNITED STATES PATENT AND TRADEMARK OFFICE
NOTICE OF RECORDATION OF ASSIGNMENT DOCUMENT**

THE ENCLOSED DOCUMENT HAS BEEN RECORDED BY THE ASSIGNMENT DIVISION OF THE U.S. PATENT AND TRADEMARK OFFICE. A COMPLETE MICROFILM COPY IS AVAILABLE AT THE ASSIGNMENT SEARCH ROOM ON THE REEL AND FRAME NUMBER REFERENCED BELOW.

PLEASE REVIEW ALL INFORMATION CONTAINED ON THIS NOTICE. THE INFORMATION CONTAINED ON THIS RECORDATION NOTICE REFLECTS THE DATA PRESENT IN THE PATENT AND TRADEMARK ASSIGNMENT SYSTEM. IF YOU SHOULD FIND ANY ERRORS OR HAVE QUESTIONS CONCERNING THIS NOTICE, YOU MAY CONTACT THE EMPLOYEE WHOSE NAME APPEARS ON THIS NOTICE AT 571-272-3350. PLEASE SEND REQUEST FOR CORRECTION TO: U.S. PATENT AND TRADEMARK OFFICE, MAIL STOP: ASSIGNMENT SERVICES DIVISION, P.O. BOX 1450, ALEXANDRIA, VA 22313.

RECORDATION DATE: 08/17/2005

REEL/FRAME: 016645/0392
NUMBER OF PAGES: 4

BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

ASSIGNOR:

KIRCHHOFFER, DANIEL K.

DOC DATE: 05/16/2005

ASSIGNOR:

WU, YAN

DOC DATE: 07/27/2005

ASSIGNEE:

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CALIFORNIA

94080-4990

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016645/0392 PAGE 2

SERIAL NUMBER: 60615657

FILING DATE: 10/04/2004

PATENT NUMBER:

ISSUE DATE:

TITLE: MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR

THERESA FREDERICK, EXAMINER

ASSIGNMENT DIVISION

OFFICE OF PUBLIC RECORDS

ASSIGNMENT

WHEREAS, Daniel K. Kirchhofer, a citizen of U.S.A., residing at 326 S. Springer Road, Los Altos, California 94024, and Yan Wu, a citizen of U.S.A., residing at 101 Challenge Court, Foster City, California 94404 (hereinafter "ASSIGNORS"), have invented a new and useful invention in

MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR

for which a provisional application Serial No. 60/615,657 (Docket No. PR2179) has been filed by them on 4 October 2004; and

WHEREAS, GENENTECH, INC., a corporation organized and existing under and by virtue of the laws of the State of Delaware, having a place of business at 1 DNA Way, South San Francisco, California 94080-4490, is desirous of acquiring an interest in and to said invention, and in and to the Letters Patents to be obtained therefor;

NOW, THEREFORE, be it known by all whom it may concern;

That for good and valuable consideration the receipt of which is hereby acknowledged, the said ASSIGNORS have and do hereby sell, assign, transfer and set over unto the said GENENTECH, INC., its successors and assigns, the full and exclusive right, title and interest including all rights under the Paris Convention for the Protection of Industrial Property, in and to said invention, and in and to any and all Letters Patents to be granted and issued therefor or any continuation, division, renewal, or substitute thereof, and as to Letters Patents any reissue or re-examination thereof, not only for, to, and in the United States of America, its territories and possessions, but for, to and in all other countries; and it has been and is hereby authorized and requested that the appropriate government agencies issue said Letters Patents to said GENENTECH, INC., in accordance with this Assignment.

Said ASSIGNORS covenant and agree to cooperate with GENENTECH, INC., to enable said GENENTECH, INC. to enjoy to the fullest extent the right, title and interest herein conveyed in the United States and foreign countries. Such cooperation by said ASSIGNORS includes prompt production of pertinent facts and documents, giving of testimony, execution of petitions, oaths, specifications, declarations or other papers, and other assistance all to the extent deemed necessary or desirable by said GENENTECH, INC., (a) for perfecting the right, title and interest herein conveyed; (b) for prosecuting any of said applications; (c) for filing and prosecuting applications for reissuance of any of said patents; (d) for interference or other priority proceedings involving said invention; and (e) for legal proceedings involving said invention and any applications therefor and any patents granted thereon, including without limitation opposition proceedings, cancellation proceedings, priority contests, public use proceedings, infringement actions and court actions; provided, however, that the expense incurred by said ASSIGNORS in providing such cooperation shall be paid for by said GENENTECH, INC.

The terms and covenants of this assignment shall inure to the benefit of said GENENTECH, INC., its successors, assigns and other legal representatives, and shall be binding upon said ASSIGNORS, their respective heirs, legal representatives and assigns.

Said ASSIGNORS hereby warrant and represent that they have not entered and will not enter into any assignment, contract, or understanding in conflict herewith.

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IN WITNESS WHEREOF we undersign as follows;

South San Francisco

Dated: 5/16/05

Daniel K. Kirchhofer

Daniel K. Kirchhofer

South San Francisco

Dated: _____

Yan Wu

332

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IN WITNESS WHEREOF we undersign as follows;

South San Francisco

Daniel K. Kirchhofer

Dated: _____

South San Francisco

Yan Wu

Yan Wu

Dated: 1-27-05

PCT REQUEST

Original (for SUBMISSION)

0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form PCT/RO/101 PCT Request Prepared Using	PCT-SAFE [EASY mode] Version 3.50 (Build 0002.173)
0-5	Petition The undersigned requests that the present International application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	United States Patent and Trademark Office (USPTO) (RO/US)
0-7	Applicant's or agent's file reference	P2179R1
I	Title of Invention	MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR
II	Applicant	
II-1	This person is	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	GENENTECH, INC.
II-5	Address	1 DNA Way South San Francisco, California 94080-4990 United States of America
II-6	State of nationality	US
II-7	State of residence	US
II-8	Telephone No.	1-650-225-1000
II-9	Facsimile No.	1-650-952-9881
III-1	Applicant and/or inventor	
III-1-1	This person is	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	KIRCHHOFFER, Daniel K.
III-1-5	Address	326 S. Springer Road Los Altos, California 94024 United States of America
III-1-6	State of nationality	US
III-1-7	State of residence	US

PCT REQUEST

Original (for SUBMISSION)

III-2	Applicant and/or inventor	
III-2-1	This person is	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	WU, Yan
III-2-5	Address	101 Challenge Court Foster City, California 94404 United States of America
III-2-6	State of nationality	US
III-2-7	State of residence	US
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	NAIK, Paul
IV-1-2	Address	Genentech, Inc. MS 49 1 DNA Way South San Francisco, California 94080 United States of America
IV-1-3	Telephone No.	650-225-5530
IV-1-4	Facsimile No.	650-952-9881
V	DESIGNATIONS	
V-1	The filing of this request constitutes under Rule 4.8(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.	
VI-1	Priority claim of earlier national application	
VI-1-1	Filing date	04 October 2004 (04.10.2004)
VI-1-2	Number	60/615,657
VI-1-3	Country	US
VI-2	Priority document request The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s)	VI-1
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)

PCT REQUEST

Original (for SUBMISSION)

VIII	Declarations	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	Check list	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	4	✓
IX-2	Description	85	-
IX-3	Claims	1	-
IX-4	Abstract	1	✓
IX-5	Drawings	22	-
IX-7	TOTAL	113	
	Accompanying items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-11	Copy of general power of attorney	✓	-
IX-17	PCT-SAFE physical media	-	✓
IX-19	Figure of the drawings which should accompany the abstract	1	
IX-20	Language of filing of the international application	English	
X-1	Signature of applicant, agent or common representative		
X-1-1	Name (LAST, First)	NAIK, Paul	
X-1-2	Name of signatory		
X-1-3	Capacity		

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FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	
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MODULATORS OF HEPATOCYTE GROWTH FACTOR ACTIVATOR

RELATED APPLICATIONS

10 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

15 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

20 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
25 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
30 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.

35 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 specifically 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.

DISCLOSURE OF THE INVENTION

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 interfering 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.

In one aspect, the invention provides antagonists that disrupt the HGF/c-met signaling pathway. For example, the invention provides 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 invention does not inhibit HGF binding to c-met. For example, in one embodiment, an antagonist molecule of the invention 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

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HB-11894 (hybridoma 1A3.3.13) or HB-11895 (hybridoma 5D5.11.6). In one embodiment, an antagonist molecule of the invention inhibits biological activities associated with HGF/c-met activation.

In one aspect, the invention provides 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. In one aspect, the invention provides 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. In one aspect, the invention provides 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 invention provides 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, 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 invention provides 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, 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 invention provides 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, 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 invention provides 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, 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 aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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.

In one aspect, the invention provides 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 invention 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.

In one aspect, the invention provides 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

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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
5 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
15 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,
20 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)
25 (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
30 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,
35 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,

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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 of the invention 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 of the invention 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 of the invention 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 of the invention 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.

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

In one aspect, an antagonist molecule of the invention is 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.

The invention also provides methods and compositions useful for modulating disease states associated with dysregulation of the HGF/c-met signaling axis. Thus, in one aspect, the invention provides 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. In one aspect, the invention provides 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 of the invention 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, in another aspect, the invention provides 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 the invention, whereby cell proliferation associated with c-met activation is inhibited. In yet another aspect, the invention provides 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 the invention, whereby angiogenesis is inhibited.

In one aspect, the invention provides use of a modulator molecule of the invention 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.

In one aspect, the invention provides use of a nucleic acid of the invention 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.

In one aspect, the invention provides use of an expression vector of the invention 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.

In one aspect, the invention provides use of a host cell of the invention 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.

In one aspect, the invention provides use of an article of manufacture of the invention 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.

In one aspect, the invention provides use of a kit of the invention 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.

In one aspect, the invention provides a method of inhibiting c-met activated cell proliferation, said method comprising contacting a cell or tissue with an effective amount of a

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modulator molecule of the invention, whereby cell proliferation associated with c-met activation is inhibited

In one aspect, the invention provides a method of treating a pathological condition associated with dysregulation of c-met activation in a subject, said method comprising administering
5 to the subject an effective amount of a modulator molecule of the invention, whereby said condition is treated.

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

In one aspect, the invention provides 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
15 of the invention, 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).

In one aspect, the invention provides 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
20 of the invention, thereby effectively treating or preventing said cell proliferative disorder. In one embodiment, said proliferative disorder is cancer.

In one aspect, the invention provides 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
25 amount of a modulator molecule of the invention, thereby effectively treating or preventing said cell proliferative disorder. In one embodiment, said proliferative disorder is cancer.

In one aspect, the invention provides 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
30 invention, 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).

In one aspect, the invention provides 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
35 amount of a modulator molecule of the invention, 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).

In one aspect, the invention provides 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 invention, 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).

In one aspect, the invention provides 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 invention, 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 invention 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 invention 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 invention is a hyperproliferative and/or hyperplastic cell. In one embodiment, a cell that is targeted in a method of the invention is a dysplastic cell. In yet another embodiment, a cell that is targeted in a method of the invention is a metastatic cell.

Methods of the invention 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 invention, 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 invention causes the death of a targeted cell. For example, contact with a modulator molecule of the invention 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 invention comprises targeting a tissue wherein one or more of HGFA, c-met and hepatocyte 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 invention, 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).

In one aspect, the invention provides compositions comprising one or more modulator molecules of the invention and a carrier. In one embodiment, the carrier is pharmaceutically acceptable.

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

In one aspect, the invention provides vectors comprising a nucleic acid of the invention.

In one aspect, the invention provides host cells comprising a nucleic acid or a vector of the invention. 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.

In one aspect, the invention provides methods for making a modulator molecule of the invention. For example, the invention provides 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 invention encoding said antibody (or fragment thereof), and recovering said antibody.

In one aspect, the invention provides 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 invention. In one embodiment, the composition comprises a nucleic acid of the invention. 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 of the invention further comprises instructions for administering the composition (for e.g., the modulator molecule) to a subject.

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In one aspect, the invention provides a kit comprising a first container comprising a composition comprising one or more modulator molecules of the invention; 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.

In one aspect the invention provides 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 invention, whereby HGFA bound by the antibody indicates presence and/or amount of HGFA in the sample. In another aspect, the invention provides 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 invention 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 invention, 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.

In one aspect, the invention provides a method of binding an antibody of the invention to HGFA present in a bodily fluid, for example blood.

In yet another aspect, the invention is directed to a method of binding an antibody of the invention 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.

In one aspect, the invention provides 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 invention, 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.

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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); clone 35 (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 ¹²⁵I-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 ¹²⁵I-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.

5 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., $K_M = 200 \mu M$) the linear rates of substrate activation were measured on a kinetic microplate reader. Inhibition of enzyme activity was expressed as fractional activity (v_i/v_o) of uninhibited activity.

10 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).

20 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.

30 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.

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

MODES FOR CARRYING OUT THE INVENTION

The invention provides 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.

5 ***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., 10 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).

15 ***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 20 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 25 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), 30 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 35 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.

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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 3 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:

$$100 \text{ times the fraction } X/Y$$

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

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 * 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},

```

20

 $\frac{1}{2}$

```
#include <ctype.h>
```

30

```
#define PINS0      8      /* penalty for a gap */
#define PINS1      4      /* penalty per residue */

struct jmp {
5      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 {
10      int      score; /* score at last jmp */
      long      offset; /* offset of prev block */
      short      ijmp; /* current jmp index */
      struct jmp  jp; /* list of jmps */
};

15      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) */
20  };

      char      *ofile; /* output file name */
      char      *namex[2]; /* seq names: getseqs() */
      char      *prog; /* prog name for err msgs */
25      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 */
30      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 */
```


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```
long      offset;          /* current offset in jmp file */
struct diag *dx;           /* holds diagonals */
struct path pp[2];         /* holds path for seqs */

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

10

/* Needleman-Wunsch alignment program
 *
 * usage: progs file1 file2
15  * where file1 and file2 are two dna or two protein sequences.
 * The sequences can be in upper- or lower-case and 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
20  * 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
 */
25  #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
30  };

static _pbval[26] = {
        1, 2|(1<<('D'-'A'))|(1<<('N'-'A')), 4, 8, 16, 32, 64,
        128, 256, 0xFFFFFFFF, 1<<10, 1<<11, 1<<12, 1<<13, 1<<14,
```

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```

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'))
};

```

```

5  main(ac, av)                                main
    int    ac;
    char   *av[];
    {
        prog = av[0];
10     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");
15     fprintf(stderr, "Output is in the file \"align.out\"\n");
            exit(1);
        }
        namex[0] = av[1];
        namex[1] = av[2];
20     seqx[0] = getseq(namex[0], &len0);
        seqx[1] = getseq(namex[1], &len1);
        xbm = (dna)? _dbval : _pbval;

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

        nw();    /* fill in the matrix, get the possible jmps */
        readjmps(); /* get the actual jmps */
        print();  /* print stats, alignment */
30
        cleanup(0); /* unlink any tmp files */
    }

```

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```

/* do the alignment, return best score: main()
 * dna: values in Fitch and Smith, PNAS, 80, 1382-1386, 1983
 * pro: PAM 250 values
5  * 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
10 {
    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 */
15  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 */
20  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));
25  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;

30  smax = -10000;
    if (endgaps) {
        for (col0[0] = dely[0] = -ins0, yy = 1; yy <= len1; yy++) {
            col0[yy] = dely[yy] = col0[yy-1] - ins1;
        }
    }
}

```

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```

        ndely[yy] = yy;
    }
    col0[0] = 0; /* Waterman Bull Math Biol 84 */
}
5  else
    for (yy = 1; yy <= len1; yy++)
        dely[yy] = -ins0;

