SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
INVENTARIO

DELEGATURA PROPIEDAD INDUSTRIAL

División de Nuevas Creaciones

PCT

SOLICITUD

PATENTE DE INVENCION

ENTRADA EN FASE NACIONAL

EXPEDIENTE No. 07-80736

TITULO INHIBICIÓN DE LA LIBERACIÓN DE HER2 ("HER2 SHEDDING") CON ANTAGONISTAS DE METALOPROTEASAS DE MATRIZ

51. **CLASIFICACION INTERNACIONAL**

71. **SOLICITANTE** GENENTECH, INC.

DOMICILIO SAN FRANCISCO DEL SUR, CALIFORNIA, ESTADOS UNIDOS DE AMÉRICA

74. **APODERADO** JUAN PABLO CADENA SARMIENTO

22. **SANTAFE DE BOGOTA, D.C.**

Oficina de Comunicaciones

(FORMA P 10)

PETITORIO

AS: 104.752

Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
No. 07-080736- -00000-0000
Fecha: 2007-08-08 16:14:23 Dep. 2020 NUECREACIONE
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 359

FORMULARIO ÚNICO DE SOLICITUD DE PATENTE PCT ENTRADA EN FASE NACIONAL

9 SEP / 2007

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, Estados Unidos de América Nacionalidad o Domicilio: Estados Unidos de América Lugar de Constitución: Estados Unidos de América Teléfono: Fax: E-mail	**IDENTIFICACIÓN** C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Cual_____ Número _____
3 REPRESENTANTE O APODERADO	Nombre: JUAN PABLO CADENA SARMIENTO Dirección: Carrera 7 # 71 – 21 Torre B – Piso 4 Bogotá, Colombia Teléfono: 744-2200 Fax: 744-2700 E-mail:	**IDENTIFICACIÓN** C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Cual_____ Número 80.410.337 TP 57492
4 INVENTOR (ES) (72)	**Nombre:** Kendall D. CAREY, Ralph H. SCHWALL, Mark X. SLIWKOWSKI. **Dirección:** 545 Miller Avenue, Apartment No. 5, South San Francisco, California 94080, USA; 400 Griffin Avenue, Pacifica, California 94044, USA; 42 Oak Creek Lane, San Carlos, California 94070, USA (Respectivamente) **Nacionalidad o Domicilio:** Estados Unidos de América (Respectivamente)	

5 PCT (86)
Solicitud Internacional No. PCT/US2006/004926 Fecha 09-02-2006
Publicación Internacional No. WO 2006/086730 A3 Fecha 17-08-2006

6 Título (54): **INHIBICIÓN DE LA LIBERACIÓN DE HER2 ("HER2 SHEDDING") CON ANTAGONISTAS DE METALOPROTEASAS DE MATRIZ**

7 Clasificación Internacional (51) : Ver hoja 221

8 Prioridad SI ☒ NO ☐	(33) País de Origen	(32) Fecha	(31) Número de Solicitud
	US	09-02-2005	60/651,348

9 Comprobante de pago No. 07- 50,869 **Fecha** 08-08-2007

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

10 **ANEXOS**

2

- [x] Comprobante de pago de la tasa de presentación de la solicitud. $482.000
- [x] Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- [x] Poderes, si fuere el caso. **Fotocopia Autenticada**
- [x] Documento de cesión del inventor al solicitante o a su causante.
- [ ] Declaraciones
- [x] Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- [x] 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.
- [x] Otros: Extracto para Publicación y Tarjeta de Archivo Temático
  Tarjeta de Propietarios
  Documentos PCT
  Listado de Secuencias

11 **FIGURA CARACTERÍSTICA:**

12 Solicito la concesión de la patente,

NOMBRE: **JUAN PABLO CADENA SARMIENTO**

FIRMA:

C.C. 80.410.337 de Usaquén T.P. 57492 del C.S.J.

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

AS 104.702

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 07-080736- -00000-0000

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Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 359

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 07 - 50,869
FECHA : JUNIO 25 DE 2007

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
BRIGARD & CASTRO LTDA	CONSIGNACION	BANCO POPULAR	050-00110-6	59098666	25/06/2007	19,580,000.00

***** C O N C E P T O *****

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._____

104. 752

7

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

(19) World Intellectual Property Organization
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C12N 15/11 (2006.01) *G01N 33/574* (2006.01)
C12N 9/64 (2006.01) *A61P 35/00* (2006.01)
A61K 39/395 (2006.01)

(21) International Application Number:
PCT/US2006/004926

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(25) Filing Language: English

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(30) Priority Data:
60/651,348 9 February 2005 (09.02.2005) US

(71) Applicant *(for all designated States except US)*: **GENENTECH, INC.** [US/US]; 1 DNA Way, MS 49, South San Francisco, California 94080 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CAREY, Kendall, D.** [US/US]; 545 Miller Avenue, Apartment No. 5, South San Francisco, California 94080 (US). **SCHWALL, Ralph, H.** [US/US]; 400 Griffin Avenue, Pacifica, California 94044 (US). **SLIWKOWSKI, Mark, X.** [US/US]; 42 Oak Creek Lane, San Carlos, California 94070 (US).

(74) Agents: **KUBINEC, Jeffrey, S.** et al.; GENENTECH, INC., 1 DNA Way, MS 49, South San Francisco, CA 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, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, 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) 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, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
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1 March 2007

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

WO 2006/086730 A3

(54) Title: INHIBITING HER2 SHEDDING WITH MATRIX METALLOPROTEASE ANTAGONISTS

(57) Abstract: The present application describes using antagonists of matrix metalloproteases (MMPs), especially of MMP-15, for inhibiting HER2 shedding.

PCT/US2006/004926

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/11 C12N9/64 A61K39/395 G01N33/574 A61P35/00

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)
C12N A61K G01N

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, WPI Data, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/096899 A1 (AOKI TAKANORI [JP] ET AL) 20 May 2004 (2004-05-20)	13,20,21
Y	whole document esp. [0002, 0004, 0006, 0027, 0042, 0074, 0108, 0111, 0208, 0213, 0217], claims 48,49,52	1-12, 14-19
X	WO 2004/096139 A2 (INCYTE CORP [US]; YAO WENQING [US]; ZHOU JINCONG [US]; XU MEIZHONG [US]) 11 November 2004 (2004-11-11)	13-16
Y	whole document esp. pages 4,7,8,9,146,152, claims 91-93	1,2, 5-12, 17-21

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[X] Further documents are listed in the continuation of Box C.

[X] 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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 2006	12/12/2006

Name and mailing address of the ISA/	Authorized officer
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	Brück, Marianne

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/087042 A2 (YEDA RES & DEV [IL]; SAGI IRIT [IL]; DANON TAMAR [IL]) 14 October 2004 (2004-10-14)	13
Y	whole document esp. pages 1, 8, 22, 23, 32, claim 20	1-12, 14-21
X	WO 02/20020 A (PHARMACIA & UPJOHN SPA [IT]; UPJOHN CO [US]; SALLE ENRICO DI [IT]; PIS) 14 March 2002 (2002-03-14)	13-16
Y	whole document esp. pages 2-3, 8, claim 6	1-12, 17-21
X	WO 03/005971 A2 (PARATEK PHARMACEUTICALS INC [US]) 23 January 2003 (2003-01-23)	13-16
Y	whole document esp. pages 8, 11, 18, 134 claims 3, 5, 30-33	1-12, 17-21
Y	US 2002/164334 A1 (ARAKAWA TSUTOMU [US] ET AL) 7 November 2002 (2002-11-07) whole document esp. [0001, 0003], claim 1	1,2,5-21
Y	WO 03/026490 A2 (ELUSYS THERAPEUTICS INC [US]; JACKSON DAVID [US]; NARDONE LINDA [US]) 3 April 2003 (2003-04-03) whole document esp. pages 3,5-7,9,52, claims 1,3,8,16	5-10, 14-16
Y	WO 98/42865 A (UNIV MELBOURNE [AU]; PRINCE HENRYS INST MED RES [AU]; LOPATA ALEXANDER) 1 October 1998 (1998-10-01) whole document esp. pages 2-5	1-21
Y	MOLINA MA ET AL: "Trastuzumab (Herceptin), a Humanized Anti-HER2 Receptor Monoclonal Antibody, Inhibits Basal and Activated HER2 Ectodomain Cleavage in Breast Cancer Cells" CANCER RESEARCH, vol. 61, 2001, pages 4744-4749, XP002408598 whole document esp. abstract, pages 4748-4749	1-21
Y	WO 2004/046354 A (BEIERSDORF AG) 3 June 2004 (2004-06-03) whole document esp. pages 3,5,6,10-11	1-21
P,X	WO 2005/037826 A (INCYTE CORP [US]; LI YUN-LONG [US]; ZHOU JINCONG [US]; BURNS DAVID [US]) 28 April 2005 (2005-04-28) whole document esp. pages 2,3,12	1,2,5-19

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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(10) International Publication Number
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(21) International Application Number:
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(22) International Filing Date: 9 February 2006 (09.02.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/651,348 9 February 2005 (09.02.2005) US

(71) Applicant *(for all designated States except US)*: **GENENTECH, INC.** [US/US]; 1 DNA Way, MS 49, South San Francisco, California 94080 (US).

(72) Inventors; and

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(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, 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.

WO 2006/086730 A2

(54) Title: INHIBITING HER2 SHEDDING WITH MATRIX METALLOPROTEASE ANTAGONISTS

(57) Abstract: The present application describes using antagonists of matrix metalloproteases (MMPs), especially of MMP-15, for inhibiting HER2 shedding.

INHIBITING HER2 SHEDDING WITH MATRIX METALLOPROTEASE ANTAGONISTS

This is a non-provisional application claiming priority under 35 USC §119 to provisional application no. 60/651,348 filed February 9, 2005, the entire disclosure of which is hereby incorporated by reference.

Field of the Invention

The present invention concerns using antagonists of matrix metalloproteases (MMPs), especially of MMP-15, for inhibiting HER2 shedding.

Background of the Invention

The HER family of receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes four distinct members including epidermal growth factor receptor (EGFR, ErbB1, or HER1), HER2 (ErbB2 or $p185^{neu}$), HER3 (ErbB3) and HER4 (ErbB4 or tyro2).

EGFR, encoded by the *erb*B1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, transforming growth factor alpha (TGF-α), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn *Pharmac. Ther.* 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-α and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, *e.g.*, Baselga and Mendelsohn., *supra*; Masui *et al. Cancer Research* 44:1002-1007 (1984); and Wu *et al. J. Clin. Invest.* 95:1897-1905 (1995).

The second member of the HER family, $p185^{neu}$, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the *neu* proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of *neu* is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon *et al.*, *Science*, 235:177-182 (1987); Slamon *et al.*, *Science*, 244:707-712 (1989); and US Pat No. 4,968,603). To date, no point mutation analogous to that in the *neu* proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. See, among others, King *et al.*, *Science*, 229:974 (1985); Yokota *et al.*, *Lancet*: 1:765-767 (1986); Fukushige *et al.*, *Mol Cell Biol.*, 6:955-958 (1986); Guerin *et al.*, *Oncogene Res.*, 3:21-31 (1988); Cohen *et al.*, *Oncogene*, 4:81-88 (1989); Yonemura *et al.*, *Cancer Res.*, 51:1034 (1991); Borst *et al.*, *Gynecol. Oncol.*, 38:364 (1990); Weiner *et al.*, *Cancer Res.*, 50:421-425 (1990); Kern *et al.*, *Cancer Res.*, 50:5184 (1990); Park *et al.*, *Cancer Res.*, 49:6605 (1989); Zhau *et al.*, *Mol. Carcinog.*, 3:254-257 (1990); Aasland *et al. Br. J. Cancer* 57:358-363 (1988); Williams *et al. Pathobiology* 59:46-52 (1991); and McCann *et al.*, *Cancer*, 65:88-92 (1990). HER2 may be overexpressed in prostate cancer (Gu

et al. Cancer Lett. 99:185-9 (1996); Ross *et al. Hum. Pathol.* 28:827-33 (1997); Ross *et al. Cancer* 79:2162-70 (1997); and Sadasivan *et al. J. Urol.* 150:126-31 (1993)).

Antibodies directed against the rat $p185^{neu}$ and human HER2 protein products have been described.

Drebin and colleagues have raised antibodies against the rat *neu* gene product, $p185^{neu}$. See, for example, Drebin *et al., Cell* 41:695-706 (1985); Myers *et al., Meth. Enzym.* 198:277-290 (1991); and WO94/22478. Drebin *et al. Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions of $p185^{neu}$ result in synergistic anti-tumor effects on *neu*-transformed NIH-3T3 cells implanted into nude mice. See also U.S. Patent 5,824,311 issued October 20, 1998.

Hudziak *et al., Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of HER2 antibodies which were characterized using the human breast tumor cell line SK-BR-3. Relative cell proliferation of the SK-BR-3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also U.S. Patent No. 5,677,171 issued October 14, 1997. The HER2 antibodies discussed in Hudziak *et al.* are further characterized in Fendly *et al. Cancer Research* 50:1550-1558 (1990); Kotts *et al. In Vitro* 26(3):59A (1990); Sarup *et al. Growth Regulation* 1:72-82 (1991); Shepard *et al. J. Clin. Immunol.* 11(3):117-127 (1991); Kumar *et al. Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis *et al. Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras *et al. Oncogene* 9:1829-1838 (1994); Vitetta *et al. Cancer Research* 54:5301-5309 (1994); Sliwkowski *et al. J. Biol. Chem.* 269(20):14661-14665 (1994); Scott *et al. J. Biol. Chem.* 266:14300-5 (1991); D'souza *et al. Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis *et al. Cancer Research* 56:1457-1465 (1996); and Schaefer *et al. Oncogene* 15:1385-1394 (1997).

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Patent No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga *et al., J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration September 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein.

Other HER2 antibodies with various properties have been described in Tagliabue *et al. Int. J. Cancer* 47:933-937 (1991); McKenzie *et al. Oncogene* 4:543-548 (1989); Maier *et al. Cancer Res.* 51:5361-5369 (1991); Bacus *et al. Molecular Carcinogenesis* 3:350-362 (1990); Stancovski *et al. PNAS (USA)* 88:8691-8695 (1991); Bacus *et al. Cancer Research* 52:2580-2589 (1992); Xu *et al. Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk *et al. Cancer Research* 52:2771-2776 (1992);Hancock *et al. Cancer Res.* 51:4575-4580 (1991); Shawver *et al. Cancer Res.* 54:1367-1373 (1994); Arteaga *et al. Cancer Res.* 54:3758-3765 (1994); Harwerth *et al. J. Biol. Chem.* 267:15160-15167 (1992); U.S. Patent No. 5,783,186; and Klapper *et al. Oncogene* 14:2099-2109 (1997).

Homology screening has resulted in the identification of two other HER receptor family members; HER3 (US Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus *et al. PNAS (USA)* 86:9193-9197 (1989)) and HER4 (EP Pat Appln No 599,274; Plowman *et al., Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); and

Plowman *et al., Nature*, 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The HER receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of HER ligands (Earp *et al. Breast Cancer Research and Treatment* 35: 115-132 (1995)). EGFR is bound by six different ligands; epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin, heparin binding epidermal growth factor (HB-EGF), betacellulin and epiregulin (Groenen *et al. Growth Factors* 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta and gamma heregulins (Holmes *et al., Science*, 256:1205-1210 (1992); U.S. Patent No. 5,641,869; and Schaefer *et al. Oncogene* 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motor neuron derived factor (SMDF). For a review, see Groenen *et al. Growth Factors* 11:235-257 (1994); Lemke, G. *Molec. & Cell. Neurosci.* 7:247-262 (1996) and Lee *et al. Pharm. Rev.* 47:51-85 (1995). Recently three additional HER ligands were identified; neuregulin-2 (NRG-2) which is reported to bind either HER3 or HER4 (Chang *et al. Nature* 387 509-512 (1997); and Carraway *et al Nature* 387:512-516 (1997)); neuregulin-3 which binds HER4 (Zhang *et al. PNAS* (USA) 94(18):9562-7 (1997)); and neuregulin-4 which binds HER4 (Harari *et al. Oncogene* 18:2681-89 (1999)) HB-EGF, betacellulin and epiregulin also bind to HER4.

While EGF and TGFα do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase. See Earp *et al., supra*. Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski *et al., J. Biol. Chem.*, 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi *et al., Journal of Neuroscience* 15: 1329-1340 (1995); Morrissey *et al., Proc. Natl. Acad. Sci. USA* 92: 1431-1435 (1995); and Lewis *et al., Cancer Res.*, 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, *Cell* 78:5-8 (1994)).

The HER signaling network is depicted in Fig. 4.

Patent publications related to HER antibodies include: US 5,677,171, US 5,720,937, US 5,720,954, US 5,725,856, US 5,770,195, US 5,772,997, US 6,165,464, US 6,387,371, US 6,399,063, US2002/0192211A1, US 6,015,567, US 6,333,169, US 4,968,603, US 5,821,337, US 6,054,297, US 6,407,213, US 6,719,971, US 6,800,738, US2004/0236078A1, US 5,648,237, US 6,267,958, US 6,685,940, US 6,821,515, WO98/17797, US 6,127,526, US 6,333,398, US 6,797,814, US 6,339,142, US 6,417,335, US 6,489,447, WO99/31140, US2003/0147884A1, US2003/0170234A1, US2005/0002928A1, US 6,573,043, US2003/0152987A1, WO99/48527, US2002/0141993A1, WO01/00245, US2003/0086924, US2004/0013667A1, WO00/69460, WO01/00238, WO01/15730, US 6,627,196B1, US6,632,979B1, WO01/00244, US2002/0090662A1, WO01/89566, US2002/0064785, US2003/0134344, WO 04/24866, US2004/0082047, US2003/0175845A1, WO03/087131, US2003/0228663, WO2004/008099A2, US2004/0106161, WO2004/048525, US2004/0258685A1, US 5,985,553, US 5,747,261, US 4,935,341, US 5,401,638, US 5,604,107, WO 87/07646, WO 89/10412, WO 91/05264, EP 412,116 B1, EP 494,135 B1, US 5,824,311, EP 444,181 B1, EP 1,006,194

A2, US 2002/0155527A1, WO 91/02062, US 5,571,894, US 5,939,531, EP 502,812 B1, WO 93/03741, EP 554,441 B1, EP 656,367 A1, US 5,288,477, US 5,514,554, US 5,587,458, WO 93/12220, WO 93/16185, US 5,877,305, WO 93/21319, WO 93/21232, US 5,856,089, WO 94/22478, US 5,910,486, US 6,028,059, WO 96/07321, US 5,804,396, US 5,846,749, EP 711,565, WO 96/16673, US 5,783,404, US 5,977,322, US 6,512,097, WO 97/00271, US 6,270,765, US 6,395,272, US 5,837,243, WO 96/40789, US 5,783,186, US 6,458,356, WO 97/20858, WO 97/38731, US 6,214,388, US 5,925,519, WO 98/02463, US 5,922,845, WO 98/18489, WO 98/33914, US 5,994,071, WO 98/45479, US 6,358,682 B1, US 2003/0059790, WO 99/55367, WO 01/20033, US 2002/0076695 A1, WO 00/78347, WO 01/09187, WO 01/21192, WO 01/32155, WO 01/53354, WO 01/56604, WO 01/76630, WO02/05791, WO 02/11677, US 6,582,919, US2002/0192652A1, US 2003/0211530A1, WO 02/44413, US 2002/0142328, US 6,602,670 B2, WO 02/45653, WO 02/055106, US 2003/0152572, US 2003/0165840, WO 02/087619, WO 03/006509, WO03/012072, WO 03/028638, US 2003/0068318, WO 03/041736, EP 1,357,132, US 2003/0202973, US 2004/0138160, US 5,705,157, US 6,123,939, EP 616,812 B1, US 2003/0103973, US 2003/0108545, US 6,403,630 B1, WO 00/61145, WO 00/61185, US 6,333,348 B1, WO 01/05425, WO 01/64246, US 2003/0022918, US 2002/0051785 A1, US 6,767,541, WO 01/76586, US 2003/0144252, WO 01/87336, US 2002/0031515 A1, WO 01/87334, WO 02/05791, WO 02/09754, US 2003/0157097, US 2002/0076408, WO 02/055106, WO 02/070008, WO 02/089842 and WO 03/86467.

The HER2 extracellular domain (ECD) is proteolytically shed from breast carcinoma cells in culture (Petch *et al., Mol. Cell. Biol.* 10:2973-2982 (1990); Scott *et al., Mol. Cell. Biol.* 13:2247-2257 (1993); and Lee and Maihle, *Oncogene* 16:3243-3252(1998)), and is found in the serum of some cancer patients (Leitzel *et al., J. Clin. Oncol.* 10:1436-1443 (1992)). HER2 ECD may be a serum marker of metastatic breast cancer (Leitzel *et al., J. Clin. Oncol.* 10:1436-1443 (1992)), and may allow escape of HER2 overexpressing tumors from immunological control (Baselga *et al., J. Clin. Oncol.* 14:737-744 (1997), Brodowicz *et al., Int. J. Cancer* 73:875-879 (1997)). Shed HER2 ECD serum levels represent an independent marker of poor clinical outcome in patients with HER2 overexpressing metastatic breast cancer (Ali *et al., Clin. Chem.* 48:1314-1320 (2002); Molina *et al., Clin. Cancer Res.* 8:347-353 (2002)).

A truncated extracellular domain of HER2 is also the product of a 2.3 kb alternative transcript generated by use of a polyadenylation signal within an intron (Scott *et al., Mol. Cell. Biol.* 13:2247-2257 (1993)). The alternative transcript was first identified in the gastric carcinoma cell line, MKN7 (Yamamoto *et al., Nature* 319:230-234 (1986); and Scott *et al., Mol. Cell. Biol.* 13:2247-2257 (1993)) and the truncated receptor was located within the perinuclear cytoplasm rather than secreted from these tumor cells (Scott *et al., Mol. Cell. Biol.* 13:2247-2257 (1993)).

Another alternatively spliced product of HER2, called "herstatin," has also been identified (Doherty *et al., Proc. Natl. Acad. Sci.* 96:10869-10874 (1999); Azios *et al., Oncogene* 20:5199-5209 (2001); Justman and Clinton, *J. Biol. Chem.* 277:20618-20624 (2002)). This protein consists of subdomains I and II from the extracellular domain followed by a unique C-terminal sequence encoded by intron 8.

Another mechanism that may account for poor clinical outcome in HER2 overexpressing tumors is suggested by the observation that, in some HER2 overexpressing tumor cells, the receptor is processed by an unknown metalloprotease (or metalloproteinase) to yield a truncated, membrane-associated receptor (sometimes

referred to as a "stub" and also known as p95), and a soluble extracellular domain (also known as ECD, ECD105, or p105).

As with other HER receptors, loss of the extracellular ligand binding domain renders the HER2 intracellular membrane-associated domain a constitutively active tyrosine kinase. It has therefore been postulated that the processing of the HER2 ECD creates a constitutively active receptor that can directly deliver growth and survival signals to the cancer cell. See, US Pat No. 6,541,214 (Clinton), and US Patent Appln No. 2004/0247602A1 (Friedman *et al.*)

Saez *et al. Clin Cancer Res.* 12(2): 424-431 (January, 2006) report that patients whose tumors express high levels of p95 have a significantly worse outcome than patients who do not. At present p95 level can only be determined by Western blot.

Summary of the Invention

In a first aspect, the invention concerns a method for inhibiting HER2 shedding comprising treating a HER2 expressing cell with a matrix metalloprotease (MMP) antagonist in an amount effective to inhibit HER2 shedding.

In addition, the invention provides a method for reducing HER2 extracellular domain (ECD) serum level in a mammal, comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to reduce the HER2 ECD serum level in the mammal.

In yet another aspect, a method for treating cancer in a mammal is provided, which comprises administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to treat the cancer.

Also, the invention concerns a method for treating a HER inhibitor-resistant cancer in a mammal comprising administering to the mammal a matrix metalloprotease (MMP) antagonist in an amount effective to treat the cancer.

In yet a further aspect, the invention relates to a method for reducing p95 HER2 level in a cell comprising exposing the cell to a matrix metalloprotease (MMP) antagonist in an amount effective to reduce the p95 HER2 level.

The invention also concerns a method of diagnosis (or prognosis) comprising evaluating MMP-15 (MT2-MMP) in a sample from a cancer patient, wherein elevated MMP-15 level or activity indicates the patient has an elevated p95 HER2 and/or shed HER2 serum level, and/or will have a poor clinical outcome. Preferably, MMP-15 level (protein or nucleic acid) is evaluated in the method and is used to identify patients with a poor prognosis, or who will have a poor clinical outcome. Optionally, the patient's cancer further displays HER expression, amplification, or activation, most preferably HER2 overexpression or amplification.

Brief Description of the Drawings

Figure 1 provides a schematic of the full length HER2 protein structure, and amino acid sequences for Domains I-IV (SEQ ID NOs. 1-4, respectively) of the extracellular domain thereof.

Figures 2A and 2B show the amino acid sequences of trastuzumab light chain (Fig. 2A; SEQ ID No. 5) and heavy chain (Fig. 2B; SEQ ID No. 6), respectively.

Figures 3A and 3B show the amino acid sequences of pertuzumab light chain (Fig. 3A; SEQ ID NO. 7) and heavy chain (Fig. 3B; SEQ ID NO. 8). CDRs are shown in bold. Calculated molecular mass of the light

chain and heavy chain are 23,526.22 Da and 49,216.56 Da (cysteines in reduced form). The carbohydrate moiety is attached to Asn 299 of the heavy chain.

Fig. 4 depicts the HER signaling network.

Fig. 5 illustrates trastuzumab inhibition of HER2 ECD shedding from HER2 overexpressing breast cancer cell lines (SKBR3, MT474) compared to nonoverexpressing breast cancer cell line (MCF-7).

Fig. 6 illustrates differences in p95 HER2 and shed HER2 ECD levels in MMTVHER2 transgenic tumors (f2:1282 tumors, trastuzumab-sensitive; and Fo5 tumors, trastuzumab-resistant).

Fig. 7 depicts the strategy used to identify HER2 sheddase.

Fig. 8 illustrates experiments which demonstrated sheddase had properties of a metalloprotease.

Fig. 9 shows expression of MMPs in MMTV-HER2 tumors and cell lines. MMP-15 is a candidate for explaining differences between f2:1282 tumors which are trastuzumab-sensitive, and Fo5 tumors which are trastuzumab-resistant.

Fig. 10 reflects interaction between flag-HER2 and MMP-15.

Fig. 11 shows interaction between flagHER2-f2:1282 and flagHER2-Fo5 and MMP-15. Differences between f2:1282 and Fo5 are not explainable by differential binding of mutants to MMP-15.

Fig. 12 illustrates somatic mutations found in MMTVHER2 transgenic mice. The sequences are: sheddase site (SEQ ID NO. 23), wild-type (WT) (SEQ ID NO. 24), splice (SEQ ID NO. 25), Fo5 (SEQ ID NO. 26), and f2:3078.10 (SEQ ID NO. 27).

Fig. 13 depicts results of the *in vitro* sheddase assay. gDHER29(DIV)-IgG is a substrate for the catalytic domains of MMP-15, MMP-16, MMP-19, and MMP-25. The sequences for protease digests are MMP-15 (SEQ ID NO. 28), MMP-16 (SEQ ID NO. 29), MMP-19 (SEQ ID NO. 30), MMP-25 (SEQ ID NO. 31), all other (SEQ ID NO. 32); and for the HER2 ECD C-terminal site (SEQ ID NO. 33).

Fig. 14 illustrates results of experiment demonstrating MMP-15 does not clip other HER receptors. The sequences demonstrating sequence variation near transmembrane domain are HER2 (SEQ ID NO. 34), EGFR (SEQ ID NO. 35), HER3 (SEQ ID NO. 36), HER4 (Jma) (SEQ ID NO. 37), and HER4 (Jmb) (SEQ ID NO. 38).

Fig. 15 shows MMP-15 "full length" clips HER2(+)-IgG.

Fig. 16 demonstrates an experiment which indicated p95HER2 is constitutively phosphorylated, but must heterdimerize with HER3 to activate Akt.

Fig. 17 shows MMP-15 RNA inhibitor (RNAi) reduces HER2 ECD shedding and p95 HER2 levels in SKBR3 and BT474 cells.

Fig. 18 illustrates how trastuzumab-mediated growth inhibition in SKBR3 cells is independent of inhibiting HER2 shedding.

Fig. 19 demonstrates that inhibition of metalloprotease activity in Fo5 xenograft tumors reduces HER2 shedding and inhibits p95HER2 levels.

Fig. 20 depicts members of the matrix metalloproteinase (MMP) family. MMP family members are grouped according to domain structure. The abbreviations used in this figure are: PRE, pre-domain; PRO, pro-domain; CAT, catalytic domain; H, hinge; HEM, hemopexin domain; F, furin-cleavage consensus domain; FN, fibronectin-like domain; GPI, glycophosphatidyl inositol anchor; TM, transmembrane domain; Ig, immunoglobulin-like domain; CA, cysteine array; CL, collagen-like domain.

Detailed Description of the Preferred Embodiments

I. Definitions

The terms "matrix metalloprotease" or "MMP" herein refer to a protein which is a member of a matrix metalloprotease (MMP) superfamily, dependent on Zn or Ca for activity. MMP herein includes the preproprotein, mature protein and variant forms thereof. See, also, Fig. 20 herein for examples of MMPs with various domains. MMPs are reviewed in Wagenaar-Miller *et al. Cancer and Metastasis Reviews* 23: 119-135 (2004).