/* fill in match matrix
10 */
for (px = seqx[0], xx = 1; xx <= len0; px++, xx++) {
    /* initialize first entry in col
    */
    if (endgaps) {
15         if (xx == 1)
            col1[0] = delx = -(ins0+ins1);
        else
            col1[0] = delx = col0[0] - ins1;
        ndelx = xx;
20     }
    else {
        col1[0] = 0;
        delx = -ins0;
        ndelx = 0;
25     }

...nw

30 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 ongoing del
 * ignore MAXGAP if weighting endgaps
 */
5
if (endgaps || ndely[yy] < MAXGAP) {
    if (col0[yy] - ins0 >= dely[yy]) {
        dely[yy] = col0[yy] - (ins0+ins1);
        ndely[yy] = 1;
10
    } else {
        dely[yy] -= ins1;
        ndely[yy]++;
    }
} else {
15
    if (col0[yy] - (ins0+ins1) >= dely[yy]) {
        dely[yy] = col0[yy] - (ins0+ins1);
        ndely[yy] = 1;
    } else
        ndely[yy]++;
20
}

/* update penalty for del in y seq;
 * favor new del over ongoing del
 */
25
if (endgaps || ndelx < MAXGAP) {
    if (col1[yy-1] - ins0 >= delx) {
        delx = col1[yy-1] - (ins0+ins1);
        ndelx = 1;
    } else {
30
        delx -= ins1;
        ndelx++;
    }
} else {
    if (col1[yy-1] - (ins0+ins1) >= delx) {

```

```

        delx = coll[yy-1] - (ins0+ins1);
        ndelx = 1;
    } else,
        ndelx++;
5      }

/* pick the maximum score; we're favoring
 * mis over any del and delx over dely
 */
10

15

...nw

id = xx - yy + len1 - 1;
if (mis >= delx && mis >= dely[yy])
20     coll[yy] = mis;
else if (delx >= dely[yy]) {
    coll[yy] = delx;
    ij = dx[id].ijmp;
    if (dx[id].jp.n[0] && (!dna || (ndelx >= MAXJMP
25     && 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);
        }
    }
}

```

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```

dx[id].jp.n[ij] = ndelx;
dx[id].jp.x[ij] = xx;
dx[id].score = delx;
    }
5      else {
        col1[yy] = dely[yy];
        ij = dx[id].ijmp;
        if (dx[id].jp.n[0] && (!dna || (ndely[yy] >= MAXJMP
10      && xx > dx[id].jp.x[ij]+MX) || mis > dx[id].score+DINS0))
        {
            dx[id].ijmp++,
            if (++ij >= MAXJMP) {
                writejumps(id);
                ij = dx[id].ijmp = 0;
15          dx[id].offset = offset;
                offset += sizeof(struct jmp) + sizeof(offset);
            }
        }
        dx[id].jp.n[ij] = -ndely[yy];
20      dx[id].jp.x[ij] = xx;
        dx[id].score = dely[yy];
    }
    if (xx == len0 && yy < len1) {
        /* last col
25      */
        if (endgaps)
            col1[yy] -= ins0+ins1*(len1-yy);
        if (col1[yy] > smax) {
            smax = col1[yy];
30          dmax = id;
        }
    }
}
if (endgaps && xx < len0)

```

```

        coll[yy-1] -= ins0+ins1*(len0-xx);
        if (coll[yy-1] > smax) {
            smax = coll[yy-1];
            dmax = id;
5          }
        tmp = col0; col0 = coll; coll = tmp;
    }
    (void) free((char *)ndely);
    (void) free((char *)dely);
10    (void) free((char *)col0);
    (void) free((char *)coll);
}

/*
 *
15  * 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()
20  * 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
25  */

#include "nw.h"

#define SPC 3

30 #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 */

```


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FILE *fx; /* output file */

print() print

{

5 int lx, ly, firstgap, lastgap; /* overlap */

if ((fx = fopen(ofile, "w")) == 0) {
fprintf(stderr, "%s: can't write %s\n", prog, ofile);
cleanup(1);

10

}
fprintf(fx, "< first sequence: %s (length = %d)\n", namex[0], len0);
fprintf(fx, "< second sequence: %s (length = %d)\n", namex[1], len1);
olen = 60;

15

lx = len0;
ly = len1;
firstgap = lastgap = 0;
if (dmax < len1 - 1) { /* leading gap in x */

20

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;
}

25

if (dmax0 < len0 - 1) { /* trailing gap in x */
lastgap = len0 - dmax0 - 1;
lx -= lastgap;
}

30

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

getmat(lx, ly, firstgap, lastgap);
pr_align();

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}

5

/*

* trace back the best path, count matches

*/

static

10

getmat(lx, ly, firstgap, lastgap)

getmat

int lx, ly; /* "core" (minus endgaps) */

int firstgap, lastgap; /* leading trailing overlap */

{

int nm, i0, i1, siz0, siz1;

15

char outx[32];

double pct;

register n0, n1;

register char *p0, *p1;

20

/* get total matches, score

*/

i0 = i1 = siz0 = siz1 = 0;

p0 = seqx[0] + pp[1].spc;

p1 = seqx[1] + pp[0].spc;

25

n0 = pp[1].spc + 1;

n1 = pp[0].spc + 1;

nm = 0;

while (*p0 && *p1) {

30

if (siz0) {

p1++;

n1++;

siz0--;

}

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```

else if (siz1) {
    p0++;
    n0++;
    siz1--;
5      }
    else {
        if (xbm[*p0-'A']&xbm[*p1-'A'])
            nm++;
        if (n0++ == pp[0].x[i0])
10          siz0 = pp[0].n[i0++];
        if (n1++ == pp[1].x[i1])
            siz1 = pp[1].n[i1++];
        p0++;
        p1++;
15      }
    }

/* pct homology:
 * if penalizing endgaps, base is the shorter seq
20  * else, knock off overhangs and take shorter core
 */
if (endgaps)
    lx = (len0 < len1)? len0 : len1;
else
25    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);
30

```

fprintf(fx, "< gaps in first sequence: %d", gapx);

...getmat

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```

    if (gapx) {
        (void) sprintf(outx, " (%d %s%s)",
            ngapx, (dna)? "base":"residue", (ngapx == 1)? "" : "s");
        fprintf(fx, "%s", outx);
5
    }

    fprintf(fx, ", gaps in second sequence: %d", gapy);
    if (gapy) {
        (void) sprintf(outx, " (%d %s%s)",
            ngapy, (dna)? "base":"residue", (ngapy == 1)? "" : "s");
10
        fprintf(fx, "%s", outx);
    }

    if (dna)
        fprintf(fx,
            "\n<score: %d (match = %d, mismatch = %d, gap penalty = %d + %d per
15 base)\n",
            smax, DMAT, DMIS, DINS0, DINS1);
    else
        fprintf(fx,
            "\n<score: %d (Dayhoff PAM 250 matrix, gap penalty = %d + %d per
20 residue)\n",
            smax, PINS0, PINS1);

    if (endgaps)
        fprintf(fx,
            "<endgaps penalized. left endgap: %d %s%s, right endgap: %d %s%s\n",
25 firstgap, (dna)? "base" : "residue", (firstgap == 1)? "" : "s",
            lastgap, (dna)? "base" : "residue", (lastgap == 1)? "" : "s");
    else
        fprintf(fx, "<endgaps not penalized\n");
}
30

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 */

```

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```

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 */
5  static char out[2][P_LINE]; /* output line */
static char star[P_LINE]; /* set by stars() */

/*
 * print alignment of described in struct path pp[]
10 */
static
pr_align()                                pr_align
{
    int      nn;      /* char count */
15    int      more;
    register i;

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

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

30    for (nn = nm = 0, more = 1; more; ) {
        for (i = more = 0; i < 2; i++) {
            /*
             * do we have more of this sequence?
            */

```

```

if (!*ps[i])
    continue;

more++;

5
if (pp[i].spc) { /* leading space */
    *po[i]++ = ' ';
    pp[i].spc--;
}
- 10
else if (siz[i]) { /* in a gap */
    *po[i]++ = '-';
    siz[i]--;
}
-
else { /* we're putting a seq element
15
    */
    *po[i] = *ps[i];
    if (islower(*ps[i]))
        *ps[i] = toupper(*ps[i]);
    po[i]++;
    ps[i]++;
20

    /*
    * are we at next gap for this seq?
    */

25
    if (ni[i] == pp[i].x[ij[i]]) {
        /*
        * we need to merge all gaps
        * at this location
        */
30
        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();
5       for (i = 0; i < 2; i++)
            po[i] = out[i];
        nn = 0;
    }
}
10 }

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

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

    ...dumpblock

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

```

```

        if (i == 1)
            nums(i);
    }
}
5  }

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

        for (pn = nline, i = 0; i < lmax+P_SPC; i++, pn++)
            *pn = ' ';
        20 for (i = nc[ix], py = out[ix]; *py; py++, pn++) {
            if (*py == ' ' || *py == '-')
                *pn = ' ';
            else {
                if (i%10 == 0 || (i == 1 && nc[ix] != 1)) {
                    25 j = (i < 0)? -i : i;
                    for (px = pn; j; j /= 10, px--)
                        *px = j%10 + '0';
                    if (i < 0)
                        *px = '-';
                    30 }
                else
                    *pn = ' ';
                i++;
            }
        }
    }

```


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```

    }
    *pn = '\0';
    nc[ix] = i;
    for (pn = nline; *pn; pn++)
5      (void) putc(*pn, fx);
    (void) putc('\n', fx);
}

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

15

                                                    ...putline

    int    i;
    register char *px;

20
    for (px = namex[ix], i = 0; *px && *px != ':'; px++, i++)
        (void) putc(*px, fx);
    for (; i < lmax + P_SPC; i++, ...)
        (void) putc(' ', fx);

25

    /* these count from 1:
       * ni[] is current element (from 1)
       * nc[] is number at start of current line
       */
30  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()
 */
5 static
stars()                                stars
{
    int          i;
    register char *p0, *p1, cx, *px;

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

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

```

```
    *px = '\0';
}

5

/*
10  * strip path or prefix from pn, return len: pr_align()
    */
    static
    stripname(pn)                                stripname
        char    *pn;    /* file name (may be path) */
15  {
        register char    *px, *py;

        py = 0,
        for (px = pn; *px; px++)
20          if (*px == '/')
                py = px + 1;
        if (py)
            (void) strcpy(pn, py);
        return(strlen(pn));
25  }

/*
30  * cleanup() -- cleanup any tmp file
    * getseq() -- read in seq, set dna, len, maxlen
    * g_calloc() -- calloc() with error checkin
    * readjumps() -- get the good jumps, from tmp file if necessary
    * writejumps() -- write a filled array of jumps to a tmp file: nw()
```

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```

    */
#include "nw.h"
#include <sys/file.h>

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

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

10  /*
    * remove any tmp file if we blow
    */
    cleanup(i)                                  cleanup
15      int    i;
    {
        if (fj)
            (void) unlink(jname);
        exit(i);
20  }

    /*
    * read, return ptr to seq, set dna, len, maxlen
    * skip lines starting with ';', '<', or '>'
25  * seq in upper or lower case
    */
    char    *
    getseq(file, len)                          getseq
30      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);
5      exit(1);
}
tlen = natgc = 0;
while (fgets(line, 1024, fp)) {
    if (*line == ';' || *line == '<' || *line == '>')
10      continue;
    for (px = line; *px != '\n'; px++)
        if (isupper(*px) || islower(*px))
            tlen++;
}
15 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';
20

...getseq

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

while (fgets(line, 1024, fp)) {
    if (*line == ';' || *line == '<' || *line == '>')
30      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++;
    }
5      }
      *py++ = '\0';
      *py = '\0';
      (void) fclose(fp);
      dna = natgc > (tlen/3);
10     return(pseq+4);
}

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

20     if ((px = calloc((unsigned)nx, (unsigned)sz)) == 0) {
         if (*msg) {
             fprintf(stderr, "%s: g_alloc() failed %s (n=%d, sz=%d)\n", prog, msg,
nx, sz);
             exit(1);
25         }
     }
     return(px);
}

30 /*
   * get final jmps from dx[] or tmp file, set pp[], reset dmax: main()
   */
readjmps()                                readjmps
{

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```

int          fd = -1;
int          siz, i0, i1;
register i, j, xx;

5      if (fj) {
        (void) fclose(fj);
        if ((fd = open(jname, O_RDONLY, 0)) < 0) {
            fprintf(stderr, "%s: can't open() %s\n", prog, jname);
            cleanup(1);
10         }
    }
    for (i = i0 = i1 = 0, dmax0 = dmax, xx = len0; ; i++) {
        while (1) {
            for (j = dx[dmax].ijmp; j >= 0 && dx[dmax].jp.x[j] >= xx; j--)
15                 ;

                                                    ...readjumps

            if (j < 0 && dx[dmax].offset && fj) {
                (void) lseek(fd, dx[dmax].offset, 0);
20                (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;
            }
25            else
                break;
        }
        if (i >= JMPS) {
            fprintf(stderr, "%s: too many gaps in alignment\n", prog);
30            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,
5      /* id = xx - yy + len1 - 1
        */
        pp[1].x[i1] = xx - dmax + len1 - 1;
        gapy++;
        ngapy -= siz;
10     /* ignore MAXGAP when doing endgaps */
        siz = (-siz < MAXGAP || endgaps)? -siz : MAXGAP;
        i1++;
    }
    else if (siz > 0) {     /* gap in first seq */
15     pp[0].n[i0] = siz;
        pp[0].x[i0] = xx;
        gapx++;
        ngapx += siz;
    /* ignore MAXGAP when doing endgaps */
20     siz = (siz < MAXGAP || endgaps)? siz : MAXGAP;
        i0++;
    }
}
else
25     break;
}

/* reverse the order of jmps
*/
30 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--) {

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```

        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)
5        (void) close(fd);
    if (fj) {
        (void) unlink(jname);
        fj = 0;
        offset = 0;
10    }
}

/*
 * write a filled jmp struct offset of the prev one (if any) nw()
15 */
writejmps(ix)                                writejmps
    int    ix;
{
    char    *mktemp();
20
    if (!fj) {
        if (mktemp(jname) < 0) {
            fprintf(stderr, "%s: can't mktemp() %s\n", prog, jname);
            cleanup(1);
25        }
        if ((fj = fopen(jname, "w")) == 0) {
            fprintf(stderr, "%s: can't write %s\n", prog, jname);
            exit(1);
        }
30    }

    (void) fwrite((char *)&dx[ix].jp, sizeof(struct jmp), 1, fj);
    (void) fwrite((char *)&dx[ix].offset, sizeof(dx[ix].offset), 1, fj);

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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, poly-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
5 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.
10 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.
15 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
20 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
25 *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
30 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
35 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.

5 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
10 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
15 NO:46)-H1-WVRQAPGKGLEWV (SEQ ID NO:47)-H2-RFTISRDN SKNTLYLQMNSLRAEDTAVYYC (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
20 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-GVPSRFSGSGSGTDFLTISLQPEDFATYYC (SEQ ID NO:52)-L3-FGQGTKVEIK (SEQ ID NO:53).

25 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. The term "wild type
30 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
35 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);
5 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).

10 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.

15 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);
20 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.

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

30 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.

35 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.

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"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.

5 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
 ~ 10 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.

15 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
 20 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,
 25 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, hypertrophic bone formation, osteoarthritis (OA), refractory ascites, polycystic ovarian
 30 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
 35 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 mellitus); 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 cryoglobulinemia 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

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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., At²¹¹, I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹², P³² and radioactive isotopes of Lu), chemotherapeutic agents e.g. methotrexate, adriamycin, 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® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide 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, scopoletin, 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 ranimustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gamma11 and calicheamicin omega11 (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 antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, 5 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, 10 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, 15 floxuridine; androgens such as calusterone, dromostanolone propionate, epitio stanol, 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 20 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®, 25 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; 30 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 35 prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovovin.

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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 triptorelin; 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®), formestane, 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 aberrant 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 I 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,

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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.

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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 λ GEM.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.

In one aspect of the invention, each cistron within the recombinant vector comprises 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 invention 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 invention, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof.

In another aspect, the production of the immunoglobulins according to the invention 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).

The present invention provides 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 invention. 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 Archaeobacteria 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 Δ fhuA (Δ tonA) ptr3 lac Iq lacL8 Δ ompT Δ (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 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.

In one aspect of the invention, antibody production is conducted in large quantity by a fermentation process. Various large-scale fed-batch fermentation procedures are available for

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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 OD₅₅₀ 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 invention, 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 invention. 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 invention.

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

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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.

5 In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the full length antibody products of the invention. Protein A is a 41kD cell wall protein from *Staphylococcus aureus* 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.

10 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:

20 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

25 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.

30 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

5 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.

10 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.

15 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.

20 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).

25 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

30 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.

35 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.

5 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.
10 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

15 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
20 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.

25 (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
30 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

35 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

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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

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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.

5 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 $\gamma 1$, $\gamma 2$,
10 or $\gamma 4$ heavy chains (Lindmark et al., J. Immunol. Meth. 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss et al., EMBO J. 5:1567-1575 (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(styrene-divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the
15 antibody comprises a C_H3 domain, the Bakerbond ABXTM 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 SEPHAROSETM chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate
20 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).

25 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
30 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
35 can be used herein include without limitation any direct or competitive binding assays using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay),

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"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 Fc(RIII) only, whereas monocytes express Fc(RI, Fc(RII and Fc(RIII. 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.

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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

In one aspect, the invention provides 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

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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.

5 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
10 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
15 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
20 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
25 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

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Original Residue	Exemplary Substitutions	Preferred Substitutions
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;

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- (3) acidic: Asp, Glu;
- (4) basic: His, Lys, Arg;
- (5) residues that influence chain orientation: Gly, Pro;
- (6) aromatic: Trp, Tyr, Phe.

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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.

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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.

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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

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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-maytansinoid conjugate was tested in vitro on the human breast cancer cell line SK-BR-3, which expresses 3×10^5 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)

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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-

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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_1^1 , α_2^1 , α_3^1 , N-acetyl- γ_1^1 , PSAG and θ^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, streptozocin, 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 ^{99m}Tc 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 ^{99m}Tc or ^{123}I , ^{186}Re , ^{188}Re and ^{111}In 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.



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 substituents 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 glucose 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 as 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, propylene glycol homopolymers, polypropylene 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

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including glucose, mannose, or dextrans; 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).

5 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.

10 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-(methylmethacrylate) 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).

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

20 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-

25 (-)-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.

30 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

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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 invention 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.

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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

In another aspect of the invention, 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 comprises 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

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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 CaCl₂) for 15 min at room temperature, after which ¹²⁵I-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 ¹²⁵I-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. 10⁶cpm/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

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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 μ M 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 Langmuir 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 μ M final conc., $K_M = 200 \mu$ M) 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 μ g/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

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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 $1\mu\text{M}$ 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 $0.67\mu\text{M}$ significantly inhibited proHGF conversion (Fig. 2). Additional experiments showed that #58 inhibited proHGF conversion at concentrations as low as $0.03\mu\text{M}$ (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 $\text{IC}_{50} > 500\text{nM}$ (Fig. 8). The 3 antibodies #42, #61, and #74 also showed relatively weak inhibition ($\text{IC}_{50} > 500\text{nM}$) despite almost complete inhibition of proHGF conversion at $0.67\mu\text{M}$ (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 $0.079\mu\text{M}$, whereas the small molecule HGFA active site binder inhibited with an IC_{50} of $0.8\mu\text{M}$ ($K_i = 0.4\mu\text{M}$) 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.

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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).
2. The antibody of claim 1, wherein the antibody binds to an active site of HGFA.
- 5 3. The antibody of claim 1, wherein the antibody binds to HGFA at a position other than an active site of HGFA.
4. The antibody of claim 1 which is a blocking antibody.
5. The antibody of claim 4 which blocks HGFA proteolytic activity.
6. The antibody of claim 5 which blocks HGFA proteolysis of single chain HGF.
- 10 7. The antibody of claim 1 which inhibits HGF/c-met signaling.
8. The antibody of claim 7 which inhibits cell proliferation.
9. The antibody of claim 7 which inhibits angiogenesis.
10. The antibody of claim 1, wherein the antibody is an allosteric inhibitor of HGFA enzymatic activity.
- 15 11. The antibody of claim 1, wherein the antibody is a complete or partial competitive inhibitor of HGFA enzymatic activity.
12. A method 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 that binds HGFA in accordance with any of the preceding claims.
- 20 13. The method of claim 10 wherein the disease is cancer.
14. The method of claim 10 wherein the disease is associated with dysregulation of angiogenesis.
15. The method of claim 10 wherein the disease is immune related.
16. An isolated antibody that specifically binds human hepatocyte growth factor activator, wherein the antibody comprises a heavy chain CDR sequence comprising the respective H1,
25 H2 and/or H3 sequences depicted in Figure 1A.
17. The antibody of claim 16, wherein the H1, H2 and/or H3 sequences are as depicted in Figures 1B, 1C and/or 1D.

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ABSTRACT

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State of California

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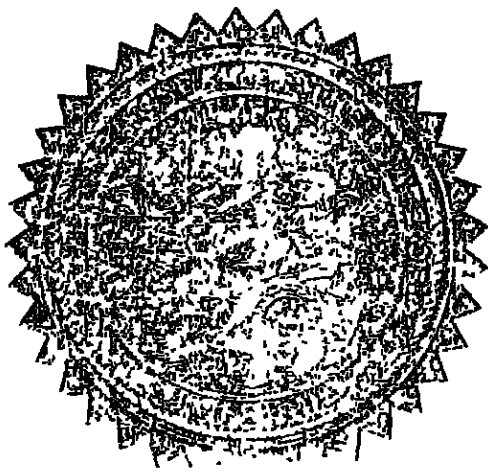
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3. acting in the capacity of Notary Public, State of California
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State of California

CERTIFIED

5. At Sacramento, California
6. the 15th day of December 2005
7. by Deputy Secretary of State, State of California
- 8 No. 267083
9. Seal/Stamp:



10 Signature

Donald R. Harris

Secretary of State

BY *[Signature]*



CALIFORNIA ALL-PURP SE ACKN WLEDGMENT

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State of California

County of

San Mateo

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On December 6, 2005 before me,

Aida Mielat Guian

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personally appeared

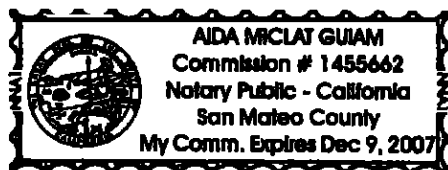
Timothy R. Schwartz

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☐ personally known to me

☒ proved to me on the basis of satisfactory evidence

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WITNESS my hand and official seal

Aida Mielat Guian
Signature of Notary Public

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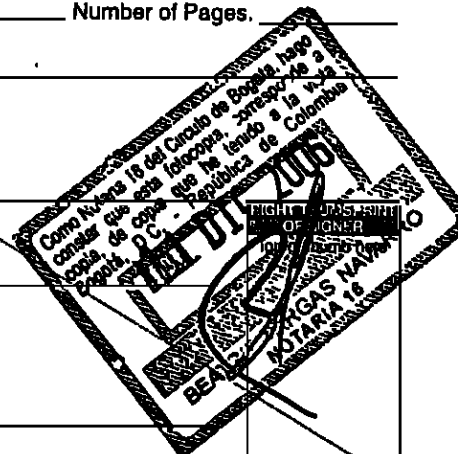
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2005
427

PODER

POWER OF ATTORNEY

Yo (nosotros)

I (We)

Genentech, Inc.

1 DNA Way

domiciliado(s) en

domiciled in

**South San Francisco, California 94080-4990
USA**

Nombro(amos) a: **JUAN PABLO CADENA SARMIENTO y/o BEATRIZ JARAMILLO y/o CARLOS H. PALACIO EASTMAN y/o HECTOR GOMEZ MEJIA**, de Bogotá, Colombia, apoderados verdaderos y legales con poder especial, amplio y suficiente, para recabar de las oficinas y autoridades colombianas administrativas, judiciales y de policía, en cualquier instancia o recurso, la obtención de patentes de invención, registros de marcas, diseños industriales, modelos de utilidad, depósitos de nombres comerciales y enseññas, registros de derechos de autor y contratos sobre los mismos, obtención de certificados de obtentor de variedades vegetales, así como sus prórrogas, renovaciones, anotaciones de traspaso, cambios de nombre o domicilio, elaborar, tramitar y registrar contratos de licencia y licencias de cualesquiera clases, intervenir como demandante o como demandado en cualquier instancia y recurso ante cualquier juez, corporación, funcionario público o autoridad en acciones judiciales, administrativas y de policía tales como: oposiciones al registro de marcas; acciones de caducidad, cancelación y nulidad; acciones reivindicatorias, acciones derivadas de la obtención o explotación de licencias, acciones de competencia desleal; demandas penales; acciones de indemnización de perjuicios, reclamos y observaciones a solicitudes de patentes, diseños industriales, modelos de utilidad, en el ejercicio de medidas cautelares y en otras acciones o medidas similares

Hereby appoints **JUAN PABLO CADENA SARMIENTO and/or BEATRIZ JARAMILLO and/or CARLOS H. PALACIO EASTMAN and/or HECTOR GOMEZ MEJIA**, of Bogotá, Colombia, my (our) true and lawful attorneys with special, ample and sufficient power to obtain from the Colombian administrative, judicial and police offices and authorities, in any instance, privileges of patents of invention, registration of trademarks, industrial designs and utility models; registration of commercial names and emblems, registration of copyright and contracts related thereto, certificates of obtainers of vegetables varieties, as well as extensions, renewals and recordal of assignments and changes of name and domicile related to the above; to prepare, prosecute and register licenses of any kind, to act as plaintiff or defendant in any instance or recourse, and before any judge, corporation, public official or authority in judicial, administrative and police actions, such as: oppositions to the registration of trademarks, caducity, cancellation and nullity actions; claim actions derived from the obtaining or exploitation of licenses, action of unfair competition, criminal proceedings, actions for payment of damages claims or observations on patent applications, models, industrial designs and utility models, in the exercise of preventive measures and in other actions or similar proceedings.

A este efecto, lo(s) faculto(amos) para elevar solicitudes, formular descripciones, enmiendas, protestas, declaraciones, oposiciones, cancelaciones, nulidades, apelaciones y reclamos; pagar impuestos, cuotas y gravámenes prescritos por la ley, renunciar o desistir a derechos concedidos o en curso de concesión; recibir toda clase de documentos y valores, dando el descargo respectivo, y, en general, para dar ante dichas autoridades todos los pasos necesarios al efecto indicado y tomar todas las medidas que creyeren conducentes al resguardo de mis (nuestros intereses), con todas las demás facultades legales necesarias

To that effect they hereby empowered to file applications, formulate descriptions, amendments, protests, declarations, oppositions, cancellations, nullities, appeals and claims; to pay taxes, quotas and assessments prescribed by law, renounce or waive the rights granted or in the process of granting; to receive all kinds of documents and valuables, giving the respective release or receipt, and, in general, to take before said authorities the necessary steps to such effect, and any measures that they may deem pertinent to the safeguard of my (our) interests, with all the other necessary legal powers.

Este poder comprende la facultad de ratificar o confirmar en mi (nuestro) nombre lo actuado así como la facultad de desistir, transigir, comprometer, y la de sustituirlo en todo o en parte y revocar sustituciones. Asimismo nombro(amos) a: **JUAN PABLO CADENA SARMIENTO y/o BEATRIZ JARAMILLO y/o CARLOS H. PALACIO EASTMAN y/o HECTOR GOMEZ MEJIA**, domiciliado(s) en la Carrera 7 No. 16-56 Piso 9, mis (nuestros) apoderados en Colombia, con facultades para recibir notificaciones y designar apoderados judiciales o extrajudiciales.

This power includes the faculty to ratify or confirm desist, settle and compromise on my (our) behalf, and to substitute this power in whole or in part and to revoke substitutions. At the same time I (We) hereby appoints **JUAN PABLO CADENA SARMIENTO and/or BEATRIZ JARAMILLO and/or CARLOS H. PALACIO EASTMAN and/or HECTOR GOMEZ MEJIA**, domiciled in Carrera 7 No 16-56, Piso 9, my (our) attorney(s) in fact, with powers to receive notifications and to designate judicial and extrajudicial attorneys.

Otorgado y firmado en

South San Francisco, California

Granted and signed in **USA**

hoy de de 200_

this 6th day of December 2005

Firma

Signature

Nombre
Cargo

Name **Timothy R. Schwartz, Ph.D.**
Title **Section Patent Counsel
Authorized Corporate Signatory
Genentech, Inc.**

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AUTHORIZATION

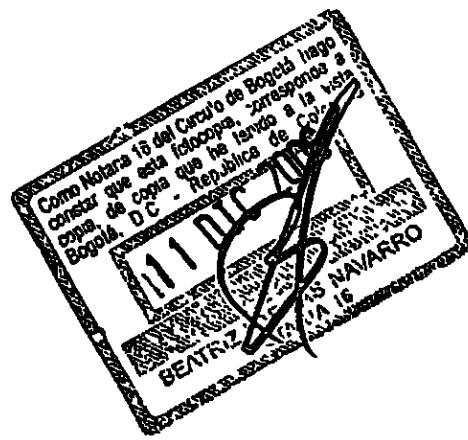
Genentech, Inc. hereby authorizes any of Janet E Hasak, Mark T. Kresnak, Jeffrey S. Kubinec and Timothy R. Schwartz, for so long as they remain employees of Genentech, Inc., to execute, on its behalf, any submissions associated with the filing, prosecution, granting, and extension and maintenance of grant of patent and trademark applications in the U.S. and foreign countries, contracts relating to biological deposits with the American Type Culture Collection, and assignments to publishers of copyrights covering works authored by Genentech employees

August 27, 2002

Dated

Sean Johnston

Sean A Johnston
Vice President, Intellectual Property
Genentech, Inc.



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- 5 en Sacramento, California
- 6 el 15 de diciembre de 2005
7. por el Secretario de Estado Delegado, Estado de California
- 8 No. 267083
- 9 Sello/Timbre

10. Firma

(Firma)

Secretario de Estado



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Estado de California)
) ss
Condado de San Mateo)

El 6 de diciembre de 2005, ante mi, AIDA MICLAT GUIAM, apareció personalmente
Thimoty R Schwartz

☐ a quien conozco personalmente

☒ quien demostró ante mi en base a
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nombre esta suscrito en el instrumento y
quien reconoció ante mi que ejecutó el
mismo en su capacidad autorizada, y que
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COMISIÓN # 14556612
Notario Publico-California
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Mi comisión expira Dic 9 de 2007

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AIDA MICLAT GUIAM

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27 de Agosto de 2002

Fechado

(Firma)

Sean A. Johnston

Vicepresidente, Propiedad Intelectual
Genentech, Inc.