A "membrane-tethered MMP" or "MT-MMP" herein is a MMP as defined above, where the MMP is capable of being attached to a cell membrane via either a transmembrane (TM) domain or a glycophosphatidyl inositol (GPI) anchor. Examples of MT-MMPs anchored by a transmembrane domain herein include MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT5-MMP (MMP-24). Examples of MT-MMPs anchored by a GPI anchor include MT4-MMP (MMP-17), and MT6-MMP (MMP-25). MMP-15 is the preferred MT-MMP herein.

"MT2-MMP" and "MMP-15"are synonyms herein and describe the preproprotein NP_002415 in the NCB1 database, the mature protein comprising amino acids 132-699 thereof, as well as variant forms thereof. Known substrates for MMP-15 include collagen, fibronectin, CD44, and complement. MMP-15 is upregulated in some cancers, and overexpression of this protease enhances tumor invasion and tumor cell growth.

A "MMP antagonist" is an agent that binds to and/or interferes to some extent with proteolytic activity of at least one MMP. Preferably, the MMP antagonist selectively binds to, or inhibits, the MMP, without significantly binding to, or inhibiting, other proteases, such as proteases in the ADAM (*a disintegrin and metalloprotease*) family. Examples of MMP antagonists herein include antibodies that bind to a MMP, small molecule inhibitors, pseudopeptides that mimic MMP substrates, nonpeptidic molecules that bind the catalytic zinc of MMPs, isolated natural tissue inhibitors of MMPs (TIMPs), nucleic acid inhibitors, such as RNA; or antisense inhibitors, etc.

A "MT-MMP antagonist" is an agent that binds to and/or interferes to some extent with proteolytic activity of at least one MT-MMP. Preferably, the MT-MMP antagonist selectively binds to, or inhibits, the MT-MMP, without significantly binding to, or inhibiting, other proteases (including other MMPs that are not membrane-tethered). Examples of MT-MMP antagonists include antibodies that bind to a MT-MMP, small molecule inhibitors, pseudopeptides that mimic MT-MMP substrates, nonpeptidic molecules that bind the catalytic zinc of a MT-MMP, isolated natural tissue inhibitors of MT-MMPs, MT-MMP nucleic acid inhibitors, such as RNA; or antisense inhibitors, etc.

A "MMP-15 antagonist" is an agent that binds to and/or interferes to some extent with proteolytic activity of MMP-15. Preferably, the MMP-15 antagonist selectively binds to, or inhibits, MMP-15 without significantly binding to, or inhibiting, other proteases (including MMPs other than MMP-15). Examples of MMP-15 antagonists include antibodies that bind to MMP-15, small molecule inhibitors, pseudopeptides that mimic MMP-15 substrates, nonpeptidic molecules that bind the catalytic zinc of MMP-15, isolated natural tissue inhibitors of MMP-15, MMP-15 nucleic acid inhibitors such as RNA; or antisense inhibitors etc.

By "elevated MMP level" is meant an amount of MMP in a biological sample, such as a tumor sample, that exceeds the normal amount of the MMP, for instance the amount in a normal, or non-tumor, sample of the

same tissue type. Such "normal amount" of MMP (*e.g.* of MMP-15) includes no or undetectable amount of MMP-15. Elevated MMP levels can be determined in various ways, including those which measure MMP protein or MMP nucleic acid.

A "HER receptor" is a receptor protein tyrosine kinase which belongs to the HER receptor family and includes EGFR, HER2, HER3 and HER4 receptors. The HER receptor includes native sequence HER receptor, and variants thereof. Preferably the HER receptor is native sequence human HER receptor.

A "full length" HER receptor comprises an extracellular domain, which may bind an HER ligand and/or dimerize with another HER receptor molecule; a lipophilic transmembrane domain; an intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated.

The terms "ErbB1," "HER1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to EGFR as disclosed, for example, in Carpenter *et al. Ann. Rev. Biochem.* 56:881-914 (1987), including variant forms thereof (*e.g.* a deletion mutant EGFR as in Humphrey *et al. PNAS (USA)* 87:4207-4211 (1990)).

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to human HER2 protein described, for example, in Semba *et al.*, *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto *et al. Nature* 319:230-234 (1986) (Genebank accession number X03363), as well as variant forms thereof, such as alternatively spliced forms (Siegel *et al. EMBO J.* 18(8):2149-2164 (1999)).

Herein, "HER2 extracellular domain" or "HER2 ECD" refers to a domain of HER2 that is outside of a cell, either anchored to a cell membrane, or in circulation, including fragments thereof. In one embodiment, the extracellular domain of HER2 may comprise four domains: "Domain I" (amino acid residues from about 1-195; SEQ ID NO:1), "Domain II" (amino acid residues from about 196-319; SEQ ID NO:2), "Domain III" (amino acid residues from about 320-488: SEQ ID NO:3), and "Domain IV" (amino acid residues from about 489-630; SEQ ID NO:4) (residue numbering without signal peptide). See Garrett *et al. Mol. Cell.*: 11: 495-505 (2003), Cho *et al. Nature* 421: 756-760 (2003), Franklin *et al. Cancer Cell* 5:317-328 (2004), and Plowman *et al. Proc. Natl. Acad. Sci.* 90:1746-1750 (1993), as well as Fig. 1 herein.

Herein, "HER2 shedding" refers to release of a soluble extracellular domain (ECD) fragment of HER2 from the cell surface of a cell which expresses HER2. Such shedding may be caused by proteolytic cleavage of cell surface HER2 resulting in release of an ECD fragment from the cell surface, or the soluble ECD or fragment thereof may be encoded by an alternate transcript.

By "shed HER2 serum level" is meant the amount of HER2 ECD present in the serum or circulation of a mammal. Such levels can be evaluated by various techniques including those described in: Ali *et al. Clin. Chem.* 48:1314-1320 (2002); Molina *et al. Clin. Cancer Res.* 8:347-353 (2002); U.S. Patent No. 4,933,294 issued June 12, 1990; WO91/05264 published April 18, 1991; U.S. Patent 5,401,638 issued March 28, 1995; or Sias *et al. J. Immunol. Methods* 132: 73-80 (1990).

Herein, "elevated shed HER2 serum level" refers to an amount of shed HER2 or HER2 ECD in the serum of a mammal (e.g. human) that exceeds the amount present in the serum a normal mammal (e.g. human). Elevated HER2 ECD serum levels may correlate with a poor prognosis and decreased responsiveness to endocrine therapy and chemotherapy in patients with advanced breast cancer.

The expression "p95 HER2" herein refers to NH2-terminal truncated HER2 protein. Generally, p95 is

a membrane-bound stub fragment which may arise from cleavage of full length HER2 by a protease or sheddase (Yuan *et al. Protein Expression and Purification* 29: 217-222 (2003)). p95 may have a M_r of about 95,000 and may be phosphorylated (Molina *et al. Cancer Research* 4744-4749 (2001)). p95 has been found in some breast cancer samples (Christianson *et al. Cancer Res.* 15:5123-5129 (1998)).

By "elevated p95 level" is meant a level of p95 in a cancer cell that exceeds the normal level, for instance the leval in a normal or non-cancerous cell of the same tissue type as the cancer cell. Such elevated p95 level may result in constitutive signaling, and nodal metastasis (Molina *et al. Clin. Cancer Research* 8:347-353 (2002); Christianson *et al. Cancer Res.* 15:5123-5129 (1998)).

By "evaluating" a marker, such as MMP-15, is intended a diagnostic and/or prognostic analysis thereof, including an analysis of the presence or absence of that marker, measurement of the amount thereof, and/or an analysis of activity thereof (*e.g.* increased activity).

A cancer patient with "a poor clinical outcome" is one with a poor prognosis, who is less likely to respond to cancer therapy, such as chemotherapy or therapy with a HER2 antibody, such as trastuzumab. The clinical outcome can be measured by standard means, such as survival, including disease free survival, etc.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in US Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus *et al. PNAS (USA)* 86:9193-9197 (1989), including variant forms thereof.

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman *et al., Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); and Plowman *et al., Nature*, 366:473-475 (1993), including variant forms thereof, such as the isoforms disclosed in WO99/19488, published April 22, 1999.

By "HER ligand" is meant a polypeptide which binds to and/or activates a HER receptor. The HER ligand of particular interest herein is a native sequence human HER ligand such as epidermal growth factor (EGF) (Savage *et al., J. Biol. Chem.* 247:7612-7621 (1972)); transforming growth factor alpha (TGF-α) (Marquardt *et al., Science* 223:1079-1082 (1984)); amphiregulin also known as schwanoma or keratinocyte autocrine growth factor (Shoyab *et al. Science* 243:1074-1076 (1989); Kimura *et al. Nature* 348:257-260 (1990); and Cook *et al. Mol. Cell. Biol.* 11:2547-2557 (1991)); betacellulin (Shing *et al., Science* 259:1604-1607 (1993); and Sasada *et al. Biochem. Biophys. Res. Commun.* 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama *et al., Science* 251:936-939 (1991)); epiregulin (Toyoda *et al., J. Biol. Chem.* 270:7495-7500 (1995); and Komurasaki *et al. Oncogene* 15:2841-2848 (1997)); a heregulin (see below); neuregulin-2 (NRG-2) (Carraway *et al., Nature* 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang *et al., Proc. Natl. Acad. Sci.* 94:9562-9567 (1997)); neuregulin-4 (NRG-4) (Harari *et al. Oncogene* 18:2681-89 (1999)); and cripto (CR-1) (Kannan *et al. J. Biol. Chem.* 272(6):3330-3335 (1997)). HER ligands which bind EGFR include EGF, TGF-α, amphiregulin, betacellulin, HB-EGF and epiregulin. HER ligands which bind HER3 include heregulins. HER ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3, NRG-4, and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide encoded by the heregulin gene product as disclosed in U.S. Patent No. 5,641,869, or Marchionni *et al., Nature*, 362:312-318 (1993). Examples of heregulins include heregulin-α, heregulin-β1, heregulin-β2 and heregulin-β3 (Holmes *et al., Science*, 256:1205-1210 (1992); and U.S. Patent No. 5,641,869); *neu* differentiation factor (NDF) (Peles *et al. Cell* 69: 205-216

(1992)); acetylcholine receptor-inducing activity (ARIA) (Falls *et al. Cell* 72:801-815 (1993)); glial growth factors (GGFs) (Marchionni *et al., Nature*, 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho *et al. J. Biol. Chem.* 270:14523-14532 (1995)); γ-heregulin (Schaefer *et al. Oncogene* 15:1385-1394 (1997)).

A "HER dimer" herein is a noncovalently associated dimer comprising at least two HER receptors. Such complexes may form when a cell expressing two or more HER receptors is exposed to an HER ligand and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski *et al., J. Biol. Chem.*, 269(20):14661-14665 (1994), for example. Examples of such HER dimers include EGFR-HER2, HER2-HER3 and HER3-HER4 heterodimers. Moreover, the HER dimer may comprise two or more HER2 receptors combined with a different HER receptor, such as HER3, HER4 or EGFR. Other proteins, such as a cytokine receptor subunit (*e.g.* gp130) may be associated with the dimer.

A cell, cancer, or biological sample which "displays HER expression, amplification, or activation" is one which, in a diagnostic test, expresses (including overexpresses) HER, has amplified HER gene, and/or otherwise demonstrates activation or phosphorylation of HER receptor(s). Such activation can be determined directly (*e.g.* by measuring HER phosphorylation) or indirectly (*e.g.* by gene expression profiling or by detecting HER heterodimers).

A cancer or tumor cell with "HER2 receptor overexpression or amplification" is one which has significantly higher levels of a HER2 protein or gene compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. HER2 overexpression or amplification may be determined in a diagnostic or prognostic assay by evaluating increased levels of the HER2 protein present on the surface of a cell (*e.g.* via an immunohistochemistry assay; IHC). Alternatively, or additionally, one may measure levels of HER2 nucleic acid in the cell, *e.g.* via fluorescent *in situ* hybridization (FISH; see WO98/45479 published October, 1998), southern blotting, or polymerase chain reaction (PCR) techniques, such as quantitative real time PCR (qRT-PCR). One may also study HER2 overexpression or amplification by measuring shed HER2 in a biological fluid such as serum (see, *e.g.*, U.S. Patent No. 4,933,294 issued June 12, 1990; WO91/05264 published April 18, 1991; U.S. Patent 5,401,638 issued March 28, 1995; and Sias *et al. J. Immunol. Methods* 132: 73-80 (1990)). Aside from the above assays, various *in vivo* assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, *e.g.* a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, *e.g.* by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

Conversely, a cancer or tumor cell which "does not overexpress or amplify HER receptor" is one which does not have higher than normal levels of HER receptor protein or gene compared to a noncancerous cell of the same tissue type. Antibodies that inhibit HER dimerization, such as pertuzumab, may be used to treat cancer which does not overexpress or amplify HER2 receptor.

A "HER inhibitor" is an agent which interferes with HER activation or function. Examples of HER inhibitors include HER antibodies (*e.g.* EGFR, HER2, HER3, or HER4 antibodies); HER dimerization inhibitors; EGFR-targeted drugs; small molecule HER antagonists; HER tyrosine kinase inhibitors; HER2 and EGFR dual tyrosine kinase inhibitors such as lapatinib/GW572016; antisense molecules (see, for example, WO2004/87207); and/or agents that bind to, or interfere with function of, downstream signaling molecules,

such as MAPK or Akt. Preferably, the HER inhibitor is an antibody or small molecule which binds to a HER receptor. Specific examples of HER inhibitors include trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, and TAK165.

A "HER dimerization inhibitor" is an agent which inhibits formation of a HER dimer. Preferably, the HER dimerization inhibitor is an antibody, for example an antibody which binds to HER2 at the heterodimeric binding site thereof. The most preferred dimerization inhibitor herein is pertuzumab or monoclonal antibody 2C4 (MAb 2C4). Other examples of HER dimerization inhibitors include antibodies which bind to EGFR and inhibit dimerization thereof with one or more other HER receptors (for example EGFR monoclonal antibody 806, MAb 806, which binds to activated or "untethered" EGFR; see Johns *et al., J. Biol. Chem.* 279(29):30375-30384 (2004)); antibodies which bind to HER3 and inhibit dimerization thereof with one or more other HER receptors; antibodies which bind to HER4 and inhibit dimerization thereof with one or more other HER receptors; peptide dimerization inhibitors (US Patent No. 6,417,168); antisense dimerization inhibitors; etc.

A "HER antibody" is an antibody that binds to a HER receptor. Optionally, the HER antibody further interferes with HER activation or function. Preferably, the HER antibody binds to the HER2 receptor. HER2 antibodies of particular interest herein are trastuzumab and pertuzumab. Examples of EGFR antibodies include cetuximab, ABX0303, EMD7200 and IMC-11F5.

"HER activation" refers to activation, or phosphorylation, of any one or more HER receptors. Generally, HER activation results in signal transduction (*e.g.* that caused by an intracellular kinase domain of a HER receptor phosphorylating tyrosine residues in the HER receptor or a substrate polypeptide). HER activation may be mediated by HER ligand binding to a HER dimer comprising the HER receptor of interest. HER ligand binding to a HER dimer may activate a kinase domain of one or more of the HER receptors in the dimer and thereby results in phosphorylation of tyrosine residues in one or more of the HER receptors and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s), such as Akt or MAPK intracellular kinases.

"Phosphorylation" refers to the addition of one or more phosphate group(s) to a protein, such as a HER receptor, or substrate thereof.

An antibody which "inhibits HER dimerization" is an antibody which inhibits, or interferes with, formation of a HER dimer. Preferably, such an antibody binds to HER2 at the heterodimeric binding site thereof. The most preferred dimerization inhibiting antibody herein is pertuzumab or MAb 2C4. Other examples of antibodies which inhibit HER dimerization include antibodies which bind to EGFR and inhibit dimerization thereof with one or more other HER receptors (for example EGFR monoclonal antibody 806, MAb 806, which binds to activated or "untethered" EGFR; see Johns *et al., J. Biol. Chem.* 279(29):30375-30384 (2004)); antibodies which bind to HER3 and inhibit dimerization thereof with one or more other HER receptors; and antibodies which bind to HER4 and inhibit dimerization thereof with one or more other HER receptors.

A "heterodimeric binding site" on HER2, refers to a region in the extracellular domain of HER2 that contacts, or interfaces with, a region in the extracellular domain of EGFR, HER3 or HER4 upon formation of a dimer therewith. The region is found in Domain II of HER2. Franklin *et al. Cancer Cell* 5:317-328 (2004).

The HER2 antibody may be one which, like trastuzumab, "inhibits HER2 ectodomain cleavage" (Molina *et al. Cancer Res.* 61:4744-4749(2001)) or may be one which, like pertuzumab, does not significantly inhibit HER2 ectodomain cleavage.

A HER2 antibody that "binds to a heterodimeric binding site" of HER2, binds to residues in domain II (and optionally also binds to residues in other of the domains of the HER2 extracellular domain, such as domains I and III), and can sterically hinder, at least to some extent, formation of a HER2-EGFR, HER2-HER3, or HER2-HER4 heterodimer. Franklin *et al. Cancer Cell* 5:317-328 (2004) characterize the HER2-pertuzumab crystal structure, deposited with the RCSB Protein Data Bank (ID Code IS78), illustrating an exemplary antibody that binds to the heterodimeric binding site of HER2.

An antibody that "binds to domain II" of HER2 binds to residues in domain II and optionally residues in other domain(s) of HER2, such as domains I and III. Preferably the antibody that binds to domain II binds to the junction between domains I, II and III of HER2.

A "native sequence" polypeptide is one which has the same amino acid sequence as a polypeptide (*e.g.*, HER receptor or HER ligand) derived from nature. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.* bispecific antibodies) formed from at least two intact antibodies, and antibody fragments, so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope(s), except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. Such monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones or recombinant DNA clones. It should be understood that the selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity *in vivo*, to create a multispecific antibody, *etc.*, and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, the monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (*e.g.*, Kohler *et al.*, *Nature*, 256:495 (1975); Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: Monoclonal Antibodies and T-Cell Hybridomas 563-681, (Elsevier, N.Y., 1981)), recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567), phage display technologies (see, *e.g.*, Clackson *et al.*, *Nature*, 352:624-

628 (1991); Marks *et al., J. Mol. Biol.*, 222:581-597 (1991); Sidhu *et al., J. Mol. Biol.* 338(2):299-310 (2004); Lee *et al., J.Mol.Biol.*340(5):1073-1093 (2004); Fellouse, *Proc. Nat. Acad. Sci. USA* 101(34):12467-12472 (2004); and Lee *et al. J. Immunol. Methods* 284(1-2):119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, *e.g.*, WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); *Bruggemann et al., Year in Immuno.*, 7:33 (1993); U.S. Patent Nos. 5,545,806; 5,569,825; 5,591,669 (all of GenPharm); U.S. Patent No. 5,545,807; WO 1997/17852; U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks *et al., Bio/Technology*, 10: 779-783 (1992); Lonberg *et al., Nature*, 368: 856-859 (1994); Morrison, *Nature*, 368: 812-813 (1994); Fishwild *et al., Nature Biotechnology*, 14: 845-851 (1996); Neuberger, *Nature Biotechnology*, 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.*, 13: 65-93 (1995).

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)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (*e.g.* Old World Monkey, Ape etc) and human constant region sequences.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragment(s).

An "intact antibody" herein is one which comprises two antigen binding regions, and an Fc region. Preferably, the intact antibody has one or more effector functions.

Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes". There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

Antibody "effector functions" refer to those biological activities attributable to an Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor; BCR), etc.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (*e.g.* Natural Killer (NK) cells, neutrophils,

and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. 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).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, *e.g.* from blood or PBMCs as described herein.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and Fcγ RIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al., Immunomethods* 4:25-34 (1994); and de Haas *et al., J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al., J. Immunol.* 117:587 (1976) and Kim *et al., J. Immunol.* 24:249 (1994)), and regulates homeostasis of immunoglobulins.

"Complement dependent cytotoxicity" or "CDC" refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (*e.g.* an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.* as described in Gazzano-Santoro *et al., J. Immunol. Methods* 202:163 (1996), may be performed.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is

aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (*e.g.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*e.g.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear

at least one free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994). HER2 antibody scFv fragments are described in WO93/16185; U.S. Patent No. 5,571,894; and U.S. Patent No. 5,587,458.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a variable heavy domain (V_H) connected to a variable light domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

"Humanized" forms of non-human (*e.g.*, rodent) 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 also will 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).

Humanized HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 or trastuzumab (HERCEPTIN®) as described in Table 3 of U.S. Patent 5,821,337 expressly incorporated herein by reference; humanized 520C9 (WO93/21319); and humanized 2C4 antibodies such as pertuzumab as described herein.

For the purposes herein, "trastuzumab," "HERCEPTIN®," and "huMAb4D5-8" refer to an antibody comprising the light and heavy chain amino acid sequences in SEQ ID NOS. 5 and 6, respectively.

Herein, "pertuzumab" and "OMNITARG™" refer to an antibody comprising the light and heavy chain amino acid sequences in SEQ ID NOS. 7 and 8, respectively.

A "naked antibody" is an antibody that is not conjugated to a heterologous molecule, such as a

cytotoxic moiety or radiolabel.

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.

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

The term "main species antibody" herein refers to the antibody structure in a composition which is the quantitatively predominant antibody molecule in the composition.

An "amino acid sequence variant" antibody herein is an antibody with an amino acid sequence which differs from a main species antibody. Ordinarily, amino acid sequence variants will possess at least about 70% homology with the main species antibody, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with the main species antibody. The amino acid sequence variants possess substitutions, deletions, and/or additions at certain positions within or adjacent to the amino acid sequence of the main species antibody.

A "glycosylation variant" antibody herein is an antibody with one or more carbohydrate moeities attached thereto which differ from one or more carbohydate moieties attached to a main species antibody.

A "deamidated"antibody is one in which one or more asparagine residues thereof has been derivitized, *e.g.* to an aspartic acid, a succinimide, or an iso-aspartic acid.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma (including medulloblastoma and retinoblastoma), sarcoma (including liposarcoma and synovial cell sarcoma), neuroendocrine tumors (including carcinoid tumors, gastrinoma, and islet cell cancer), mesothelioma, schwannoma (including acoustic neuroma), meningioma, adenocarcinoma, melanoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (*e.g.* epithelial squamous cell cancer), lung cancer including small-cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung and squamous carcinoma of the

lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer (including metastatic breast cancer), colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, testicular cancer, esophagael cancer, tumors of the biliary tract, as well as head and neck cancer.

A mammal with a "HER inhibitor-resistant cancer" is one who has progressed while receiving HER inhibitor-based therapy (*i.e.* the patient is "HER inhibitor refractory"), or the mammal has progressed within 12 months (for instance, within 6 months) after completing a HER inhibitor-based therapy regimen. The HER inhibitor-based therapy includes therapy with naked or conjugated HER inhibitor, where the HER inhibitor is administered as a single-agent, or in combination with other anti-tumor drug(s). The HER inhibitor may be trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, or TAK165, but preferably is trastuzumab.

A "mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

A "tumor sample" herein is a sample derived from, or comprising tumor cells from, a patient's tumor. Examples of tumor samples herein include, but are not limited to, tumor biopsies, circulating tumor cells, circulating plasma proteins, ascitic fluid, primary cell cultures or cell lines derived from tumors or exhibiting tumor-like properties, as well as preserved tumor samples, such as formalin-fixed, paraffin-embedded tumor samples or frozen tumor samples.

A "fixed" tumor sample is one which has been histologically preserved using a fixative.

A "formalin-fixed" tumor sample is one which has been preserved using formaldehyde as the fixative.

An "embedded" tumor sample is one surrounded by a firm and generally hard medium such as paraffin, wax, celloidin, or a resin. Embedding makes possible the cutting of thin sections for microscopic examination or for generation of tissue microarrays (TMAs).

A "paraffin-embedded" tumor sample is one surrounded by a purified mixture of solid hydrocarbons derived from petroleum.

Herein, a "frozen" tumor sample refers to a tumor sample which is, or has been, frozen.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially a HER expressing cancer cell either *in vitro* or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of HER expressing 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 topo 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.

Examples of "growth inhibitory" antibodies are those which bind to HER2 and inhibit the growth of cancer cells overexpressing HER2. Preferred growth inhibitory HER2 antibodies inhibit growth of SK-BR-3 breast tumor cells in cell culture by greater than 20%, and preferably greater than 50% (*e.g.* from about 50% to about 100%) at an antibody concentration of about 0.5 to 30 μg/ml, where the growth inhibition is determined six days after exposure of the SK-BR-3 cells to the antibody (see U.S. Patent No. 5,677,171 issued October 14, 1997). The SK-BR-3 cell growth inhibition assay is described in more detail in that patent and hereinbelow. The preferred growth inhibitory antibody is a humanized variant of murine monoclonal antibody 4D5, *e.g.*, trastuzumab.

An antibody which "induces apoptosis" is one which induces programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which overexpresses the HER2 receptor. Preferably the cell is a tumor cell, *e.g.* a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. *In vitro*, the cell may be a SK-BR-3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an annexin binding assay using BT474 cells (see below). Examples of HER2 antibodies that induce apoptosis are 7C2 and 7F3.

The "epitope 2C4" is the region in the extracellular domain of HER2 to which the antibody 2C4 binds. In order to screen for antibodies which bind to the 2C4 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Preferably the antibody blocks 2C4's binding to HER2 by about 50% or more. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 2C4 epitope of HER2. Epitope 2C4 comprises residues from Domain II in the extracellular domain of HER2. 2C4 and pertuzumab binds to the extracellular domain of HER2 at the junction of domains I, II and III. Franklin *et al. Cancer Cell* 5:317-328 (2004).

The "epitope 4D5" is the region in the extracellular domain of HER2 to which the antibody 4D5 (ATCC CRL 10463) and trastuzumab bind. This epitope is close to the transmembrane domain of HER2, and within Domain IV of HER2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of HER2 (*e.g.* any one or more residues in the region from about residue 529 to about residue 625, inclusive of the HER2 ECD, residue numbering including signal peptide).

The "epitope 7C2/7F3" is the region at the N terminus, within Domain I, of the extracellular domain of HER2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be

performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on HER2 (*e.g.* any one or more of residues in the region from about residue 22 to about residue 53 of the HER2 ECD, residue numbering including signal peptide).

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disease as well as those in which the disease is to be prevented. Hence, the patient to be treated herein may have been diagnosed as having the disease or may be predisposed or susceptible to the disease.

The term "effective amount" refers to an amount of a drug effective to treat cancer in the patient. The effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. The effective amount may extend progression free survival, result in an objective response (including a partial response, PR, or complete response, CR), increase overall survival time, and/or improve one or more symptoms of cancer.

By "complete response" or "complete remission" is intended the disappearance of all signs of cancer in response to treatment. This does not always mean the cancer has been cured.

"Partial response" refers to a decrease in the size of one or more tumors or lesions, or in the extent of cancer in the body, in response to treatment.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.* At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; TLK 286 (TELCYTA™); acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; bisphosphonates, such as clodronate; 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)) and anthracyclines such as annamycin, AD 32, alcarubicin, daunorubicin, dexrazoxane, DX-52-1, epirubicin, GPX-100, idarubicin, KRN5500, menogaril, dynemicin, including dynemicin A, an esperamicin, neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, liposomal doxorubicin, and deoxydoxorubicin), esorubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, and zorubicin; folic acid analogues such as denopterin, pteropterin, and trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, and floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, and testolactone; anti-adrenals such as aminoglutethimide, mitotane, and trilostane; folic acid replenisher such as folinic acid (leucovorin); aceglatone; anti-folate anti-neoplastic agents such as ALIMTA®, LY231514 pemetrexed, dihydrofolate reductase inhibitors such as methotrexate, anti-metabolites such as 5-fluorouracil (5-FU) and its prodrugs such as UFT, S-1 and capecitabine, and thymidylate synthase inhibitors and glycinamide ribonucleotide formyltransferase inhibitors such as raltitrexed (TOMUDEX^{RM}, TDX); inhibitors of dihydropyrimidine dehydrogenase such as eniluracil; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids and taxanes, *e.g.*, TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® docetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; antimetabolite chemotherapeutic agent such as gemcitabine (GEMZAR®), 5-fluorouracil (5-FU), capecitabine (XELODA™), 6-mercaptopurine, methotrexate, 6-thioguanine, pemetrexed, raltitrexed, arabinosylcytosine ARA-C cytarabine (CYTOSAR-U®), dacarbazine (DTIC-DOME®), azocytosine, deoxycytosine, pyridmidene, fludarabine (FLUDARA®), cladrabine, and 2-deoxy-D-glucose; 6-thioguanine; mercaptopurine; platinum-based chemotherapeutic agent such as carboplatin, cisplatin, or oxaliplatinum; vinblastine (VELBAN®); etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); vinca alkaloid; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; xeloda; 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 or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON® toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

An "anti-angiogenic agent" refers to a compound which blocks, or interferes with to some degree, the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or antibody that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. The preferred anti-angiogenic factor herein is an antibody that binds to vascular endothelial growth factor (VEGF), such as bevacizumab (AVASTIN®).