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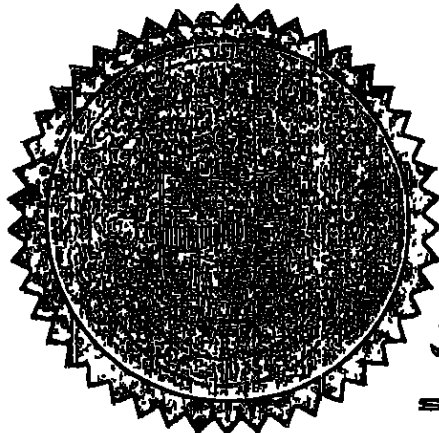
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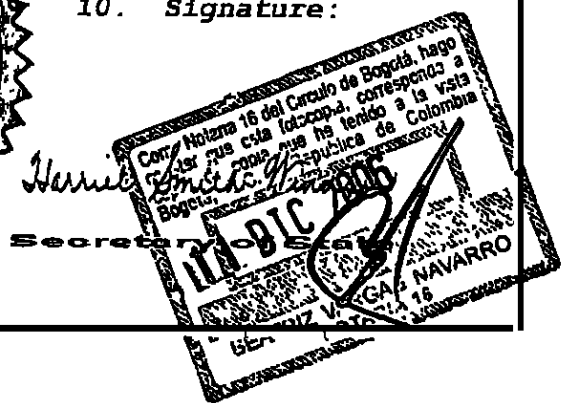
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5. at Dover, Delaware
6. the twenty-fourth day of May, A.D. 2005
7. by Secretary of State, Delaware Department of State
8. No. 0254030
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10. Signature:

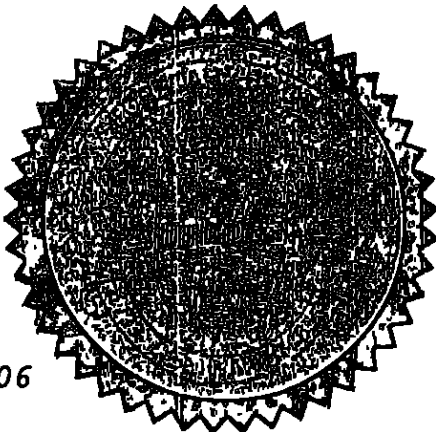


Delaware

PAGE 1

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE RESTATED CERTIFICATE OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE TWENTY-SECOND DAY OF JULY, A.D. 1999, AT 9 O'CLOCK A.M. .



2112549

050426506



Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State
AUTHENTICATION: 5900384

DATE: 05-24-05

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AMENDED AND RESTATED
CERTIFICATE OF INCORPORATION
GENENTECH, INC.

Genentech, Inc. (the "Corporation"), a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware, does hereby amend the Certificate of Incorporation of the Corporation, which was originally filed on December 23, 1986 under the name "Genentech Delaware, Inc.", and subsequently amended.

The Certificate of Incorporation of the Corporation is hereby restated and further amended to read in its entirety as follows:

ARTICLE 1

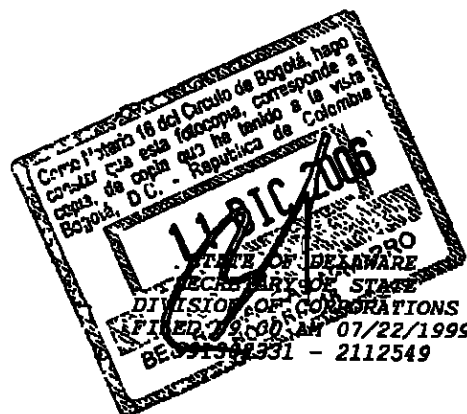
The name of the Corporation is: Genentech, Inc.

ARTICLE 2

The address of the registered office of the Corporation in the State of Delaware is Corporation Service Company, 1013 Centre Road, City of Wilmington, County of New Castle, Delaware 19805. The name of its registered agent at such address is Corporation Service Company.

ARTICLE 3

The purpose of the Corporation is to engage in any lawful act or activity for which corporations may be organized under the General Corporation Law of the State of Delaware as the same exists or may hereafter be amended ("Delaware Law").



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ARTICLE 4

SECTION 4.01. *Capital Stock.* (u) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is four hundred million (400,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value \$0.02 per share ("Preferred Stock"). Three hundred million (300,000,000) shares shall be designated common stock, par value \$0.02 per share ("Common Stock") The Common Stock of the Corporation shall be all of one class.

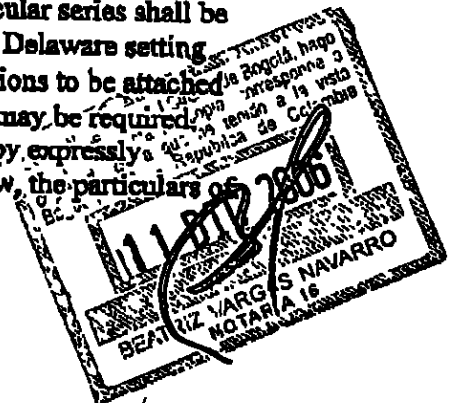
(b) The number of authorized shares of any class or classes of stock may be increased or decreased (but not below the number of shares thereof then outstanding) by the affirmative vote of a majority in voting power of the outstanding stock of the Corporation entitled to vote thereon irrespective of the provisions of Section 242(b)(2) of Delaware Law.

SECTION 4.02. *Common Stock.* (a) *Issuance and Consideration.* Any unissued or treasury shares of the Common Stock may be issued for such consideration as may be fixed in accordance with applicable law from time to time by the Board of Directors.

(b) *Dividends.* Subject to the rights of holders of the Preferred Stock, the holders of the Common Stock shall be entitled to receive, when and as declared by the Board of Directors, out of the assets of the Corporation which are by law available therefor, dividends payable either in cash, in property, or in shares of stock and the holders of the Preferred Stock shall not be entitled to participate in any such dividends (unless otherwise provided by the Board of Directors in any resolution providing for the issue of a series of Preferred Stock).

SECTION 4.03. *Preferred Stock.*

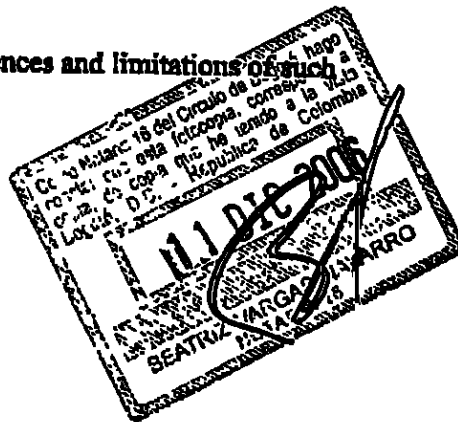
(a) *Series and Limits of Variations between Series.* Any unissued or treasury shares of the Preferred Stock may be issued from time to time in one or more series for such consideration as may be fixed from time to time by the Board of Directors. Before any shares of Preferred Stock of any particular series shall be issued, a certificate shall be filed with the Secretary of State of Delaware setting forth the designation, rights, privileges, restrictions, and conditions to be attached to the Preferred Stock of such series and such other matters as may be required and the Board of Directors shall fix and determine, and is hereby expressly empowered to fix and determine, in the manner provided by law, the particulars of



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the shares of such series (so far as not inconsistent with the provisions of this Article 4 applicable to all series of Preferred Stock), including, but not limited to, the following:

- (i) the distinctive designation of such series and the number of shares which shall constitute such series, which number may be increased (except where otherwise provided by the Board of Directors in creating such series) or decreased (but not below the number of shares thereof then outstanding) from time to time by like action of the Board of Directors;
- (ii) the annual rate of dividends payable on shares of such series, the conditions upon which such dividends shall be payable and the date from which dividends shall be cumulative in the event the Board of Directors determines that dividends shall be cumulative;
- (iii) whether such series shall have voting rights, in addition to the voting rights provided by law and, if so, the terms of such voting rights;
- (iv) whether such series shall have conversion privileges and, if so, the terms and conditions of such conversion, including, but not limited to, provision for adjustment of the conversion rate upon such events and in such manner as the Board of Directors shall determine;
- (v) whether or not the shares of such series shall be redeemable and, if so, the terms and conditions of such redemption, including the date or dates upon or after which they shall be redeemable, and the amount per share payable in case of redemption, which amount may vary under different conditions and at different redemption dates;
- (vi) whether such series shall have a sinking fund for the redemption or purchase of shares of that series and, if so, the terms and amount of such sinking fund;
- (vii) the rights of the shares of such series in the event of voluntary or involuntary liquidation, dissolution or winding up of the Corporation, and the relative rights of priority, if any, of payment of shares of that series; and
- (viii) any other relative rights, preferences and limitations of such series.



ARTICLE 5

SECTION 5.01. *Amendment of Bylaws by Directors.* (a) In furtherance and not in limitation of the powers conferred by statute, the Board of Directors is expressly authorized to make, alter, amend, repeal and rescind the bylaws of the Corporation, subject to Sections 5.01(b) and 5.01(c) hereof.

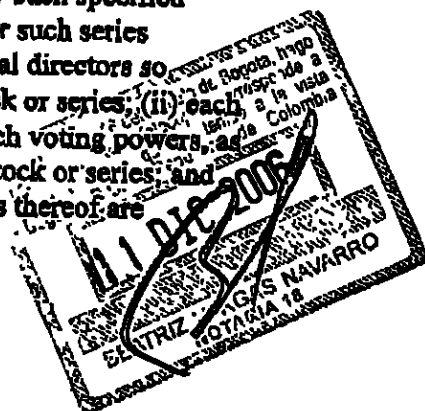
(b) Sections 2.02, 3.02, 3.03(a), 3.03(b), 3.04, 3.05, 3.14, and 5.06 of the bylaws may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 60% of the shares entitled to be voted thereon.

(c) Sections 3.03(c) and 3.15 of the bylaws may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 90% of the shares entitled to be voted thereon.

SECTION 5.02. *Amendment of Certificate of Incorporation.* Section 5.01(b) of this Certificate of Incorporation may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 60% of the shares entitled to be voted thereon. Section 5.01(c) and Articles 8 and 9 of this Certificate of Incorporation may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 90% of the shares entitled to be voted thereon.

ARTICLE 6

SECTION 6.01. *Election of Directors by Holders of Preferred Stock.* During any period when the holders of any Preferred Stock or any one or more series thereof, voting as a class, shall be entitled to elect a specified number of directors, by reason of dividend arrearages or other provisions giving them the right to do so, then and during such time as such right continues (i) the then otherwise authorized number of directors shall be increased by such specified number of directors, and the holders of such Preferred Stock or such series thereof, voting as a class, shall be entitled to elect the additional directors so provided for, pursuant to the provisions of such Preferred Stock or series; (ii) each such additional director shall serve for such term, and have such voting powers, and shall be stated in the provisions pertaining to such Preferred Stock or series; and (iii) whenever the holders of any such Preferred Stock or series thereof are



divested of such rights to elect a specified number of directors, voting as a class, pursuant to the provisions of such Preferred Stock or series, the terms of office of all directors elected by the holders of such Preferred Stock or series, voting as a class pursuant to such provisions or elected to fill any vacancies resulting from the death, resignation or removal of directors so elected by the holders of such Preferred Stock or series, shall forthwith terminate and the authorized number of directors shall be reduced accordingly.

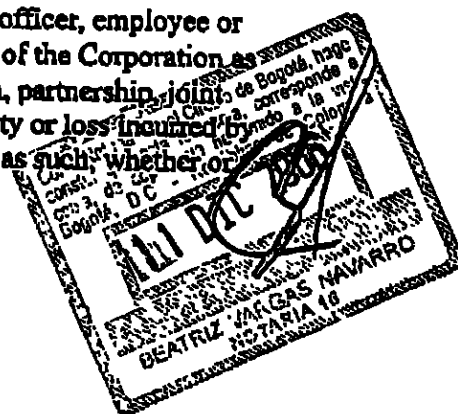
SECTION 6.02. *Ballots.* Elections of directors at an annual or special meeting of stockholders need not be by written ballot unless the bylaws of the Corporation shall provide otherwise.

SECTION 6.03. *Elimination of Certain Personal Liability of Directors.* (a) A director of the Corporation shall not be liable to the Corporation or its stockholders for monetary damages for breach of fiduciary duty as a director to the fullest extent permitted by Delaware Law.

(b) Each person (and the heirs, executors or administrators of such person) who was or is a party or is threatened to be made a party to, or is involved in any threatened, pending or completed action, suit or proceeding, whether civil, criminal, administrative or investigative, by reason of the fact that such person is or was a director of the Corporation or is or was serving at the request of the Corporation as a director of another corporation, partnership, joint venture, trust or other enterprise, shall be indemnified and held harmless by the Corporation to the fullest extent permitted by Delaware Law. The right to indemnification conferred in this Section 6.03 shall also include the right to be paid by the Corporation the expenses incurred in connection with any such proceeding in advance of its final disposition to the fullest extent authorized by Delaware Law. The right to indemnification conferred in this Section 6.03 shall be a contract right.

(c) The Corporation may, by action of its Board of Directors, provide indemnification to such of the officers, employees and agents of the Corporation to such extent and to such effect as the Board of Directors shall determine to be appropriate and authorized by Delaware Law.

(d) The Corporation shall have power to purchase and maintain insurance on behalf of any person who is or was a director, officer, employee or agent of the Corporation, or is or was serving at the request of the Corporation as a director, officer, employee or agent of another corporation, partnership, joint venture, trust or other enterprise against any expense, liability or loss incurred by such person in any such capacity or arising out of his status as such, whether or not



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not the Corporation would have the power to indemnify him against such liability under Delaware Law.

(e) The rights and authority conferred in this Section 6.03 shall not be exclusive of any other right which any person may otherwise have or hereafter acquire.

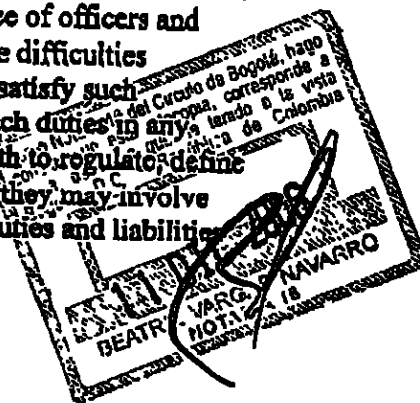
(f) Neither the alteration, amendment, repeal or rescission of this Section 6.03, nor the adoption of any provision of this Certificate of Incorporation or the bylaws of the Corporation, nor, to the fullest extent permitted by Delaware Law, any modification of law, shall eliminate or reduce the effect of this Section 6.03 in respect of any acts or omissions occurring prior to such alteration, amendment, repeal, rescission, adoption or modification.

ARTICLE 7

SECTION 7.01. *Section 203 of Delaware Law.* The Corporation hereby elects not to be governed by Section 203 of Delaware Law.

ARTICLE 8

SECTION 8.01. *Relationship With Roche.* In anticipation that the Corporation will cease to be a wholly owned subsidiary of Roche Holdings, Inc., but that Roche (as defined in Section 8.05) will remain a stockholder of the Corporation, and in anticipation that the Corporation (as defined in Section 8.05) and Roche may engage in the same or similar activities or lines of business and have an interest in the same areas of corporate opportunities, and in recognition of (i) the benefits to be derived by the Corporation through its continued contractual, corporate and business relations with Roche (including service of officers and directors of Roche as directors of the Corporation) and (ii) the difficulties attendant to any director, who desires and endeavors fully to satisfy such director's fiduciary duties, in determining the full scope of such duties in any particular situation, the provisions of this Article 8 are set forth to regulate, define and guide the conduct of certain affairs of the Corporation as they may involve Roche and its officers and directors, and the powers, rights, duties and liabilities



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of the Corporation and its officers, directors and stockholders in connection therewith.