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

As used herein, the term "EGFR-targeted drug" refers to a therapeutic agent that binds to EGFR and, optionally, inhibits EGFR activation. Examples of such agents include antibodies and small molecules that bind to EGFR. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455

(ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, US Patent No. 4,943, 533, Mendelsohn *et al.*) and variants thereof, such as chimerized 225 (C225 or Cetuximab; ERBUTIX®) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.); IMC-11F8, a fully human, EGFR-targeted antibody (Imclone); antibodies that bind type II mutant EGFR (US Patent No. 5,212,290); humanized and chimeric antibodies that bind EGFR as described in US Patent No. 5,891,996; and human antibodies that bind EGFR, such as ABX-EGF (see WO98/50433, Abgenix); EMD 55900 (Stragliotto *et al. Eur. J. Cancer* 32A:636-640 (1996)); EMD7200 (matuzumab) a humanized EGFR antibody directed against EGFR that competes with both EGF and TGF-alpha for EGFR binding; and mAb 806 or humanized mAb 806 (Johns *et al., J. Biol. Chem.* 279(29):30375-30384 (2004)). The anti-EGFR antibody may be conjugated with a cytotoxic agent, thus generating an immunoconjugate (see, *e.g.*, EP659,439A2, Merck Patent GmbH). Examples of small molecules that bind to EGFR include ZD1839 or Gefitinib (IRESSA™; Astra Zeneca); CP-358774 or Erlotinib (TARCEVA™; Genentech/OSI); and AG1478, AG1571 (SU 5271; Sugen); EMD-7200.

A "tyrosine kinase inhibitor" is a molecule which inhibits tyrosine kinase activity of a tyrosine kinase such as a HER receptor. Examples of such inhibitors include the EGFR-targeted drugs noted in the preceding paragraph; small molecule HER2 tyrosine kinase inhibitor such as TAK165 available from Takeda; CP-724,714, an oral selective inhibitor of the ErbB2 receptor tyrosine kinase (Pfizer and OSI); dual-HER inhibitors such as EKB-569 (available from Wyeth) which preferentially binds EGFR but inhibits both HER2 and EGFR-overexpressing cells; GW572016 (available from Glaxo) an oral HER2 and EGFR tyrosine kinase inhibitor; PKI-166 (available from Novartis); pan-HER inhibitors such as canertinib (CI-1033; Pharmacia); Raf-1 inhibitors such as antisense agent ISIS-5132 available from ISIS Pharmaceuticals which inhibits Raf-1 signaling; non-HER targeted TK inhibitors such as Imatinib mesylate (Gleevac™) available from Glaxo; MAPK extracellular regulated kinase I inhibitor CI-1040 (available from Pharmacia); quinazolines, such as PD 153035,4-(3-chloroanilino) quinazoline; pyridopyrimidines; pyrimidopyrimidines; pyrrolopyrimidines, such as CGP 59326, CGP 60261 and CGP 62706; pyrazolopyrimidines, 4-(phenylamino)-7H-pyrrolo[2,3-d] pyrimidines; curcumin (diferuloyl methane, 4,5-bis (4-fluoroanilino)phthalimide); tyrphostines containing nitrothiophene moieties; PD-0183805 (Warner-Lamber); antisense molecules *(e.g.* those that bind to HER-encoding nucleic acid); quinoxalines (US Patent No. 5,804,396); tryphostins (US Patent No. 5,804,396); ZD6474 (Astra Zeneca); PTK-787 (Novartis/Schering AG); pan-HER inhibitors such as CI-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); Imatinib mesylate (Gleevac; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); CI-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Sugen); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-1C11 (Imclone); or as described in any of the following patent publications: US Patent No. 5,804,396; WO99/09016 (American Cyanimid); WO98/43960 (American Cyanamid); WO97/38983 (Warner Lambert); WO99/06378 (Warner Lambert); WO99/06396 (Warner Lambert); WO96/30347 (Pfizer, Inc); WO96/33978 (Zeneca); WO96/3397 (Zeneca); and WO96/33980 (Zeneca).

II. Inhibiting HER2 Shedding

The present application concerns a method for inhibiting HER2 shedding comprising treating, or exposing, a HER2 expressing cell with or to a matrix metalloprotease (MMP) antagonist in an amount effective to inhibit HER2 shedding. Preferably, the MMP antagonist is a membrane-tethered MMP (MT-MMP) antagonist, such as MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT5-MMP (MMP-

24), MT4-MMP (MMP-17), or MT6-MMP (MMP-25). Most preferably, the MT-MMP is MMP-15, and desirably, the antagonist binds selectively or preferentially to MMP-15, without significantly binding other proteases or MMPs other than MMP-15, and/or the antagonist interferes with MMP-15 proteolytic function without significantly interfering with function of other proteases or MMPs other than MMP-15.

In the preferred embodiment, the treated cell displays HER and/or MMP expression, amplification, or activation. For example, the cell may display HER2 and/or MMP-15 overexpression or amplification.

The activity of the MMP antagonist may be enhanced by combining it with another anti-tumor drug, HER inhibitor, or HER2 antibody (such as trastuzumab or pertuzumab). Examples of HER inhibitors that may be combined with the MMP antagonist include trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, and TAK165.

The invention also concerns a method for reducing HER2 extracellular domain (ECD) serum level in a mammal, comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to reduce the HER2 ECD serum level in the mammal. The mammal optionally has an elevated MMP level.

The invention also provides a method for treating cancer in a mammal comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to treat the cancer. The cancer may display HER and/or MMP expression, amplification, or activation. For example, the cancer may display HER2 or MMP-15 overexpression or amplification. In one embodiment, the treated mammal has an elevated shed HER2 serum level or elevated p95 HER2 level.

This invention also relates to a method for treating a HER inhibitor-resistant cancer in a mammal comprising administering to the mammal a matrix metalloprotease (MMP) antagonist in an amount effective to treat the cancer. For example, the mammal may be resistant to a HER2 antibody, such as trastuzumab.

Also provided is a method for reducing p95 HER2 level in a cell comprising exposing the cell to a matrix metalloprotease (MMP) antagonist in an amount effective to reduce the p95 HER2 level.

Various MMP antagonists may be used, but preferably the MMP antagonist is a small molecule inhibitor or an antibody. Methods for making antibodies are described hereinbelow.

III. Production of Antibodies

A description follows as to exemplary techniques for the production of antibodies used in accordance with the present invention. The antigen to be used for production of antibodies may be, *e.g.*, a soluble form of the antigen or a portion thereof, containing the desired epitope. Alternatively, cells expressing the antigen at their cell surface (*e.g.* NIH-3T3 cells transformed to overexpress HER2; or a carcinoma cell line such as SK-BR-3 cells, see Stancovski *et al. PNAS (USA)* 88:8691-8695 (1991)) can be used to generate antibodies. Other forms of antigen useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide

(through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies

Various methods for making monoclonal antibodies herein are available in the art. For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); and Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al., Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies 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 murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy chain and light chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; and Morrison, *et al.*, *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has 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.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-327 (1988); Verhoeyen *et al.*, *Science*, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the

corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

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

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 a preferred 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.

(iv) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and U.S. Patent Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty *et al., Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and

displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, *e.g.*, Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, *Nature*, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), or Griffith *et al.*, *EMBO J.* 12:725-734 (1993). See, also, U.S. Patent Nos. 5,565,332 and 5,573,905.

As discussed above, human antibodies may also be generated by *in vitro* activated B cells (see U.S. Patents 5,567,610 and 5,229,275).

Human HER2 antibodies are described in U.S. Patent No. 5,772,997 issued June 30, 1998 and WO 97/00271 published January 3, 1997.

(v) Antibody fragments

Various techniques have been developed for the production of antibody fragments comprising one or more antigen binding regions. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.* , *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan *et al.*, *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Patent No. 5,571,894; and U.S. Patent No. 5,587,458. The antibody fragment may also be a "linear antibody", *e.g.*, as described in U.S. Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the HER2 protein. Other such antibodies may combine a HER2 binding site with binding site(s) for EGFR, HER3 and/or HER4. Alternatively, a HER2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the HER2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express HER2. These antibodies possess a HER2-binding arm and an arm which binds the cytotoxic agent (*e.g.* saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies).

WO 96/16673 describes a bispecific HER2/FcγRIII antibody and U.S. Patent No. 5,837,234 discloses a bispecific HER2/FcγRI antibody IDM1 (Osidem). A bispecific HER2/Fcα antibody is shown in WO98/02463. U.S. Patent No. 5,821,337 teaches a bispecific HER2/CD3 antibody. MDX-210 is a bispecific HER2-FcγRIII Ab.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al., Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al., EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al., Methods in Enzymology*, 121:210 (1986).

According to another approach described in U.S. Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al.*, *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

(vii) Other amino acid sequence modifications

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 changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites.

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 *Science*, 244:1081-1085 (1989). 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 antibody variants 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 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.

Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred 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 substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion 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 human HER2. 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.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that lacks fucose attached to an Fc region of the antibody are described in US Pat Appl No US 2003/0157108 A1, Presta, L. See also US 2004/0093621 A1 (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetylglucosamine (GlcNAc) in the carbohydrate attached to an Fc region of the antibody are referenced in WO03/011878, Jean-Mairet *et al.* and US Patent No. 6,602,684, Umana *et al.* Antibodies with at least one galactose residue in the oligosaccharide attached to an Fc region of the antibody are reported in WO97/30087, Patel *et al.* See, also, WO98/58964 (Raju, S.) and WO99/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof.

It may be desirable to modify the antibody of the invention with respect to effector function, *e.g.* so as to enhance antigen-dependent cell-mediated cyotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al., J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al. Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:219-230 (1989).

WO00/42072 (Presta, L.) describes antibodies with improved ADCC function in the presence of human effector cells, where the antibodies comprise amino acid substitutions in the Fc region thereof.

Preferably, the antibody with improved ADCC comprises substitutions at positions 298, 333, and/or 334 of the Fc region. Preferably the altered Fc region is a human IgG1 Fc region comprising or consisting of substitutions at one, two or three of these positions.

Antibodies with altered C1q binding and/or complement dependent cytotoxicity (CDC) are described in WO99/51642, US Patent No. 6,194,551B1, US Patent No. 6,242,195B1, US Patent No. 6,528,624B1 and US Patent No. 6,538,124 (Idusogie *et al.*). The antibodies comprise an amino acid substitution at one or more of amino acid positions 270, 322, 326, 327, 329, 313, 333 and/or 334 of the Fc region thereof (using Eu numbering of Fc region residues as in Kabat).

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in US Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

Antibodies with improved binding to the neonatal Fc receptor (FcRn), and increased half-lives, are described in WO00/42072 (Presta, L.) and US2005/0014934A1 (Hinton *et al.*). These antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. For example, the Fc region may have substitutions at one or more of positions 238, 250, 256, 265, 272, 286, 303, 305, 307, 311, 312, 314, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424, 428 or 434 thereof (using Eu numbering of Fc region residues as in Kabat). The preferred Fc region-comprising antibody variant with improved FcRn binding comprises amino acid substitutions at one, two or three of positions 307, 380 and 434 of the Fc region thereof.

Engineered antibodies with three or more (preferably four) functional antigen binding sites are also contemplated (US Appln No. US2002/0004587 A1, Miller *et al.*).

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.

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.* a small molecule toxin or an enzymatically active toxin of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, a maytansine (U.S. Patent No. 5,208,020), a trichothene, and CC1065 are also contemplated herein.

In one preferred embodiment of the invention, the antibody is conjugated to one or more maytansine molecules (*e.g.* about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody (Chari *et al. Cancer Research* 52: 127-131 (1992)) to generate a maytansinoid-antibody immunoconjugate.

Another immunoconjugate of interest comprises an antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ^I_1 (Hinman *et al. Cancer Research* 53: 3336-3342 (1993) and Lode *et al. Cancer Research* 58: 2925-2928 (1998)). See, also, US Patent Nos. 5,714,586; 5,712,374; 5,264,586; and 5,773,001 expressly incorporated herein by reference.

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).

A variety of radioactive isotopes are available for the production of radioconjugated HER2 antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) 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 glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 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, dimethyl linker or disulfide-containing linker (Chari *et al. Cancer Research* 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, *e.g.* by recombinant techniques or peptide synthesis.

Other immunoconjugates are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, 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, Oslo, A., Ed., (1980).

The antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA*, 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published October 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al. J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon *et al. J. National Cancer Inst.*81(19)1484 (1989).

IV. Pharmaceutical Formulations

Therapeutic formulations of the MMP antagonists used in accordance with the present invention are prepared for storage by mixing a MMP antagonist having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Lyophilized antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

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. Various drugs which can be combined with the MMP antagonist are described in the method of treatment section below. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, 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).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

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

V. Treatment

Examples of various cancers that can be treated with the MMP antagonist are listed in the definition section above. Administration of the MMP antagonist will result in an improvement in the signs or symptoms of cancer.

The MMP antagonist is administered to a human patient in accord with known methods, such as intravenous administration, *e.g.*, as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

For the prevention or treatment of disease, the dose of MMP antagonist will depend on the type of cancer to be treated, as defined above, the severity and course of the cancer, 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.

While the MMP antagonist may be the sole anti-tumor drug administered, the patient is optionally treated with a combination of the MMP antagonist, and one or more other anti-tumor agent(s). The combined administration includes coadministration or concurrent administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Thus, the other anti-tumor agent may be administered prior to, or following, administration of the MMP antagonist. In this embodiment, the timing between at least one administration of the MMP antagonist and at least one administration of the other anti-tumor agent is preferably approximately 1 month or less, and most preferably approximately 2 weeks or less. Alternatively, the MMP antagonist and other anti-tumor agent are administered concurrently to the patient, in a single formulation or separate formulations. Treatment with the combination of the MMP antagonist and the other anti-tumor agent may result in a synergistic, or greater than additive, therapeutic benefit to the patient.

Examples of second anti-tumor agents that may be combined with the MMP antagonist include: one or more chemotherapeutic agent(s); a HER inhibitor (*e.g.* trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, TAK165, etc); Raf and/or ras inhibitor (see, for example, WO 2003/86467); a growth inhibitory HER2 antibody such as trastuzumab; a HER dimerization inhibitor such as Pertuzumab; a HER2 antibody which induces apoptosis of a HER2-overexpressing cell, such as 7C2, 7F3 or humanized variants thereof; an antibody directed against a tumor

associated antigen, such as EGFR, HER3, HER4; anti-hormonal compound, *e.g.*, an anti-estrogen compound such as tamoxifen, or an aromatase inhibitor; a cardioprotectant (to prevent or reduce any myocardial dysfunction associated with the therapy); a cytokine; an EGFR-targeted drug (such as TARCEVA®, IRESSA® or Cetuximab); an anti-angiogenic agent (especially bevacizumab sold by Genentech under the trademark AVASTIN™); a tyrosine kinase inhibitor; a COX inhibitor (for instance a COX-1 or COX-2 inhibitor); non-steroidal anti-inflammatory drug, Celecoxib (CELEBREX®); farnesyl transferase inhibitor (for example, Tipifarnib/ZARNESTRA® R115777 available from Johnson and Johnson or Lonafarnib SCH66336 available from Schering-Plough); antibody that binds oncofetal protein CA 125 such as Oregovomab (MoAb B43.13); HER2 vaccine (such as HER2 AutoVac vaccine from Pharmexia, or APC8024 protein vaccine from Dendreon, or HER2 peptide vaccine from GSK/Corixa); doxorubicin HCl liposome injection (DOXIL®); topoisomerase I inhibitor such as topotecan; taxane; HER2 and EGFR dual tyrosine kinase inhibitor such as lapatinib/GW572016; TLK286 (TELCYTA®); EMD-7200; a temperature-reducing medicament such as acetaminophen, diphenhydramine, or meperidine; hematopoietic growth factor, etc.

Suitable dosages for any of the above coadministered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and MMP antagonist.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

Aside from administration of protein MMP antagonists to the patient, the present application contemplates administration of MMP antagonists by gene therapy. See, for example, WO96/07321 published March 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells; *in vivo* and *ex vivo*. For *in vivo* delivery the nucleic acid is injected directly into the patient, usually at the site where the antibody is required. For *ex vivo* treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, *e.g.* U.S. Patent Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for *ex vivo* delivery of the gene is a retrovirus.

The currently preferred *in vivo* nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.* capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated

endocytosis is described, for example, by Wu *et al., J. Biol. Chem.* 262:4429-4432 (1987); and Wagner *et al., Proc. Natl. Acad. Sci. USA* 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson *et al., Science* 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

VI. Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	October 17, 1996
7F3	ATCC HB-12216	October 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	April 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1

The following example investigated the role of matrix metalloproteases (MMPs) in HER2 shedding.

MATERIALS AND METHODS

Cell Culture and Transfections

All cell lines used in this study were obtained from the American Type Tissue Culture Collection (ATCC, Manassas, VA). BT 474, MCF-7, and SKBR-3 cells were maintained in high-glucose DMEM:Ham's F12 (50:50) supplemented with 10% heat-inactivated FBS and 2mM L-Glutamine. Cos-7 cells were maintained in High-Glucose DMEM supplemented with 10% heat-inactivated FBS and 2mM L-Glutamine. Cell lines were maintained at 37^{0}C in a humidified incubator supplied with 5% CO_2. BT 474 and SKBR-3 cells were transiently transfected with constructs as described by electroporation using kit V according to the manufacturer's instructions (AMAXA™). Cos-7 cells were transfected as described using LIPOFECTAMINE™ 2000 (Invitrogen) according to the manufacturer's recommendations.

Fo5 and f2:1282 Mouse Xenograft Tumor lines

The Fo5 and f2:1282 lines have been previously described (Finkle *et al., Clin. Cancer Res.* 10: 2499-2511 (2004)). These lines were derived from primary tumors from a MMTV HER2 transgenic mouse and passaged in the mammary fat pad of FVB mice.

GM6001 injections

Fo5 tumors were transplanted into FVB mice and allowed to grow to an average size of 400-600 mm^3 prior to initiating the study. GM6001 (3-[N-hydroxycarbomyl]-[2R]-isobutylpropionyl-L-tryptophan methylamide) was obtained from Calbiochem and reconstituted in a slurry of 4% carboxymethylcellulose/0.9% PBS and administered daily by intraperitoneal injection at 100mg/kg body weight for 3 days. Twenty-four

hours after the third day of injection, animals were sacrificed and serum collected by cardiac puncture. Tumors size was measured at day 0 and at day 4. Tumors were removed and flash frozen for further analysis.

RNA preparation and AFFYMETRIX™ arrays

Total RNA was isolated from flash frozen tumor samples using a RNAEASY™ kit (Qiagen, Chatsworth, CA). Residual genomic DNA was removed by DNAse I treatment (Roche Molecular Biochemicals). Microarray experiments were performed and analyzed as previously described (Jin, H *et al., Circulation* 103:736-742 (2001)). Samples were hybridized to the AFFYMETRIX™ mouse genome single array (MOE430P) and known genes array (MOE430A) (AFFYMETRIX™, Inc., Santa Clara, CA). Experiments were done in three replicates for three Fo5 and three f2:1282 tumor samples. A Mann-Whitney pairwise comparison was performed and those genes with a 2-fold increase in expression having a concordance of greater than 80% were considered significant. The GeneLogic Bioexpress Database (GeneLogic, Gaithersburg, MD), a collection of gene expression data from AFFYMETRIX™ microarray analysis of cell lines and tissues, was used to examine the expression of proteases identified from the Fo5-f2:1282 tumor differential screen for expression in SKBR-3 and BT 474 cell lines.

HER2 ECD ELISA

Serum from mice harboring Fo5 or f2:1282 xenograft tumors was collected by cardiac puncture and HER2 ECD levels detected using a previously described HER2 ELISA (Finkle *et al. Clin. Cancer Res.* 10: 2499-2511 (2004)). Serum was diluted 1:50 using assay buffer (PBS/0.5% BSA, 0.05% TWEEN® 20/10 ppm PROCLIN® 300/0.2% BGG/0.25% CHAPS/0.15 M NaCl/5mM EDTA (pH 7.4) followed by additional 1:2 serial dilutions. Shed HER2 was captured using a goat anti-HER2 polyclonal antibody (Genentech) onto NUNC® Maxisorp plates and detected using a biotin-conjugated rabbit anti-HER2 polyclonal antibody (Genentech) followed by a AMDEX™-strepavidin-horseradish peroxidase antibody (Amersham Pharmacia Biotech) following a procedure described previously (Sias et *al., J. Immunol. Meth.* 109:219-27 (1990)). For cell culture experiments, cells were treated as indicated and conditioned media was collected and diluted in assay buffer serially 1:2. The concentration of shed HER2 in serum or in conditioned media was determined from a four-parameter fit of a standard curve using purified recombinant HER2 ECD protein (Genentech) as a standard.

DNA constructs and Protein Purification

Human MMP-15 was cloned into pRK5 by PCR using an origen clone as a template. A C-terminally V5His tagged full length version was generated by PCR using primers
5'-GCCGAAGCTTGCCACCATGGGCAGCGACCCGAGCGCG-3' (SEQ ID NO. 9) and
5'-GCTGTCTAGAGTTCACCGTCCGTGCCAGTGCCACCTCCTCC-3' (SEQ ID NO. 10)
and cloned in pcDNA3.1V5His.

A soluble version of MMP-15, which lacks the transmembrane domain, was generated by PCR using primers
5'-GCCGAAGCTTGCCACCATGGGCAGCGACCCGAGCGCG-3' (SEQ ID NO. 11) and
5'-GCTGTCTAGAGACCCACTCCTGCAGCGAGCG-3' (SEQ ID NO. 12).

A catalytically dead mutant of MMP-15 was generated by substituting an alanine residue for glutamine at amino acid 260 of the mature protein by site-directed mutagenesis.

pRK5.gDHER2-Fc (pRK5.gDHER2-IgG) was generated by PCR using primers 5'-CGAGTCGACCTCGAGGCCAGCCCTCTGACGTCC-3' (SEQ ID NO. 13) and 5'-GGCACGCGTCGTCAGAGGGCTGGCTCTCTG-3' (SEQ ID NO. 14) and cloned into the XhoI-MluI digested pRK5.gDHER2-Fc (Fitzpatrick *et. al., FEBBS Lett.* 431(1):102-6 (1998)). This introduced the putative cleavage site of HER2 identified from purified HER2 ECD isolated from SKBR-3 conditioned media (Yuan et. *al., Protein Expression and Purification* 29:217-222 (2003)).

A truncated version of gDHER2-Fc was also generated that only contains domain IV of HER2 using PCR primers 5'-GGCCTCGAGGGCCTGGCCTGCCACCAG-3' (SEQ ID NO. 15) and 5'-GGCACGCGTCGTCAGAGGGCTGGCTCTCTG-3' (SEQ ID NO. 16).

pRK5.HER3-Fc and pRK5.HER4-Fc have been previously described (Fitzpatrick *et al., FEBBS Lett.* 431(1): 102-6 (1998)). A FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; (SEQ ID NO. 17)) was introduced into the N-terminus of pRK5. HER-Fc fusion proteins were transfected into 293 cells and purified from serum free conditioned media as previously described (Fitzpatrick *et. al., FEBBS Lett.,* 431(1):102-6 (1998)). Soluble MMP-15V5His and sMMP-15V5His(E260A) protein was generated from transiently transfected 293 cell conditioned medium and purified using Ni-NTA agarose according to manufacturer's recommendations (Qiagen).

shRNA knockdown of MMP-15

Several different shRNAs were evaluated for the ability to reduce MMP-15 protein levels when introduced into cells. The most effective shRNA directed against human MMP-15 was found to recognize bp 5'-CCACCATCTGACCTTTAGCTT-3' (SEQ ID NO. 18) corresponding to nt 1396-1414 (GENBANK™ accession number NM_002428). The RNAi to MMP-15 was introduced and into the BamH1-EcoRI sites pSIREN vector (BD Biosciences) as oligos:

5'-GATCCGCCACCATCTGACCTTTAGCTTCAAGAGAGCTAAAGGTCAGATGGTGGTTTTTTGCTAGCG-3' (SEQ ID NO. 19) and

5'-AATTCGCTAGCAAAAAACCACCATCTGACCTTTAGCTCTCTTGAAGCTAAAGGTCAGATGGTGGCG-3' (SEQ ID NO. 20) which contain an internal hairpin to form a shRNA. A shRNA to MMP-25 was purchased from OPENBIOSYSTEMS™. The pSIREN shRNA construct(s) or vector alone were transiently transfected into SKBR-3 and BT 474 cells using AMAXA™ kit V following manufacturer's recommendations. After 48 hours, conditioned media was collected and assayed for shed HER2 by HER2 ELISA and the cells were lysed directly on the plate using 2xSample buffer. MMP-15, full length HER2, and p95 HER2 proteins were detected by Western blotting using a rabbit polyclonal anti-MMP-15 antibody (Labvision cat. no. RB-1546-P) or mouse monoclonal anti-HER2 antibody Ab-15 (Labvision, cat. no. MS-599-P0).

In Vitro **HER2 cleavage assay**

The ability of candidate proteases to cleave HER2 ECD was determined *in vitro* using purified gDHER2-Fc fusion protein as a substrate. The purified catalytic domains of MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-19), and MT5-MMP (MMP-25) were purchased from R&D systems. MMP purified proteins were mixed with gDHER2-Fc, or other HER-Fc proteins as described, in assay buffer (100mM Tris (pH=7.4), 100 mM NaCl, 2.5 μM $ZnCl_2$, 10 mM $CaCl_2$, 0.001% Brij35) in a ratio of 1:100, enzyme:substrate, and incubated at 37^{0}C for 20 minutes. Reactions were stopped using an equal volume of 2x Sample buffer (Invitrogen), and boiled for 5 min. prior to loading onto a 4-20% Tris-glycine gradient gel (Invitrogen). Proteins were visualized by staining with GEL CODE BLUE™ stain reagent (Pierce cat. no. 24592) following manufacturer's recommendations. The MMP cleavage site was determined by N-terminal protein sequencing by automated Edman degradation using an automated protein sequencer (Kishiyama, A., *Anal. Chem.* 72(21):5431-6 (2000)).

Immunoprecipitation assays and Western Blotting

Cos-7 cells were co-transfected with pRK5.Flag-HER2 and pcDNA3.MMP-15 constructs or pcDNA3.1 vector alone using LIPOFECTAMINE™ 2000. Cells were allowed to recover for 48 hrs. Transfected cells were rinsed with PBS and cells were ruptured in lysis buffer (1%TRITON X-100™, 50mMTris (pH=7.4), 150mM NaCl, 1mM PMSF, 10μg/ml leupeptin, 10U/ml aprotinin, and 2mM Na_2VO_4). Lysates were cleared of insoluble material by centrifugation and total protein levels were determined using a BCA protein assay kit (Pierce cat. no. 23229). Two-hundred micrograms of total cellular protein was added to lysis buffer to a final volume of 1 ml and Flag-HER2 was immunoprecipitated using anti-Flag M2-agarose (Sigma) or with polyclonal anti-MMP-15 antibody complexed to ProteinA/G agarose. Immune complexes were washed twice with lysis buffer and resuspended in SDS sample buffer and boiled. Samples were separated on a 4-12% Tris-glycine gradient gel (Invitrogen) and transferred to nitrocellulose membranes. Blots were blocked in 5%BSA/TBST and probed with antibodies to either MMP-15 or HER2 followed by a peroxidase conjugated anti-mouse or rabbit secondary antibody as described. Blots were developed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Fo5 and f2:1282 tumors were resuspended in lysis buffer with protease inhibitors and homogenized using a POLYTRON TISSUEMIZER™ (PT2100) on ice. Tumor lysates were cleared of insoluble material by centrifugation and total protein levels were determined using a BCA protein assay kit. HER2 was immunoprecipitated from 1 mg total protein from at least three independent tumor lysates using mouse monoclonal antibody Ab-15 (Labvision, cat. no. MS-599-P0) complexed to Protein A/G sepharaose overnight at 4^{0}C. Complexes were pelleted by centrifugation, washed twice with lysis buffer and resuspended in SDS sample buffer and boiled. Samples were separated on a 4-12% Tris-Glycine gel and transferred to nitrocellulose membranes. Blots were probed with mouse monoclonal antibody Ab-18 to detect phosphorylated HER2 (Labvision, cat. no. MS-1072-P) or with Ab-15 (Labvsion cat. no., MS-599-P0). Expression of MMP-14, MMP-15, and MMP-25 was evaluated by Western blotting from 50 ug of tumor lysate or from 50 ug from BT 474, or MCF-7, or SKBR-3 cellular lysate using rabbit polyclonal Ab-1 (Labvision cat. no. RB-1544-P) to detect MMP-14, rabbit polyclonal Ab-1 (Labvision cat. no. RB-1546-P) to detect MMP-15,or rabbit polyclonal Ab-1 (Oncogene Research Products cat. no. PC499) to detect MMP-25.

Activated MAPK (Cell Signaling Technology cat. no. 9101), PhosphoAkt Ser473 (Cell Signaling Technology cat. no. 9271), total Akt (Cell Signaling Technology cat. no. 9272), total Erk (Cell Signaling Technology cat. no. 9102, or phosphoHER3 (Cell Signaling Technology cat. no. 4791) was determined by Western blotting of 50 µg of cellular lysate.

Proliferation assays

Cells were seeded into 96-well dishes (Nunc) at a density of 10^4 cells per well in quadruplicate and allowed to adhere overnight. Cells were treated as indicated, or transfected as indicated. Following a 3 day incubation, 25µl of Alamar blue reagent (Trek Diagnostic Systems, cat. no. 00-100) was added to each well, and incubated for an additional 3 hours. Plates were read in a flourometer with excitation wavelength of 530nm and emission wavelength of 590nm according to manufacturer's recommendations. Proliferation relative to non-stimulated or control transfected cells was determined as previously described (Lewis *et al., Cancer Res.* 56(6):1457-65 (1996)).