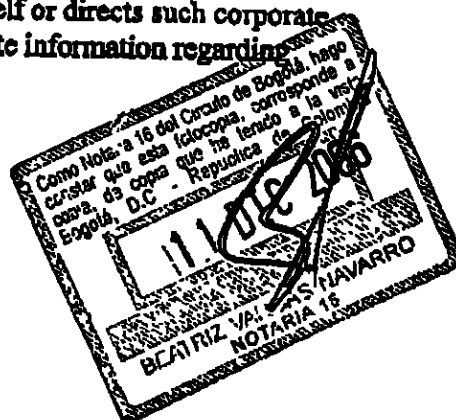
SECTION 8.02. *Similar Business Activities.* Except as Roche Holdings, Inc. may otherwise agree in writing,

(a) Roche shall not have a duty to refrain from engaging directly or indirectly in the same or similar business activities or lines of business as the Corporation, and

(b) neither Roche nor any officer or director thereof shall be liable to the Corporation or its stockholders for breach of any fiduciary duty by reason of any such activities of Roche or of such person's participation therein.

In the event that Roche acquires knowledge of a potential transaction or matter that may be a corporate opportunity for both Roche and the Corporation, Roche shall have no duty to communicate or offer such corporate opportunity to the Corporation and shall not be liable to the Corporation or its stockholders for breach of any fiduciary duty as a stockholder of the Corporation or controlling person of a stockholder by reason of the fact that Roche pursues or acquires such corporate opportunity for itself, directs such corporate opportunity to another person or entity, or does not communicate information regarding, or offer, such corporate opportunity to the Corporation.

SECTION 8.03. *Corporate Opportunities.* In the event that a director, officer or employee of the Corporation who is also a director, officer or employee of Roche acquires knowledge of a potential transaction or matter that may be a corporate opportunity for the Corporation and Roche (whether such potential transaction or matter is proposed by a third-party or is conceived of by such director, officer or employee of the Corporation), such director, officer or employee shall be entitled to offer such corporate opportunity to the Corporation or Roche as such director, officer or employee deems appropriate under the circumstances in his sole discretion, and no such director, officer or employee shall be liable to the Corporation or its stockholders for breach of any fiduciary duty or duty of loyalty or failure to act in (or not opposed to) the best interests of the Corporation or the derivation of any improper personal benefit by reason of the fact that (i) such director, officer or employee offered such corporate opportunity to Roche (rather than the Corporation) or did not communicate information regarding such corporate opportunity to the Corporation or (ii) Roche pursues or acquires such corporate opportunity for itself or directs such corporate opportunity to another person or does not communicate information regarding such corporate opportunity to the Corporation.



SECTION 8.04. Deemed Consent of Stockholders. Any person or entity purchasing or otherwise acquiring any interest in any shares of capital stock of the Corporation shall be deemed to have notice of and to have consented to the provisions of this Article 8.

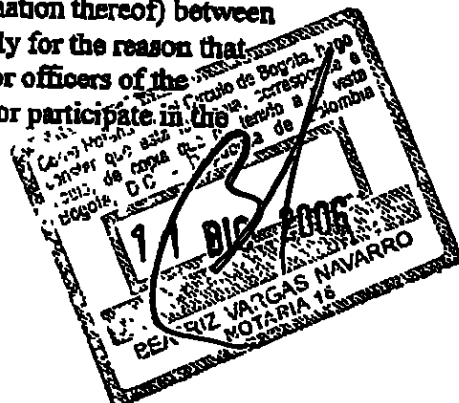
SECTION 8.05. Definitions. For purposes of this Article 8 and Article 9 only, (i) the term "Corporation" shall mean the Corporation and all corporations, partnerships, joint ventures, associations and other entities in which the Corporation beneficially owns (directly or indirectly) fifty percent or more of the outstanding voting stock, voting power or similar voting interests, and (ii) the term "Roche" shall mean Roche Holdings, Inc. and all corporations, partnerships, joint ventures, associations and other entities (other than the Corporation, defined in accordance with clause (i) of this Section 8.05) that are "affiliates" (as defined in Rule 12b-2 under the Securities Exchange Act of 1934, as amended) of Roche Holdings, Inc.

SECTION 8.06. Termination: Binding Effect. Notwithstanding anything in this Certificate of Incorporation to the contrary, the foregoing provisions of this Article 8 shall expire on the date that Roche ceases to own beneficially Common Stock representing at least 5% of the number of outstanding shares of Common Stock of the Corporation and no person who is a director or officer of the Corporation is also a director or officer of Roche. Neither such expiration, nor the alteration, amendment, change or repeal of any provision of this Article 8 nor the adoption of any provision of this Amended and Restated Certificate of Incorporation inconsistent with any provision of this Article 8 shall eliminate or reduce the effect of this Article 8 in respect of any matter occurring, or any cause of action, suit or claim that, but for this Article 8, would accrue or arise, prior to such expiration, alteration, amendment, repeal or adoption.

SECTION 8.07. Article 9. The provisions of this Article 8 are in addition to the provisions of Article 9.

ARTICLE 9

SECTION 9.01. Contracts Not Void. No contract, agreement, arrangement or transaction (or any amendment, modification or termination thereof) between the Corporation and Roche shall be void or voidable solely for the reason that Roche is a party thereto, or solely because any directors or officers of the Corporation who are affiliated with Roche are present at or participate in this



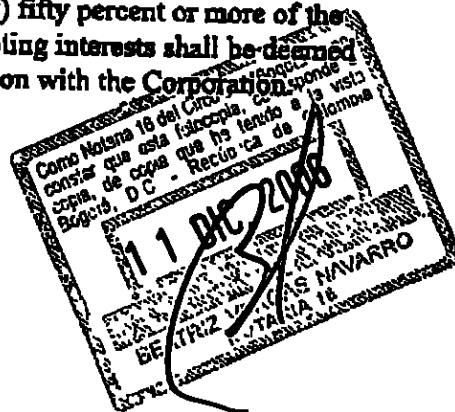
meeting of the Board of Directors or committee thereof which authorizes the contract, agreement, arrangement, transaction, amendment, modification or termination or solely because his or their votes are counted for such purpose, but any such contract, agreement, arrangement or transaction (or any amendment, modification or termination thereof) shall be governed by the provisions of this Amended and Restated Certificate of Incorporation, the Corporation's Bylaws, Delaware Law and other applicable law. For purposes of this Article 9, the terms the "Corporation" and "Roche" have the meanings set forth in Section 8.05.

SECTION 9.02. *Quorum.* Directors of the Corporation who are also directors or officers of Roche may be counted in determining the presence of a quorum at a meeting of the Board of Directors or of a committee that authorizes or approves any such contract, agreement, arrangement or transaction (or amendment, modification or termination thereof). Outstanding shares of Common Stock owned by Roche may be counted in determining the presence of a quorum at a meeting of stockholders that authorizes or approves any such contract, agreement, arrangement or transaction (or amendment, modification or termination thereof).

SECTION 9.03. *No Liability For Good Faith Actions.* Neither Roche nor any officer or director thereof shall be liable to the Corporation or its stockholders for breach of any fiduciary duty or duty of loyalty or failure to act in (or not opposed to) the best interests of the Corporation or the derivation of any improper personal benefit by reason of the fact that Roche or an officer or director thereof in good faith takes any action or exercises any rights or gives or withholds any consent in connection with any agreement or contract between Roche and the Corporation. No vote cast or other action taken by any person who is an officer, director or other representative of Roche, which vote is cast or action is taken by such person in his capacity as a director of this Corporation, shall constitute an action of or the exercise of a right by or a consent of Roche for the purpose of any such agreement or contract.

SECTION 9.04. *Deemed Consent by Stockholders.* Any person or entity purchasing or otherwise acquiring any interest in any shares of capital stock of the Corporation shall be deemed to have notice of and to have consented to the provisions of this Article 9.

SECTION 9.05. *Contracts Covered.* For purposes of this Article 9, any contract, agreement, arrangement or transaction with the Corporation or any corporation, partnership, joint venture, association or other entity in which the Corporation beneficially owns (directly or indirectly) fifty percent or more of the outstanding voting stock, voting power or similar voting interests shall be deemed to be a contract, agreement, arrangement or transaction with the Corporation.



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SECTION 9.06. *Binding Effect.* Neither the alteration, amendment, change or repeal of any provision of this Article 9 nor the adoption of any provision inconsistent with any provision of this Article 9 shall eliminate or reduce the effect of this Article 9 in respect of any matter occurring, or any cause of action, suit or claim that, but for this Article 9, would accrue or arise, prior to such alteration, amendment, change, repeal or adoption.

SECTION 9.07. *Article 8.* The provisions of this Article 9 are in addition to the provisions of Article 8.



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IN WITNESS WHEREOF, this Amended and Restated Certificate of Incorporation, having been duly adopted in accordance with Sections 242 and 245 of the General Corporation Law of the State of Delaware and by the written consent of the sole stockholder of the Corporation in accordance with Section 228 of the General Corporation Law of the State of Delaware, has been executed this 22nd day of July, 1999.

GENENTECH, INC.

By: /s/ Stephen G. Juelsgaard
Name: Stephen G. Juelsgaard
Title: Senior Vice President,
General Counsel and Secretary

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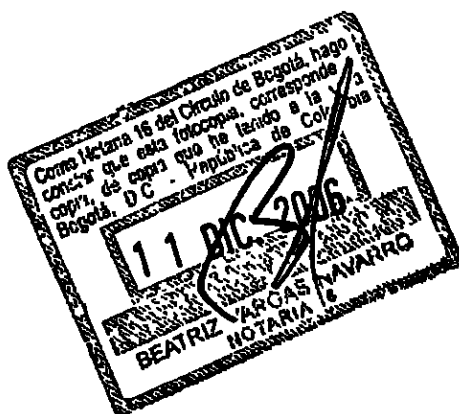
(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No 0254030
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado



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preferenciales, por un valor de \$0.02 por acción ("Acción Preferencial"). Trecientos millones (300,000,000) de acciones serán designadas acciones comunes, por un valor de \$0.02 por acción ("Acción Común"). La acción común de la Corporación será toda de una misma clase.

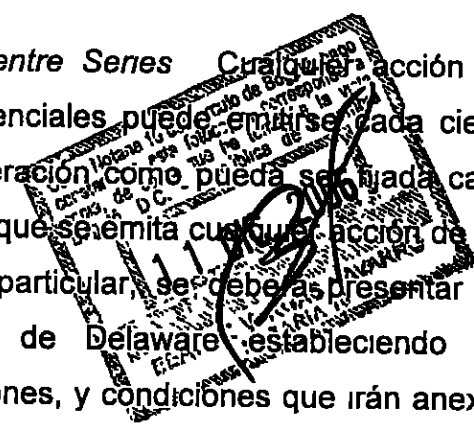
(b) El número de acciones autorizadas de cualquier clase o clases de acciones puede incrementarse o disminuirse (pero no por debajo del número del número de acciones de las mismas que en su momento están vigentes) por el voto afirmativo de una mayoría con poder de votación de las acciones vigentes de la Corporación con derecho a votar por la misma independiente de las consideraciones de la Sección 242(b)(2) de la Ley de Delaware.

SECCION 4.02. *Acción Común.* (a) *Emisión y Consideración.* Cualquier acción sin emitir o del tesoro de las Acciones Comunes puede emitirse para tal consideración como pueda fijarse de acuerdo con la ley aplicable cada cierto tiempo por la Junta Directiva.

(b) *Dividendos* Sujeto a los derechos de los accionistas de las Acciones Preferenciales, los accionistas de Acciones Comunes deberán tener el derecho a recibir, cuando y como se declare por la Junta Directiva, de los activos de la Corporación los cuales están disponibles para ello por ley, dividendos pagables ya sea en dinero, propiedad, o acciones y los accionistas de la Acción Preferencial no tendrán derecho a participar en tales dividendos (a menos que la Junta Directiva determine lo contrario en cualquier resolución que condiciona la emisión de una serie de Acciones Preferenciales).

SECCIÓN 4.03 *Acción Preferencial*

(a) *Serie y Límites de Variaciones entre Series* Cualquier acción no emitida o del tesoro de las Acciones Preferenciales puede emitirse cada cierto tiempo en una o más series para tal consideración como pueda ser fijada cada cierto tiempo por la Junta Directiva. Antes de que se emita cualquier acción de las Acciones Preferenciales de cualquier serie particular, se deberá presentar un certificado ante el Secretario de Estado de Delaware estableciendo las designaciones, derechos, privilegios, restricciones, y condiciones que irán anexas a las Acciones Preferenciales de tal serie y tales otras cuestiones como puedan requerirse, y la Junta Directiva deberá fijar y determinar, y por la presente está expresamente autorizada para fijar y determinar, en la forma provista por la ley, los



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CORREGIDO Y VUELTO A REDACTAR
CERTIFICADO DE INCORPORACIÓN
GENENTECH, INC.

Genentech, Inc (la "Corporación"), una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware, por la presente corrige el Certificado de Incorporación, el fue presentado originalmente el 23 de diciembre de 1986, bajo el nombre "Genentech Delaware, Inc.", y corregido subsiguientemente

Por la presente se vuelve a redactar el certificado de incorporación de la Corporación y se corrige aun más para que diga en su totalidad lo que sigue:

ARTICULO 1

El nombre de la Corporación es: Genentech, Inc.

ARTICULO 2

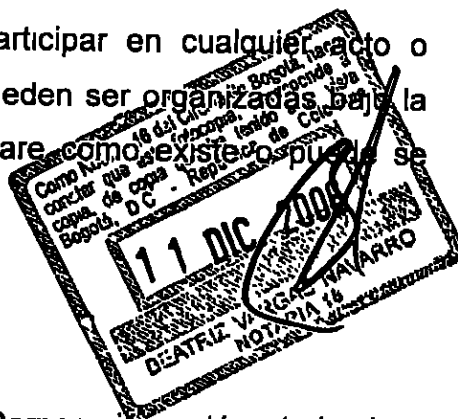
La dirección de la oficina registrada de la Corporación en el Estado de Delaware es Corporation Service Company, 1013 Centre Road, Ciudad de Wilmington, Condado de New castle, Delaware 19805 El nombre de su agente registrado en tal dirección es Corporation Service Company.

ARTICULO 3

El propósito de a Corporación es el de participar en cualquier negocio o actividad legal para las cuales las corporaciones pueden ser organizadas bajo la Ley General de Corporación del Estado de Delaware como existe o puede ser modificada la misma en adelante ("Delaware Law").

ARTICULO 4

SECCION 4.01 *Acciones de Capital* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de cuatrocientos millones (400,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones



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particulares de las acciones de tal serie (siempre y cuando no sean inconsistentes con las condiciones de este Artículo 4 aplicables a todas las series de Acciones Preferenciales), incluyendo, pero sin limitarse a, las siguientes:

(i) la designación distintiva de tal serie y el número de las acciones que han de constituir tal serie, numero que puede incrementarse (excepto cuando lo determine de otro modo la Junta Directiva al crear tal serie) o reducida (pero no por debajo del número de acciones de las mismas que están vigentes) cada cierto tiempo por una acción semejante de la Junta Directiva;

(ii) la tasa anual de dividendos a pagar sobre las acciones de tal serie, las condiciones sobre las cuales se pagarán tales dividendos y le fecha a partir de la cual los dividendos serán acumulativos en el evento que la Junta Directiva determine que los dividendos serán acumulativos;

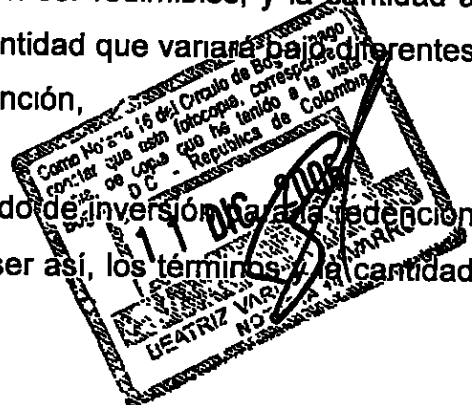
(iii) si la serie tendrá o no derechos de voto, además de los derechos de voto provistos por la ley, y de ser así, los términos de tales derechos de voto,

(iv) si tal serie tendrá o no privilegios de conversión y, de ser así, los términos y condiciones de tal conversión, incluyendo, pero sin estar limitado a, la condición para ajuste de la tasa de conversión en tales eventos y de tal forma como la Junta Directiva lo determine,

(v) si las acciones de tal serie serán o no redimibles y, de ser así, los términos y condiciones de tal redención, incluyendo la fecha o fechas en las cuales o después de las cuales pueden ser redimibles, y la cantidad a pagar por acción en caso de redención, cantidad que variará bajo diferentes condiciones y en diferentes fechas de redención,

(vi) si tal serie tendrá o no un fondo de inversión para la redención o compra de acciones de esa serie y, de ser así, los términos y la cantidad de tal fondo de inversión,

(vii) los derechos de las acciones de tal serie en caso de una liquidación, disolución o cierre voluntaria o involuntaria de la Corporación, y



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(viii) cualquier otro derecho relativo, preferencia y limitación de tal

ARTICULO 5

SECCION 5.01 *Enmiendas a los estatutos por los Directores* (a) En adición y no limitando los poderes conferidos por estatutos, la Junta Directiva esta expresamente autorizada para hacer, alterar, enmendar, rechazar y rescindir los estatutos de la Corporación, sujetos a las secciones 5.01(b) y 5.01(c) a continuación.

(b) Las secciones 2.02, 3.02, 3.03(a), 3.04, 3.05, 3.14 y 5.06 de los estatutos solo pueden alterarse, enmendarse, repelerse, o rescindirse por el voto afirmativo de los accionistas de capital con derecho a votar las mismas que representen más del 60% de las acciones con derecho a votar las mismas.

(c) Las secciones 3.03(c) y 3.15 de los estatutos solo pueden ser alteradas, enmendadas, repelidas, o rescindidas por el voto afirmativo de los accionistas de capital con derecho a votar las mismas que representen más del 90% de las acciones con derecho a votar las mismas.

SECCION 5.02 *Enmienda del Certificado de Incorporación.* La sección 5.01 (b) de este Certificado de Incorporación solo puede ser alterada, enmendada, repelida, o rescindida por el voto afirmativo de los accionistas de capital con derecho a voto sobre la misma que representen a más del 60% de las acciones con derecho a votar la misma. La sección 5.01(c) y los Artículos 8 y 9 de este Certificado de Incorporación solo pueden alterarse, enmendarse, repelirse, o rescindirse por el voto afirmativo de los accionistas de capital con derecho a votar la misma que representen más del 90% de las acciones con derecho a votar la misma.

ARTICULO 6

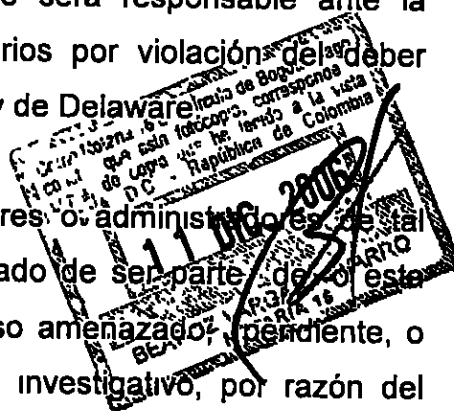
SECCION 6 01. *Elección del Directorio por los Accionistas de Acción Preferencial.* Durante cualquier periodo cuando los accionistas de cualquier

acción preferencial cualquiera de una o más series de las mismas, que voten como una clase, tengan derecho a elegir un número específico de directores, por razón a atrasos en los dividendos u otras condiciones que les dan el derecho a hacerlo, entonces y durante el tiempo que continúe el derecho (i) el número de directores autorizado de otro modo será incrementado por tal número de directores especificado, y los accionistas de tales Acciones Preferenciales o tales series de las mismas, que votan como una clase, tendrán derecho a elegir los directores adicionales para lo que se provee, de acuerdo con las condiciones de tales Acciones Preferenciales o series; (ii) cada uno de tales directores adicionales servirá durante tal termino, y tendrá tales poderes de voto, como se han de otorgar en las provisiones pertinentes a tales Acciones Preferenciales o series, y (iii) siempre que los accionistas de cualquiera de tales Acciones Preferenciales o series de las mismas están desprovistos de tales derechos a elegir un número específico de directores, votando como clase, de acuerdo con las condiciones de tales Acciones preferenciales o series, los términos de oficio de todos los directores elegidos por los accionistas de tales Acciones preferenciales o series, que votan como clase, de acuerdo con las condiciones o elegidos para llenar cualquier vacante resultante de la muerte, renuncia o remoción de directores así elegidos por los accionistas de tales Acciones Preferenciales o series, terminarán en adelante y el número autorizado de directores será reducido en concordancia

SECCION 6.02 *Votaciones.* Las elecciones de directores en una reunión anual o especial de accionistas necesaria no serán por votos escritos a menos que los estatutos de la Corporación digan lo contrario

SECCION 6.03. *Eliminación de cierta responsabilidad personal de los directores* (a) Un director de la Corporación no será responsable ante la Corporación o sus accionistas por daños monetarios por violación del deber fiduciario como director hasta donde lo permita la Ley de Delaware

(b) Cada persona (y sus herederos, ejecutores o administradores de tal persona) quien fue o es una parte o esta amenazado de ser parte de este involucrado en cualquier acción, demanda o proceso amenazado, pendiente, o completado, ya sea civil, criminal, administrativo o investigativo, por razón del hecho de que esa persona es o fue un director de la Corporación o es o estaba sirviendo por solicitud de la Corporación como un director de otra corporación, sociedad, sociedad de riesgo compartido, fideicomiso u otra empresa, será indemnizado y completamente protegido por la Corporación hasta donde lo



permita la Ley de Delaware El derecho a la indemnización concedido en esta sección 6.03 también debe incluir el derecho a que la Corporación le pague los gastos incurridos en conexión cuna cualquiera de tales procesos por adelantado de su decisión final hasta donde los autoriza la Ley de Delaware El derecho a indemnización conferido en esta sección 6.03 será un derecho contractual

(c) La Corporación puede, por acción de su Junta Directiva, proporcionar indemnización a tales funcionarios, empleados y agentes de la Corporación hasta tal punto y a tal efecto como lo determine apropiado la Junta Directiva y lo autorice la Ley de Delaware.

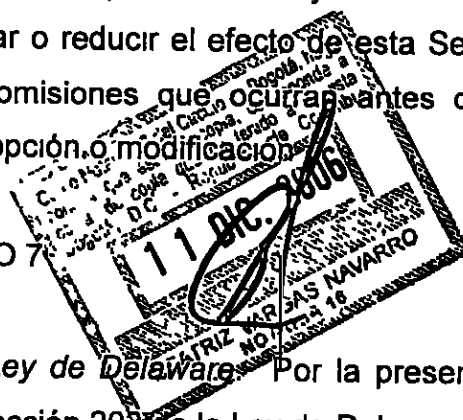
(d) La Corporación tendrá el poder de comprar y mantener un seguro a nombre de cualquier persona quien es o fue director, funcionario, empleado o agente de la corporación, o esta o estuvo en servicio por pedido de la Corporación como director, funcionario, empleado o agente de otra corporación, sociedad, sociedad de riesgo compartido, fideicomiso u otra empresa contra cualquier gasto, responsabilidad o pérdida incurrida por tal persona en cualquier capacidad o que surge de su estado como tal, ya sea si la Corporación tiene o no el poder de indemnizarlo contra tal responsabilidad bajo la ley de Delaware.

(e) Los derechos y autoridad conferidos en esta Sección 6.03 no serán exclusivos de ningún otro derecho el cual cualquier otra persona pueda tener o adquirir de aquí en adelante.

(f) Ni la alteración, enmienda, rechazo o rescisión de esta Sección 6.03, ni la adopción de ninguna condición de este Certificado de Incorporación o de los Estatutos de la Corporación, ni, hasta donde lo permite la Ley de Delaware, ninguna modificación de la ley, debe eliminar o reducir el efecto de esta Sección 6.03 respecto a ninguno de los actos u omisiones que ocurran antes de tal alteración, enmienda, rechazo, rescisión, adopción o modificación.

ARTICULO 7

SECCION 7 01, Sección 203 de la Ley de Delaware. Por la presente la Corporación elige no ser gobernada por la Sección 203 de la Ley de Delaware.



ARTICULO 8

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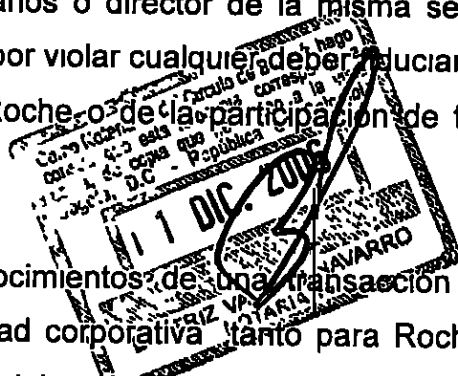
SECCION 8.01 *Relación con Roche.* Anticipándose a que la Corporación cese de ser un subsidiaria completamente de propiedad de Roche Holdings, Inc., pero que Roche (como se define en la sección 8.05) permanezca siendo un accionista de la Corporación, y anticipándose a que la Corporación (como se define en la Sección 8.05) y Roche puede entrar en actividades o líneas de negocios semejantes o similares y tener un interés en las mismas áreas de oportunidades corporativas, y en reconocimiento de (i) los beneficios a ser derivados por la Corporación a través de sus relaciones contractuales, corporativas y de negocios con Roche (incluyendo el servicio de funcionarios y directores de Roche como directores de la Corporación) y (ii) las dificultades concomitantes a cualquier director, quien desea y se propone satisfacer completamente tales deberes fiduciarios del director, al determinar el alcance completo de tales deberes en cualquier situación particular, las condiciones de este artículo 8 se establecen para regular, definir y guiar la conducta de ciertas cuestiones de la Corporación ya que pueden involucrar a Roche y sus funcionarios y directores, y los poderes, derechos, deberes y responsabilidades de la Corporación y sus funcionarios, directores y accionistas en conexión con éste

SECCION 8.02 *Actividades en Negocios Similares.* Excepto que Roche Holdings, Inc. pueda de otra forma acordarlo por escrito,

(a) Roche no tendrá el deber de abstenerse de entrar directamente o indirectamente en actividades de negocios o líneas de negocios semejantes o similares a las de la Corporación, y

(b) ni Roche ni ninguno de sus funcionarios o director de la misma será responsable a la Corporación o sus accionistas por violar cualquier deber fiduciario por razón de alguna de tales actividades de Roche o de la participación de tal persona en las mismas.

En el evento que Roche adquiera conocimientos de una transacción o materia potencial que pueda ser una oportunidad corporativa tanto para Roche como para la Corporación, Roche no tendrá el deber de comunicar u ofrecer tal oportunidad corporativa a la Corporación y no será responsable ante la Corporación o sus accionistas por violar ningún deber fiduciario como accionista de la Corporación o la persona que controla a un accionista por razón del hecho

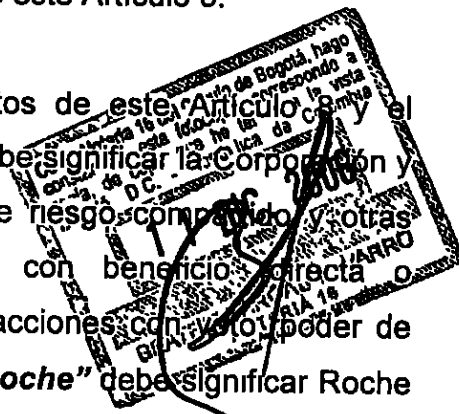


que Roche prosigue o adquiere tal oportunidad corporativa para si misma, dirige tal oportunidad corporativa a otra persona o entidad, o no comunica información respecto, u ofrece, tal oportunidad corporativa a la Corporación.

SECCION 8 03. *Oportunidades Corporativas.* En el evento que un director, funcionario o empleado de la Corporación quien también es director, funcionario o empleado de Roche adquiera conocimiento de una transacción o materia potencial que pueda ser una oportunidad corporativa para la Corporación y Roche (ya se si esta transacción o materia potencial es propuesta por un tercero o es concebida por tal director, funcionario o empleado de la Corporación o no), tal director, funcionario o empleado debe tener el derecho a ofrecer tal oportunidad corporativa a la Corporación o Roche como tal director, funcionario o empleado los considere apropiado bajo las circunstancias de su discreción, y tal director, funcionario o empleado no será responsable a la Corporación o sus accionistas por violar ningún deber fiduciario o deber de lealtad o faltar en actuar en (o no oponerse a) los mejores intereses de la Corporación o la derivación de ningún beneficio personal inapropiado por razón del hecho que (i) tal director, funcionario o empleado ofreció tal oportunidad corporativa a Roche (en lugar de a la Corporación) o no comunicó información respecto a tal oportunidad corporativa a la Corporación o (ii) Roche busca o adquiere tal oportunidad corporativa para si misma o dirige tal oportunidad corporativa a otra persona o no comunica información respecto a tal oportunidad corporativa a la Corporación

SECCION 8 04. *Consentimiento estimado de los accionistas* Se considerará que cualquier persona o entidad que compre o de otro modo adquiera algún interés en cualquiera de las acciones de capital de la Corporación ha tenido noticia o ha estado de acuerdo con las condiciones de este Artículo 8.