RESULTS

The ECD of HER2 is proteolytically shed from breast carcinoma cells in culture and has been detected in the serum of patients with metastatic breast cancer, where it is associated with a poor clinical prognosis (Molina *et al. Cancer Res.* 61: 4744-4749 (2001)). However, the biological role of HER2 shedding is unclear. The experiments herein were carried out in order to identify the HER2 sheddase.

Identification of the HER2 sheddase may also be important as trastuzumab has previously been demonstrated to inhibit shedding in breast carcinoma cell lines (Molina *et al. Cancer Res.* 61:4744-4749 (2001)). Consistent with previously reported results, trastuzumab reduced shedding in the breast cancer cell lines BT 474 and SKBR-3 cells by more than 60% at a concentration of 10 µg/ml (Fig. 5, left panel) and reduced cellular proliferation in these cell lines by 50% (Fig. 5, right panel).

An animal model system where mammary tumors derived from a MMTVHER2 transgenic mouse (Finkle *et al. Clin. Cancer Res.* 10:2499-2511 (2004)) was used to identify the HER2 sheddase. This animal model system reproducibly shed different levels of HER2 into serum and exhibited differential sensitivity to trastuzumab (Fig. 6, right panel). This difference in the level of serum HER2 also correlated well with the presence of a protein fragment immunoprecipitated from three independent Fo5 tumor cell lysates having an apparent molecular weight of 95 kD, consistent with the size of p95 HER2 (Figure 6, lower left panel). This fragment is constitutively phosphorylated in the Fo5 tumor cell lysates, indicating that this fragment may have biological activity in these tumors. The increased levels of a HER2 sheddase may contribute to the trastuzumab resistance of this line, since the epitope of trastuzumab would be lost by proteolytic cleavage (Cho *et al. Nature* 421: 756-760 (2003)).

Since the Fo5 tumor line sheds higher levels of HER2 and has high levels of p95HER2 fragment, differential expression analysis was used as an approach to identify transcripts that are upregulated in Fo5 tumors relative to f2:1282 tumors. RNA was prepared from 3 individual tumor samples from either Fo5 tumors or f2:1282 tumors with an average tumor size of 300-600mm^3. RNA from each independent tumor sample was hybridized in triplicate to AFFYMETRIX™ mouse genome single array chip (MOE430P) and known gene array (MOE430A). A Mann-Whitney pairwise comparison was performed identifying 638 upregulated

transcripts in Fo5 tumor samples. Since BT 474 and SKBR-3 cell lines also shed HER2 ECD into conditioned medium (Fig. 5, left panel), this list was compared to transcripts that are expressed in both cell lines using the Genelogic Bioexpress database. The approach taken to identify the HER2 sheddase is illustrated in Fig. 7.

In order to narrow down the number of candidate genes, the general metalloprotease inhibitor GM6001 was used to evaluate whether MMP activity plays a role in regulating HER2 shedding. GM6001, but not its stereoisomer, dose dependently inhibited HER2 shedding in SKBR-3 cells as determined by a HER2 ECD ELISA (Fig. 8, left panel). This result is consistent with previously published reports that the general metalloprotease inhibitor BB-94 inhibited HER2 ECD shedding in breast carcinoma cell lines (Codony-Servat *et al. Cancer Res.* 59:1196-1201 (1999)). Timp-1, Timp-2 and Timp-3 are naturally occurring peptides that regulate metalloprotease activity (Overall and Lopez-Otin *Nature Reviews Cancer* 2:657-672 (2002)). Adding purified Timp-1, Timp-2, or Timp-3 to the conditioned media of SKBR-3 cells at a concentration of 1 μg/ml reduced HER2 ECD levels (Fig. 8, right panel). However, Timp-2 reduced HER2 ECD shedding the most efficiently (Fig. 8, right panel). Timp-2 is known to efficiently inhibit the MT-MMP subfamily of metalloproteases (Overall and Lopez-Otin *Nature Reviews Cancer* 2:657-672 (2002)). These data suggested that the protease responsible for HER2 shedding in SKBR-3 and BT 474 cells is a metalloprotease, significantly reducing the number of candidates identified from the bioinformatics screen.

Several members of the MT-MMP family are expressed in SKBR-3 and BT 474 cells based on the GENELOGIC™ database. Both MT1-MMP and MT2-MMP are also expressed in Fo5 tumors from the AFFYMETRIX™ microarray data. To determine if these transcripts are expressed, Fo5 and f2:1282 tumor and breast carcinoma cell line lysates were immunoblotted with polyclonal antibodies to these MT-MMPs that recognize both human and mouse proteins (Fig. 9). The cell lines all express MMP-14, MMP-15, and MMP-25, at different levels. While MMP-14 is expressed in both f2:1282 and Fo5 tumor cell lysates, MMP-15 is abundantly expressed in Fo5 tumors lysates. This result was consistent with the Mann-Whitney comparison which indicated a 2-fold higher level of mRNA expression of MMP-15 in Fo5 tumors. To verify that MMP-15 was expressed in the epithelial cells of the tumor, and not the surrounding stromal cells, Fo5 and f2:1282 tumors were sectioned and stained with either an anti-HER2 antibody (Dako) or an anti-MMP-15 antibody (Ab-1). Both f2:1282 and Fo5 tumors express human HER2, but MMP-15 is more abundantly expressed in the Fo5 tumors than the f2:1282 tumors.

Substrates for members of the MT-MMPs have traditionally been thought to be extracellular matrix proteins. A biochemical assay was set up to test candidates identified from the microarray data to cleave full length HER2 *in vitro*. This assay uses a purified recombinant fusion protein as a substrate for candidate proteases and consists of a N-terminally gD epitope-tagged HER2 ECD in-frame with the Fc heavy chain of human IgG. Several different proteins were used in these studies. gDHER2(+)-IgG contains amino acids 2-656 of HER2 ECD (GenBank accession AAA75493) and gDHER2(-)-IgG contains amino acids 2-626 of HER2 ECD. The gDHER2(+)-IgG recombinant protein contains juxtamembrane sequence that have previously been shown to contain a putative HER2 sheddase site (PA/EQR/ASP; SEQ ID NO. 23) while the gDHER2(-)-IgG protein lacks this sequence (Yuan *et al. Protein Expression and Purification* 29:217-222 (2003)). A truncated version gDHER2(+)-IgG was also used in this assay, gDHER2(DIV)-IgG, which has only domain IV (DIV) of HER2 in-frame with human Fc. Purified catalytic domains of MMP-14, MMP-15, MMP-16, MMP-19, and MMP-25 were incubated with gDHER2(DIV)-IgG for 20 minutes at 37^{0}C. All MT-MMPs except MMP-14

efficiently cleave gDHER2(DIV)-IgG substrate (Fig. 13, left panel). The cleaved protein products were excised and N-terminally sequenced to identify the cleavage site. All four MT-MMPs cleaved HER2 near the published sequence site (Yuan *et al. Protein Expression and Purification* 29: 217-222 (2003)) (Fig. 13, right panel).

HER2-Fc fusion proteins that have the sequence PINCTHSCVDLDDKGCPAEQRASPASPLTSIV (SEQ ID NO. 21) are substrates for these four MMPs *in vitro* (Fig. 14). This data suggest that HER2 is a substrate for MMP-15.

MT-MMP substrate recognition may be influenced by the hemopexin domain (Overall and Lopez-Otin *Nature Reviews Cancer* 2:657-672 (2002)). A soluble form of MMP-15 with a C-terminal V5 His epitope was transiently expressed in 293 cells and purified from 293 conditioned media by affinity chromatography. As shown in Fig. 15, this soluble form of MMP-15 can also cleave gDHER2(+)-IgG in the *in vitro* sheddase assay.

To determine if MMP-15 and HER2 can associate in the membrane, co-immunoprecipitation assays were performed using transiently transfected Cos-7 cells. pRK5.FlagHER2 was co-transfected with either vector, pcDNA3.MMP-15V5His, pcDNA3.sMMP-15V5His, or pcDNA3.MMP-15(E260A). FlagHER2 was immunoprecipitated with an anti-FLAG resin. Fig. 10 shows that both soluble MMP-15 and a catalytically dead (E260A) mutant can associate with Flag-HER2. This is consistent with the notion that MMP-15 can cleave HER2 in membranes since the wild-type version of the protease, pcDNA3.MMP-15V5His, would remove the FlagHER2 ECD and a HER2-MMP-15 complex would not be co-immunoprecipitated.

Somatic point mutations and deletions and splice variants have been identified mammary tumors from MMTVHER2 transgenic animals (Siegel *et al. EMBO J.* 18:2149-2164 (1999)); and Finkle *et al. Clin. Cancer Res.* 10:2499-2511 (2004)). These mutations most frequently occur in the HER2 extracellular domain near the transmembrane domain. The Fo5 tumor line has a five amino acid deletion DLDDK (SEQ ID NO. 22) that is adjacent to the HER2 cleavage site while the f2:1282 tumor line has a single point mutation C for R (Fig. 12). These mutations do not influence MMP-15 association (Fig. 11). However, it is possible that the proximity of the DLDDK (SEQ ID NO. 22) deletion to the HER2 MMP cleavage site may influence enzymatic activity of the protease.

RNA inhibition (RNAi) is a method that can downregulate target transcript and protein levels. To examine the biological role of MMP-15 in SKBR-3 and BT 474 cells, an anti-MMP-15 RNAi construct was used. When introduced into SKBR-3 or BT 474 cells, this shRNAi expression plasmid reduced endogenous protein levels of MMP-15 in both cell types (Fig. 17, left upper panel). Transfection of this shRNA also reduced the amount of p95 HER2 in both cell lines (Fig. 17, left lower panel). Loss of MMP-15 protein in these cells significantly reduced HER2 ECD shedding in both lines (Fig. 17, right panel). However, no significant reduction in HER2 ECD shedding was observed when a shRNAi to MMP-25 was introduced into either cell line (Fig. 17, right panel). These experiments strongly suggest that loss of MMP-15 dramatically effects HER2 ECD shedding.

Reducing MMP-15 protein levels by RNAi also has an effect on the growth rates of SKBR-3 and BT 474 cells (Fig. 18, left panel). This effect is also duplicated by a shRNAi to MMP-25. This suggests that reducing HER2 ECD shedding also downregulates the amount of p95 HER2 in BT 474 and SKBR-3 cells, thereby reducing cell growth rates. When a gD-tagged p95 HER2 construct was introduced into these cell lines, an increase in cell growth rates was detected in both cell types relative to control transfected cells (Fig. 18, left panel). This construct is constituively phosphorylated and activates MAPK when introduced into Cos-7 cells in

a ligand-independent manner (Fig. 16). However, this construct must still heterodimerize with HER3 or EGFR in order to activate Akt signaling pathways consistent with a previously published study (Xia *et al. Oncogene* 23:646-653 (2004)). Overexpression of gDp95HER2 in SKBR-3 cells did not significantly inhibit trastuzumab-mediated growth inhibition in these cells (Fig. 18, right panel).

A pharmacological approach was used to determine if MMP activity plays a role in regulating HER2 shedding and p95 HER2 levels in Fo5 tumors. FVB mice with Fo5 tumors with average size of 400-600 mm^3 were injected with GM6001 intraperitoneally or control vehicle daily over the course of three days. On day four, serum was collected and assayed for HER2 ECD by ELISA, and tumors collected and weighed. HER2 was immunoprecipitated from tumor cell lysates and assayed for p95 HER2 levels by Western blotting. GM6001 treated animals showed a significant reduction in the amount of p95 HER2 as compared to control animals (Fig. 19, right panel). Serum HER2 ECD levels were reduced in both vehicle and GM6001 treated animals (Fig. 19, left panel), however, a greater reduction was observed in GM6001 treated animals.

SUMMARY

The experiments above demonstrate that the matrix metalloprotease MMP-15 cleaves HER2 *in vitro* at a site that is consistent with purified shed ECD from SKBR3 cells, and interacts with full length HER2. Reducing levels of this MMP correlates well with reduced HER2 receptor shedding, and decreases basal proliferation levels in SKBR3 and BT474 cell lines. Trastuzumab appears to inhibit HER2 receptor shedding by indirectly regulating MMP-15 activity, but does not compete with MMP-15 for substrate binding and cleavage. Reducing HER2 shedding with a MMP antagonist, such as an MMP-15 antagonist, represents a therapeutic approach for treating cancer, including trastuzumab-resistant cancer.

What is claimed is:

1. A method for inhibiting HER2 shedding comprising treating a HER2 expressing cell with a matrix metalloprotease (MMP) antagonist in an amount effective to inhibit HER2 shedding.
2. The method of claim 1 wherein the MMP antagonist is a membrane-tethered MMP (MT-MMP) antagonist.
3. The method of claim 2 wherein the MT-MMP is selected from the group consisting of MMP-15 (MT2-MMP), MMP-16 (MT3-MMP), MMP-24 (MT5-MMP), MMP-17 (MT4-MMP), and MMP-25 (MT6-MMP).
4. The method of claim 3 wherein the MT-MMP is MMP-15.
5. The method of claim 1 wherein the cell displays HER2 overexpression, amplification, or activation.
6. The method of claim 5 wherein the cell displays HER2 overexpression or amplification.
7. The method of claim 1 further comprising treating the cell with a HER inhibitor.
8. The method of claim 7 wherein the HER inhibitor is a HER2 antibody.
9. The method of claim 8 wherein the HER2 antibody is trastuzumab or pertuzumab.
10. The method of claim 7 wherein the HER inhibitor is selected from the group consisting of trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, and TAK165.
11. A method for reducing HER2 extracellular domain (ECD) serum level in a mammal, comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to reduce the HER2 ECD serum level in the mammal.
12. The method of claim 11 wherein the mammal has an elevated MMP level.
13. A method for treating cancer in a mammal comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to treat the cancer.
14. The method of claim 13 wherein the cancer displays HER expression, amplification, or activation.
15. The method of claim 14 wherein the cancer displays HER2 overexpression or amplification.
16. The method of claim 13 wherein the mammal has an elevated shed HER2 serum level or elevated p95 HER2 level.
17. A method for treating a HER inhibitor-resistant cancer in a mammal comprising administering to the mammal a matrix metalloprotease (MMP) antagonist in an amount effective to treat the cancer.
18. The method of claim 17 wherein the HER inhibitor is trastuzumab.
19. A method for reducing p95 HER2 level in a cell comprising exposing the cell to a matrix metalloprotease (MMP) antagonist in an amount effective to reduce the p95 HER2 level.
20. A method of diagnosis comprising evaluating MMP-15 (MT2-MMP) in a sample from a cancer patient, wherein elevated MMP-15 level or activity indicates the patient has an elevated p95 HER2 or shed HER2 serum level, or will have a poor clinical outcome.
21. The method of claim 20 wherein an elevated MMP-15 level indicates the patient will have a poor clinical outcome.