SECCION 8 05, *Definiciones.* Para propósitos de este Artículo 8 y el Artículo 9 solamente, (i) el término "**Corporación**" debe significar la Corporación y todas las corporaciones, sociedades, sociedades de riesgo compartido y otras entidades en las cuales la Corporación posee con beneficio directo o indirectamente) cincuenta por ciento o más de las acciones con voto o poder de voto o intereses de voto similares, y (ii) el término "**Roche**" debe significar Roche Holdings, Inc y todas las corporaciones, sociedades, sociedades de riesgo compartido, asociaciones y otras entidades (diferentes a la Corporación, definida de acuerdo con la cláusula (i) de esta sección 8.05) que son "**afiliados**" (como se



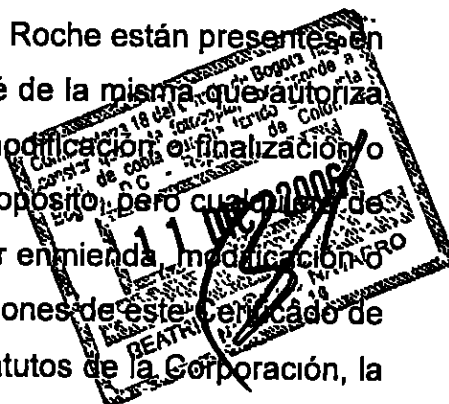
define en la Regla 12b-2 bajo el Acta de Intercambio de Valores de 1934, como se enmendó) de Roche Holdings, Inc

SECCION 8.06, *Finalización: Efecto Obligante* Son consideración con nada en este Certificado de Incorporación que lo contradiga, las condiciones anteriores de este Artículo 8 deberán expirar en la fecha que Roche cese de poseer con beneficios Acciones Comunes que representen al menos 5% del número de acciones vigentes de las Acciones Comunes de la Corporación y nadie que se director o funcionario de la Corporación sea también director o funcionario de Roche. Ni tal vencimiento, ni la alteración, enmienda, cambio o rechazo de alguna de las condiciones de este Artículo 8 así como tampoco ninguna condición de este Certificado de Incorporación Enmendado y Vuelto a Redactar inconsistente con alguna de las condiciones de este Artículo 8 eliminará o reducirá el efecto de este Artículo 8 respecto a cualquier materia, o cualquier causa de acción, demanda o reclamo que, excepto por este Artículo 8, pueda acaecer o surgir, antes de tal vencimiento, alteración, enmienda o adopción

SECCION 8 07. *Artículo 9.* Las condiciones de este Artículo son adicionales a las condiciones del Artículo 9

ARTICULO 9

SECCION 9.01 *Contratos no nulos.* Ningún contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización de los mismos) entre la Corporación y Roche será nulo u anulable solamente por razón de que Roche es parte del mismo, o solamente porque cualquiera de los directores o funcionarios de la Corporación quienes están afiliados a Roche están presentes en o participan en la reunión de la Junta Directiva o comité de la misma que autoriza el contrato, acuerdo, arreglo, transacción, enmienda, modificación o finalización o solamente porque su o sus votos se cuentan para tal propósito, pero cualquier tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo) estará gobernado por las condiciones de este Certificado de Incorporación Enmendado y vuelto a Redactar, los Estatutos de la Corporación, la Ley de Delaware y otras leyes aplicables. Para propósitos de este Artículo 9, los términos "**Corporación**" y "**Roche**" tienen los significados establecidos en la Sección 8 05



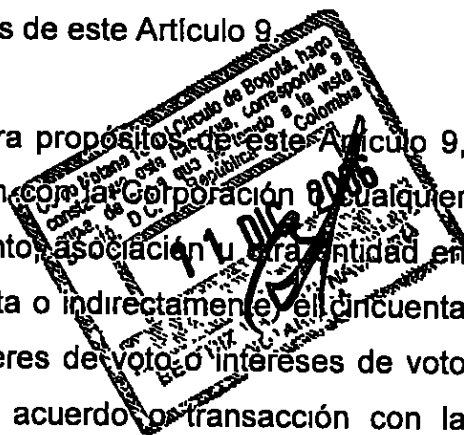
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SECCION 9.02 *Quórum.* Los Directores de la Corporación que son también directores o funcionarios de Roche pueden contarse al determinar la presencia de un quórum en una reunión de la Junta Directiva o de un comité que autoriza o aprueba cualquiera de tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo) Las acciones vigentes de las Acciones Comunes de propiedad de Roche pueden contarse al determinar la presencia de un quórum en una reunión de accionistas que autorice o apruebe tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo).

SECCION 9 03. *No responsabilidad por acciones de buena fe.* Ni Roche ni ningún funcionario o director de la misma será responsable ante la Corporación o sus accionistas por violar cualquiera de los deberes fiduciarios o deberes de lealtad o por fallar en actuar en (o no oponerse a) los mejores intereses de la Corporación o por derivación de cualquier beneficio personal por razón del hecho que Roche o un funcionario o director de la misma de buena fe tome cualquier acción o ejerza cualquier derecho o de o retenga cualquier consentimiento en conexión con cualquier acuerdo o contrato entre Roche y la Corporación. Ningún voto dado u otra acción tomada por cualquier persona que es un funcionario, director o representante de Roche, donde el voto o acción es tomado por tal persona en su capacidad como director de esta Corporación, constituirá una acción de o el ejercicio de un derecho o un consentimiento de Roche para los propósitos de cualquiera de tal acuerdo o contrato

SECCION 9.04 *Consentimiento estimado de los accionistas.* Se considerará que cualquier persona o entidad que compre o de otro modo adquiera algún interés en cualquiera de las acciones de capital de la Corporación ha tenido noticia o ha estado de acuerdo con las condiciones de este Artículo 9.

SECCION 9 05 *Contratos cubiertos* Para propósitos de este Artículo 9, cualquier contrato, acuerdo, arreglo o transacción con la Corporación o cualquier corporación, sociedad, sociedad de riesgo conjunto, asociación u otra entidad en la cual la Corporación posee con beneficio (directa o indirectamente) el cincuenta por ciento o más de las acciones con voto, poderes de voto o intereses de voto similares vigentes se considerará un contrato, acuerdo o transacción con la Corporación.



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SECCION 9.06. *Efecto obligante.* Ni la alteración, enmienda, cambio o rechazo de alguna de las condiciones de este Artículo 9 ni la adopción de alguna condición inconsistente con cualquier condición de este Artículo 9 eliminará o reducirá el efecto de este Artículo 9 respecto a cualquier materia que ocurra, o cualquier causa de acción, demanda o reclamo que, a no ser por este Artículo 9, pueda acaecer o surgir, antes de tal alteración, enmienda, cambio, rechazo o adopción.

SECCION 9 07 *Artículo 8.* Las condiciones de este Artículo 9 son adicionales a las condiciones del Artículo 8.

EN TESTIMONIO DE LO CUAL, este Certificado de Incorporación Enmendado y vuelto a Redactar, el cual ha sido adoptado de acuerdo con las secciones 242 y 245 de la Ley General de Corporación del Estado de Delaware y por consentimiento escrito de el único accionista de la Corporación de acuerdo con la sección 228 de Ley General de Corporación del Estado de Delaware, ha sido ejecutado este 22 de Julio de 1999.

GENENTECH, INC.

Por: /s/ Stephen G. Juelsgaard

Nombre: Stephen G. Juelsgaard

Título: Vicepresidente

Consejo General y Secretaría



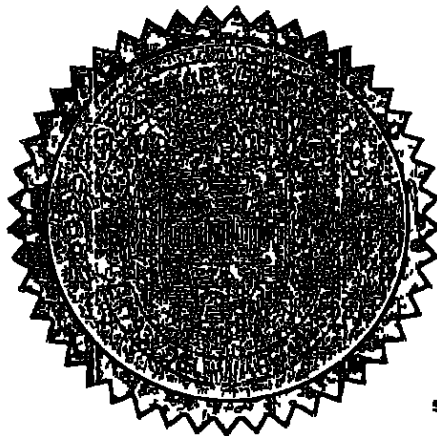
Apostille

(Convention de La Haye du 5 Octobre 1961)

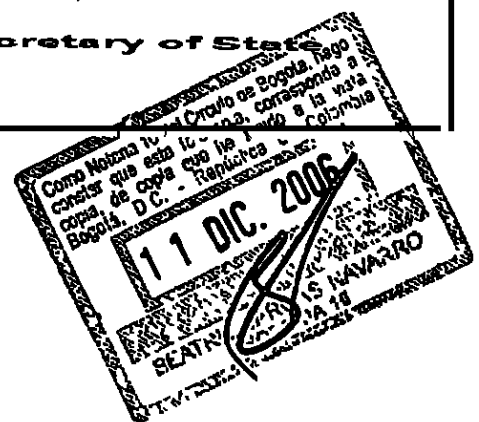
1. Country: United States of America
2. This public document:
has been signed by Harriet Smith Windsor
3. acting in the capacity of Secretary of State of Delaware
4. bears the seal/stamp of Office of Secretary of State

Certified

5. at Dover, Delaware
6. the twenty-fourth day of May, A.D. 2005
7. by Secretary of State, Delaware Department of State
8. No. 0254031
9. Seal/Stamp:  10. Signature



Harriet Smith Windsor
Secretary of State



Delaware

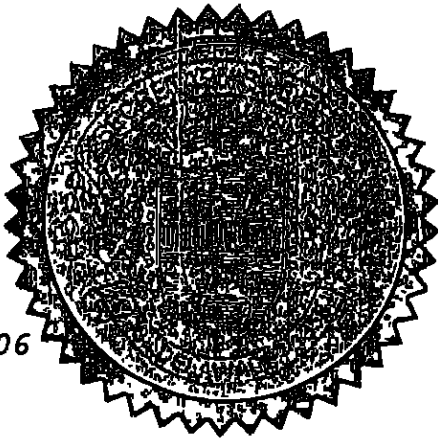
PAGE 1

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF AMENDMENT OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE FIFTEENTH DAY OF MAY, A.D. 2000, AT 5 O'CLOCK P.M.

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050426506



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CERTIFICATE OF AMENDMENT
OF AMENDED AND RESTATED
CERTIFICATE OF INCORPORATION

Genentech, Inc a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware.

DOES HEREBY CERTIFY.

FIRST: That at a meeting of the Board of Directors of Genentech, Inc. resolutions were duly adopted setting forth a proposed amendment of the Amended and Restated Certificate of Incorporation of said corporation, declaring said amendment to be advisable and calling a meeting of the stockholders of said corporation for consideration thereof. The resolution setting forth the proposed amendment is as follows.

RESOLVED, that the Amended and Restated Certificate of Incorporation of this corporation be amended by changing "Section 4.01(a)" of the Article thereof numbered "4" so that, as amended, said Section of said Article shall be and read as follows.

"Section 4.01. *Capital Stock* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is seven hundred million (700,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value \$0.02 per share ("Preferred Stock"). Six hundred million (600,000,000) shares shall be designated common stock, par value \$0.02 per share ("Common Stock") The Common Stock of the Corporation shall be all of one class."

SECOND. That thereafter, pursuant to resolution of its Board of Directors, the regular meeting of the stockholders of said corporation was duly called and held upon notice in accordance with Section 222 of the General Corporation Law of the State of Delaware at which meeting the necessary number of shares as required by statute were voted in favor of the amendment.

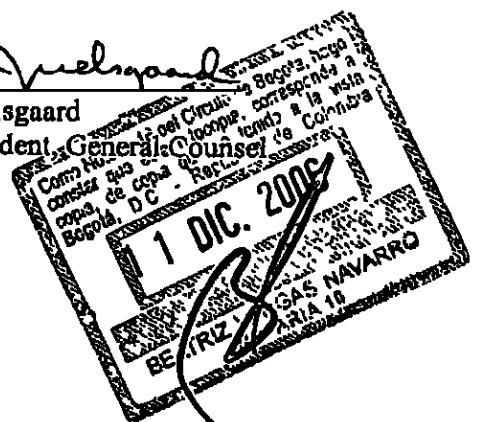
THIRD: That said amendment was duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware.

FOURTH: That the capital of said corporation shall not be reduced under or by reason of said amendment

IN WITNESS WHEREOF, said Genentech, Inc has caused this Certificate of Amendment to be signed by Stephen G Juelsgaard, an Authorized Officer, this 15th day of May, 2000.

GENENTECH, INC.

By: Stephen Juelsgaard
Name: Stephen G. Juelsgaard
Title: Senior Vice President, General Counsel
and Secretary



APOSTILLA

(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No. 0254031
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado



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DELAWARE
EL PRIMER ESTADO

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO VUELTO A REDACTAR DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL DECIMOQUINTO DIA DE MAYO, A. D. 2000, A LAS 5 EN PUNTO DE LA TARDE



Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado
AUTENTICACIÓN. 3900385

FECHA 05 - 24 - 05

DELAWARE
EL PRIMER ESTADO

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO VUELTO A REDACTAR DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL VIGÉSIMO SEGUNDO DÍA DE JULIO, A D. 1999, A LAS 9 EN PUNTO DE LA MAÑANA.



Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado
AUTENTICACIÓN 3900384

FECHA: 05 - 24 - 05

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CERTIFICADO DE ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR

Genentech, Inc. una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware.

CERTIFICA POR LA PRESENTE:

PRIMERO. Que en una reunión de la Junta Directiva de Genentech, Inc. se adoptaron debidamente resoluciones que establecen una enmienda propuesta del certificado de Incorporación Corregido y vuelto a Redactar de dicha corporación, declarando dicha enmienda como aconsejable y llamando a una reunión de accionistas de dicha corporación para consideración de la misma. La resolución establece la enmienda propuesta de la siguiente manera

RESUELVE, que el Certificado de Incorporación Corregido y vuelto a Redactar de esta corporación se enmienda cambiando "la Sección 4.01(a)" del Artículo allí numerado "4" tal que, como se enmienda, dicha Sección de dicho Artículo se lea de la siguiente manera.

SECCION 4.01. *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de setecientos millones (700,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones preferenciales, por un valor de \$0.02 por acción ("Acción Preferencial"). Seiscientos millones (600,000,000) de acciones serán designadas acciones comunes, por un valor de \$0.02 por acción ("Acción Común") La acción común de la Corporación será toda de una misma clase.



SEGUNDO: Que por lo tanto, de acuerdo con la resolución de su Junta Directiva, la reunión ordinaria de accionistas de dicha corporación se convocó debidamente y se sostuvo al ser anunciada de acuerdo con la sección 222 de la Ley General de Corporación del Estado de Delaware, en donde el número necesario de acciones requerido por los estatutos votaron a favor de la enmienda.

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TERCERO. Que dicha enmienda se adoptó debidamente de acuerdo con lo previsto en la Sección 242 de la Ley General de Corporación del Estado de Delaware.

CUARTO Que el capital de dicha corporación no será reducido bajo o por razón de dicha enmienda

EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc. ha causado este Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un Funcionario Autorizado, este 15 de Mayo de 2000.

GENENTECH, INC.

Por. (Firma)

Nombre: Stephen G. Juelsgaard

Título: Vicepresidente, Consejo
y Secretaría General.



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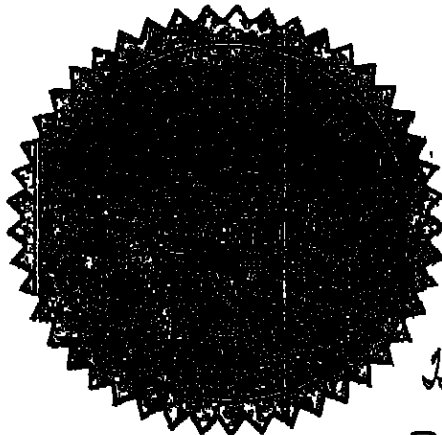
Apostille

(Convention de La Haye du 5 Octobre 1961)

1. Country: United States of America
2. *This public document:*
has been signed by Harriet Smith Windsor
3. acting in the capacity of Secretary of State of Delaware
4. bears the seal/stamp of Office of Secretary of State

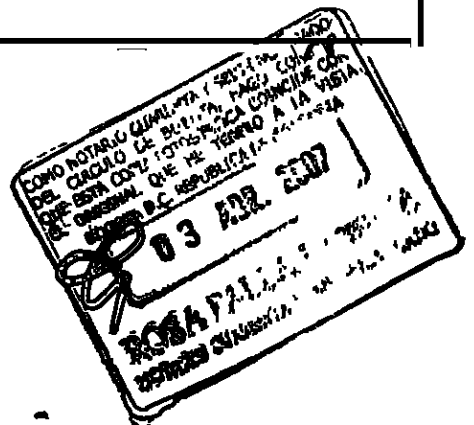
Certified

5. at Dover, Delaware
6. the twenty-fourth day of May, A.D. 2005
7. by Secretary of State, Delaware Department of State
8. No. 0254033
9. Seal/Stamp:



10. Signature:

Harriet Smith Windsor
Secretary of State



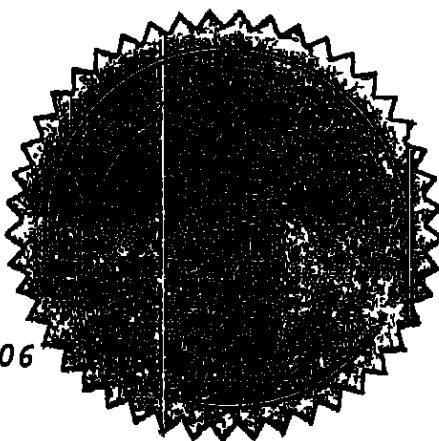
Delaware

PAGE 1

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The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF AMENDMENT OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE SIXTEENTH DAY OF APRIL, A.D. 2004, AT 7:39 O'CLOCK P.M.



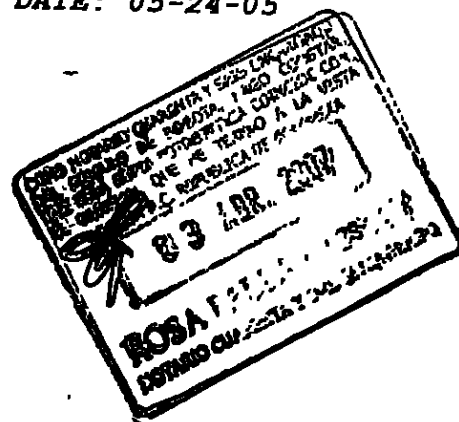
2112549

050426506

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State
AUTHENTICATION: 3900387

DATE: 05-24-05



CERTIFICATE OF THIRD AMENDMENT
OF AMENDED AND RESTATED
CERTIFICATE OF INCORPORATION

Genentech, Inc., a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware,

DOES HEREBY CERTIFY:

FIRST: That at a meeting of the Board of Directors of Genentech, Inc. resolutions were duly adopted setting forth a proposed amendment of the Amended and Restated Certificate of Incorporation of said corporation, declaring said amendment to be advisable and calling a meeting of the stockholders of said corporation for consideration thereof. The resolution setting forth the proposed amendment is as follows:

RESOLVED, that the Amended and Restated Certificate of Incorporation of this corporation be amended by changing "Section 4.01(a)" of the Article thereof numbered "4" so that, as amended, said Section of said Article shall be and read as follows.

Section 4.01 *Capital Stock* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is three billion one hundred million (3,100,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value \$0.02 per share ("Preferred Stock"). Three billion (3,000,000,000) shares shall be designated common stock, par value \$0.02 per share ("Common Stock"). The common stock of the Corporation shall be all of one class.

SECOND: That thereafter, pursuant to resolution of its Board of Directors, the regular meeting of the stockholders of said corporation was duly called and held upon notice in accordance with Section 222 of the General Corporation Law of the State of Delaware at which meeting the necessary number of shares as required by statute were voted in favor of the amendment.

THIRD: That said amendment was duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware.

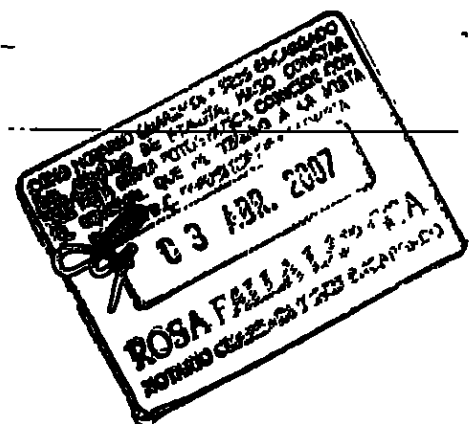
FOURTH: That the capital of said corporation shall not be reduced under or by reason of said amendment.

IN WITNESS WHEREOF, said Genentech, Inc. has caused this Third Certificate of Amendment to be signed by Stephen G. Juelsgaard, an Authorized Officer, this 16th day of April, 2004.

GENENTECH, INC.

By: Stephen G. Juelsgaard
Name: Stephen G. Juelsgaard
Title: Executive Vice President, General Counsel
and Secretary

#153087



TRADUCCION AL ESPAÑOL del Certificado de Enmienda de Genentech, Inc.

468

APOSTILLA

(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

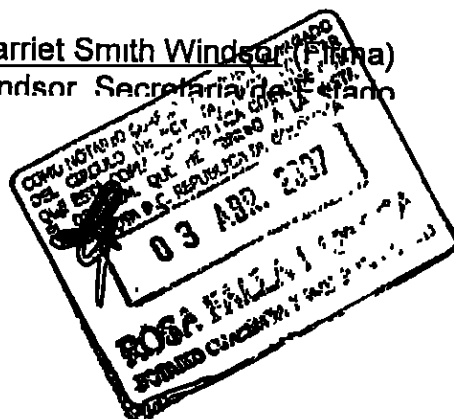
5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No 0254033
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado



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Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado



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CERTIFICADO DE TERCERA ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR

Genentech, Inc. una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware

CERTIFICA POR LA PRESENTE

PRIMERO: Que en una reunión de la Junta Directiva de Genentech, Inc. se adoptaron debidamente resoluciones que establecen una enmienda propuesta del certificado de Incorporación Corregido y vuelto a Redactar de dicha corporación, declarando dicha enmienda como aconsejable y llamando a una reunión de accionistas de dicha corporación para consideración de la misma. La resolución establece la enmienda propuesta de la siguiente manera:

RESUELVE, que el Certificado de Incorporación Corregido y vuelto a Redactar de esta corporación se enmienda cambiando "la Sección 4.01(a)" del Artículo allí numerado "4" tal que, como se enmienda, dicha Sección de dicho Artículo se lea de la siguiente manera:

SECCION 4.01. *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de tres mil cien millones (3,100,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones preferenciales, por un valor de \$0.02 por acción ("Acción Preferencial"). Tres mil millones (3,000,000,000) de acciones serán designadas acciones comunes, por un valor de \$0.02 por acción ("Acción Común"). La acción común de la Corporación será toda de una misma clase.

SEGUNDO. Que por lo tanto, de acuerdo con la resolución de su Junta Directiva, la reunión ordinaria de accionistas de dicha corporación se convocó debidamente.



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TERCERO. Que dicha enmienda se adoptó debidamente de acuerdo con lo previsto en la Sección 242 de la Ley General de Corporación del Estado de Delaware.

CUARTO. Que el capital de dicha corporación no será reducido bajo o por razón de dicha enmienda

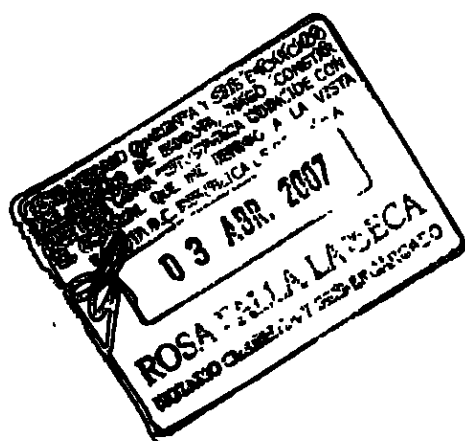
EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc ha causado este Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un Funcionario Autorizado, este 16 de Abril de 2004

GENENTECH, INC.

Por: (Firma)

Nombre: Stephen G. Juelsgaard

Título: Vicepresidente, Consejo
y Secretaría General.



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Apostille

(Convention de La Haye du 5 Octobre 1961)

1. Country: United States of America

This public document:

2. has been signed by Harriet Smith Windsor

3. acting in the capacity of Secretary of State of Delaware

4. bears the seal/stamp of Office of Secretary of State

Certified

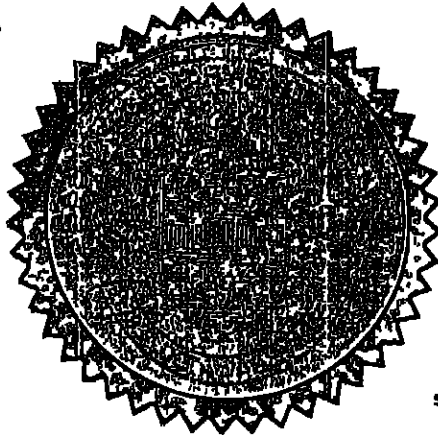
5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

8. No. 0254032

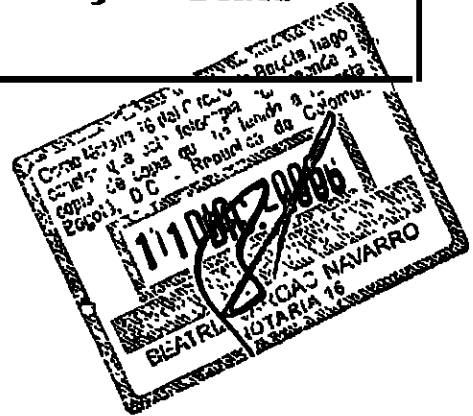
9. Seal/Stamp:



10. Signature:

Harriet Smith Windsor

Secretary of State



Delaware

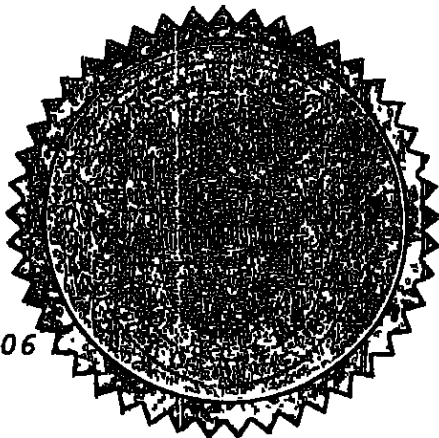
PAGE 1

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF AMENDMENT OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE TENTH DAY OF MAY, A.D. 2001, AT 2:05 O'CLOCK P.M.

2112549

050426506



Harriet

Harriet Smith Windsor, Secretary of State

AUTHENTICATION: 139836

DATE: 05/10/01

BEATRIZ

NAVARRO



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CERTIFICATE OF AMENDMENT
OF AMENDED AND RESTATED
CERTIFICATE OF INCORPORATION

Genentech, Inc. a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware

DOES HEREBY CERTIFY

FIRST: That at a meeting of the Board of Directors of Genentech, Inc. resolutions were duly adopted setting forth a proposed amendment of the Amended and Restated Certificate of Incorporation of said corporation, declaring said amendment to be advisable and calling a meeting of the stockholders of said corporation for consideration thereof. The resolution setting forth the proposed amendment is as follows:

RESOLVED, that the Amended and Restated Certificate of Incorporation of this corporation be amended by changing "Section 4.01(a)" of the Article thereof numbered "4" so that, as amended, said Section of said Article shall be and read as follows:

"Section 4.01. *Capital Stock* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is one billion three hundred million (1,300,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value \$0.02 per share ("Preferred Stock"). One billion two hundred million (1,200,000,000) shares shall be designated common stock, par value \$0.02 per share ("Common Stock") The Common Stock of the Corporation shall be all of one class."

SECOND. That thereafter, pursuant to resolution of its Board of Directors, the regular meeting of the stockholders of said corporation was duly called and held upon notice in accordance with Section 222 of the General Corporation Law of the State of Delaware at which meeting the necessary number of shares as required by statute were voted in favor of the amendment

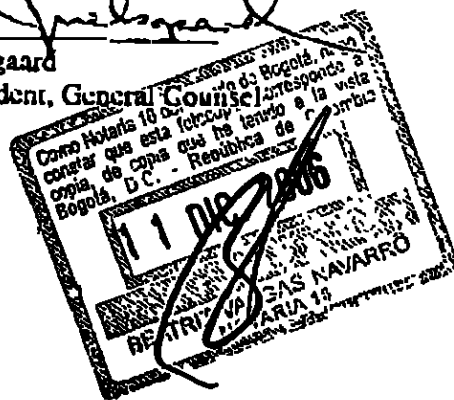
THIRD: That said amendment was duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware.

FOURTH. That the capital of said corporation shall not be reduced under or by reason of said amendment.

IN WITNESS WHEREOF, said Genentech, Inc. has caused this Certificate of Amendment to be signed by Stephen G. Juelsgaard, an Authorized Officer, this 10th day of May, 2001

GENENTECH, INC.

By: Stephen Juelsgaard
Name: Stephen G. Juelsgaard
Title: Senior Vice President, General Counsel and Secretary



APOSTILLA

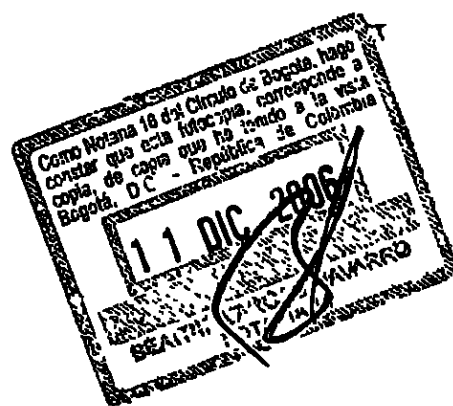
(Convención de La Haya del 5 de Octubre de 1961)

1. País Estados Unidos de América
Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

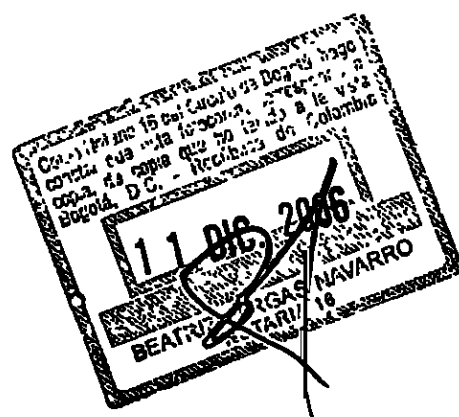
5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No 0254032
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado



DELAWARE
EL PRIMER ESTADO

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO DE ENMIENDA DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL DECIMO DIA DE MAYO, A. D 2001, A LAS 2.05 EN PUNTO DE LA TARDE.



Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado
AUTENTICACIÓN 3900386

FECHA. 05 - 24 - 05

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CERTIFICADO DE TERCERA ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR

Genentech, Inc una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware.

CERTIFICA POR LA PRESENTE:

PRIMERO: Que en una reunión de la Junta Directiva de Genentech, Inc. se adoptaron debidamente resoluciones que establecen una enmienda propuesta del certificado de Incorporación Corregido y vuelto a Redactar de dicha corporación, declarando dicha enmienda como aconsejable y llamando a una reunión de accionistas de dicha corporación para consideración de la misma. La resolución establece la enmienda propuesta de la siguiente manera:

RESUELVE, que el Certificado de Incorporación Corregido y vuelto a Redactar de esta corporación se enmienda cambiando "la Sección 4.01(a)" del Artículo allí numerado "4" tal que, como se enmienda, dicha Sección de dicho Artículo se lea de la siguiente manera:

SECCION 4 01 *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de mil trescientos millones (1,300,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones preferenciales, por un valor de \$0.02 por acción ("Acción Preferencial"). Mil doscientos millones (1,200,000,000) de acciones serán designadas acciones comunes, por un valor de \$0.01 por acción ("Acción Común") La acción común de la Corporación será toda de una misma clase



SEGUNDO: Que por lo tanto, de acuerdo con la resolución de su Junta Directiva, la reunión ordinaria de accionistas de dicha corporación se convocó debidamente y se sostuvo al ser anunciada de acuerdo con la sección 222 de la Ley General de Corporación del Estado de Delaware, en donde el número necesario de acciones requerido por los estatutos votaron a favor de la enmienda

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TERCERO. Que dicha enmienda se adoptó debidamente de acuerdo con lo previsto en la Sección 242 de la Ley General de Corporación del Estado de Delaware.

CUARTO. Que el capital de dicha corporación no será reducido bajo o por razón de dicha enmienda

EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc. ha causado este Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un Funcionario Autorizado, este 10 de Mayo de 2001.

GENENTECH, INC.

Por: (Firma)

Nombre: Stephen G Juelsgaard

Título. Vicepresidente, Consejo
y Secretaría General