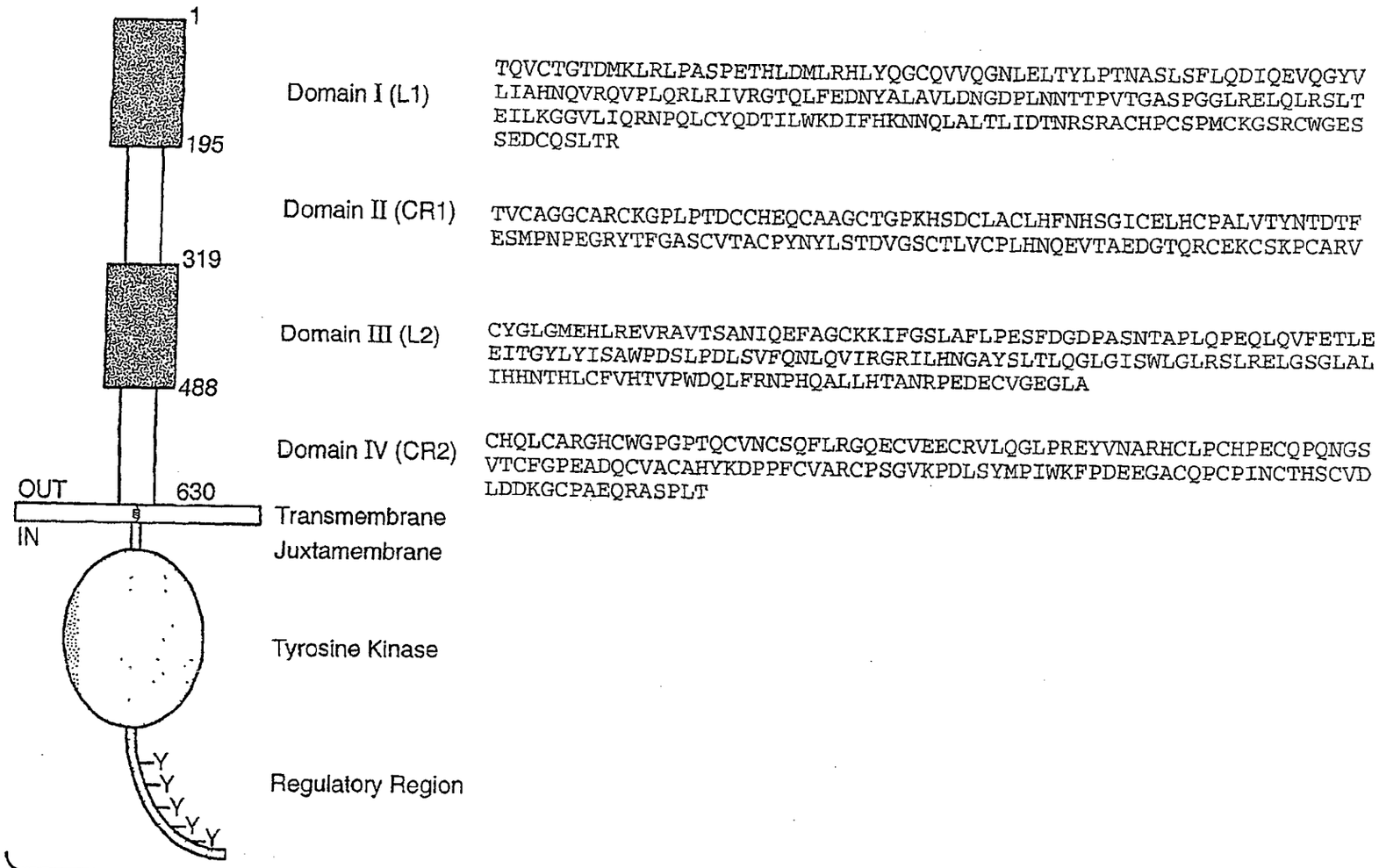

1
195
319
488
630
OUT
IN
Domain I (L1)
TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQGYV
LIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLT
EILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGES
SEDCQSLTR
Domain II (CR1)
TVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTF
ESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARV
Domain III (L2)
CYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLE
EITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLAL
IHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLA
Domain IV (CR2)
CHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGS
VTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVD
LDDKGCPAEQRASPLT
Transmembrane
Juxtamembrane
Tyrosine Kinase
Regulatory Region
Y
Y
Y
Y
Y

FIG. 1

Light Chain

```
1   D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q D V N T A V A W Y Q Q K P G K A P K
46  L L I Y S A S F L Y S G V P S R F S G S R S G T D F T L T I S S L Q P E D F A T Y Y C Q Q
91  H Y T T P P T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L
136 L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T
181 L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
```

FIG. 2A

Heavy Chain

```
1                               15                              30                              45
E V Q L V E S G G G L V Q P G G S L R L S C A A S G F N I K D T Y I H W V R Q A P G K G L

46                              60                              75                              90
E W V A R I Y P T N G Y T R Y A D S V K G R F T I S A D T S K N T A Y L Q M N S L R A E D

91                              105                             120                             135
T A V Y Y C S R W G G D G F Y A M D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S

136                             150                             165                             180
K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S

181                             195                             210                             225
G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K

226                             240                             255                             270
T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S

271                             285                             300                             315
H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D

316                             330                             345                             360
W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E

361                             375                             390                             405
M T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G

406                             420                             435                             449
S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
```

FIG. 2B

Amino Acid Sequence for Pertuzumab Light Chain

```
1         10        20        30        40        50        60
|         |         |         |         |         |         |
DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRYTGVPS

          70        80        90        100       110       120
          |         |         |         |         |         |
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVAAPSVFIFPP

          130       140       150       160       170       180
          |         |         |         |         |         |
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT

          190       200       210
          |         |         |
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
```

FIG. 3A

Amino Acid Sequence for Pertuzumab Heavy Chain

```
1         10        20        30        40        50        60
|         |         |         |         |         |         |
EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNPNSGGSIY

          70        80        90        100       110       120
          |         |         |         |         |         |
NQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFDYWGQGTLVTVSSA

          130       140       150       160       170       180
          |         |         |         |         |         |
STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG

          190       200       210       220       230       240
          |         |         |         |         |         |
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGP

          250       260       270       280       290       300
          |         |         |         |         |         *|
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS

          310       320       330       340       350       360
          |         |         |         |         |         |
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM

          370       380       390       400       410       420
          |         |         |         |         |         |
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ

          430       440       448
          |         |         |
QGNVFSCSVMHEALHNHYTQKSLSLSPG
```

FIG. 3B

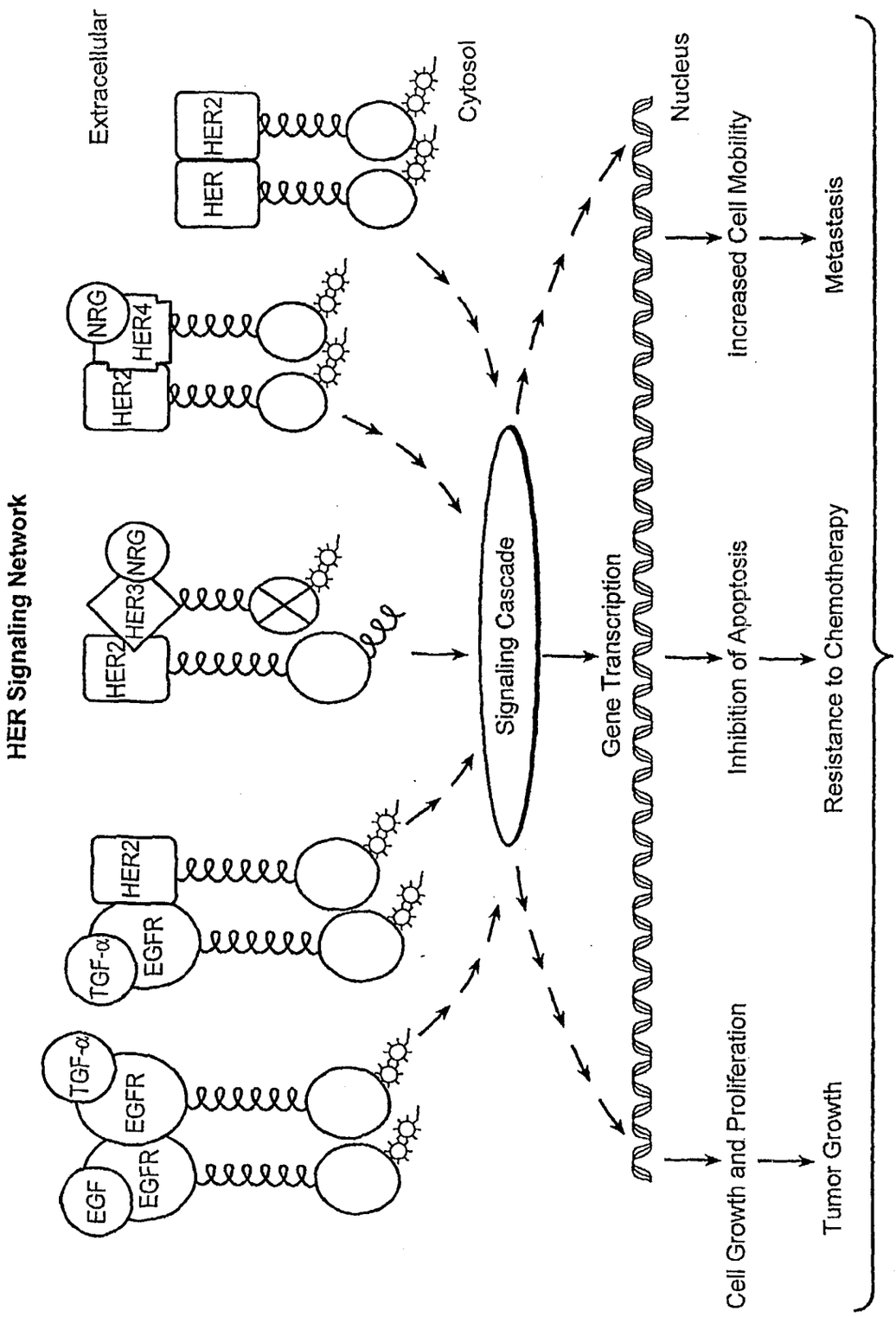

HER Signaling Network
Extracellular
Cytosol
HER
HER2
NRG
HER4
HER2
HER2
HER3
NRG
HER2
TGF-α
EGFR
EGF
EGFR
Signaling Cascade
Gene Transcription
Nucleus
Cell Growth and Proliferation
Inhibition of Apoptosis
increased Cell Mobility
Tumor Growth
Resistance to Chemotherapy
Metastasis

FIG. 4

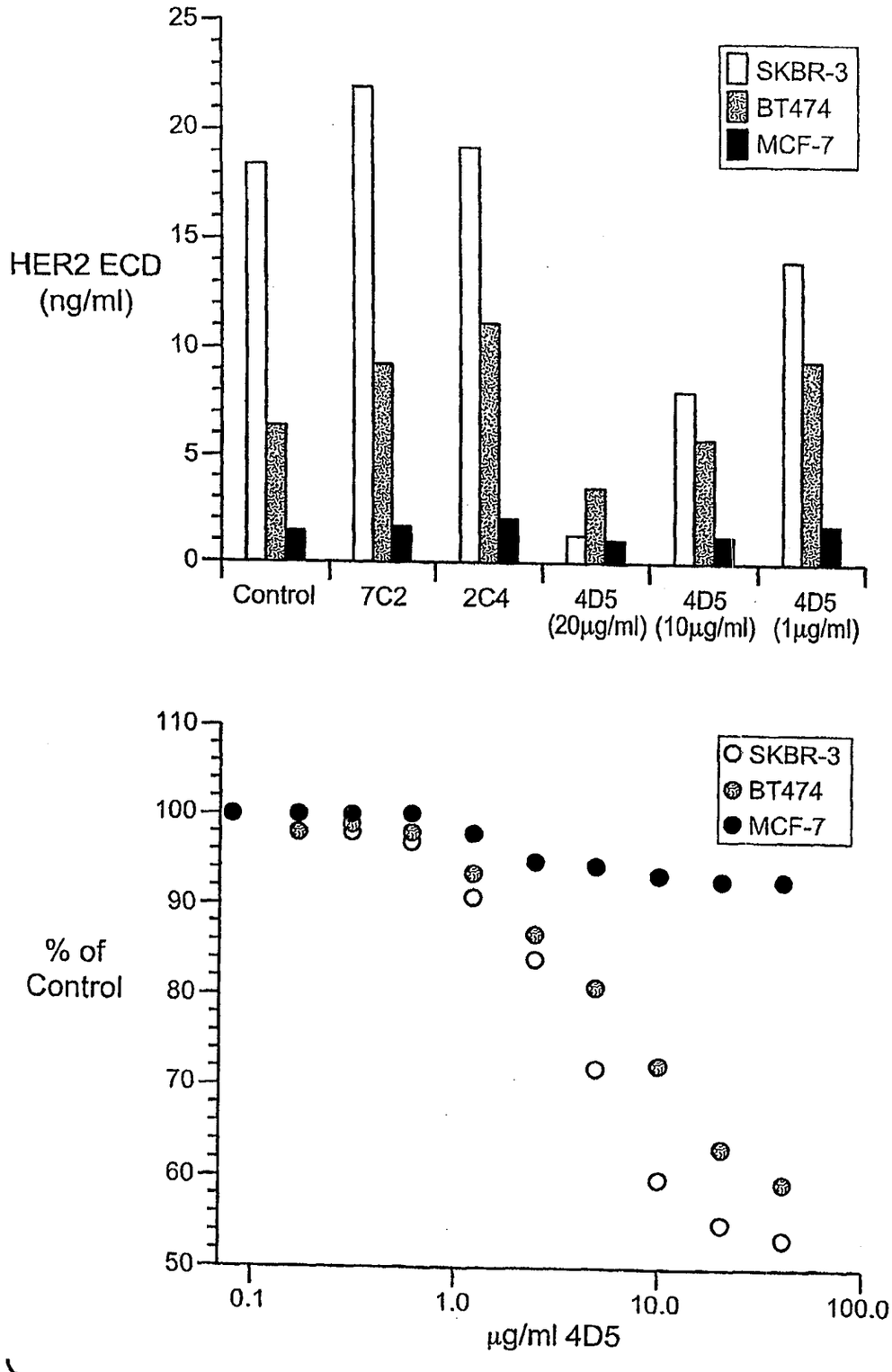

HER2 ECD (ng/ml)
25
20
15
10
5
0
SKBR-3
BT474
MCF-7
Control
7C2
2C4
4D5 (20µg/ml)
4D5 (10µg/ml)
4D5 (1µg/ml)
% of Control
110
100
90
80
70
60
50
0.1
1.0
10.0
100.0
µg/ml 4D5

FIG. 5

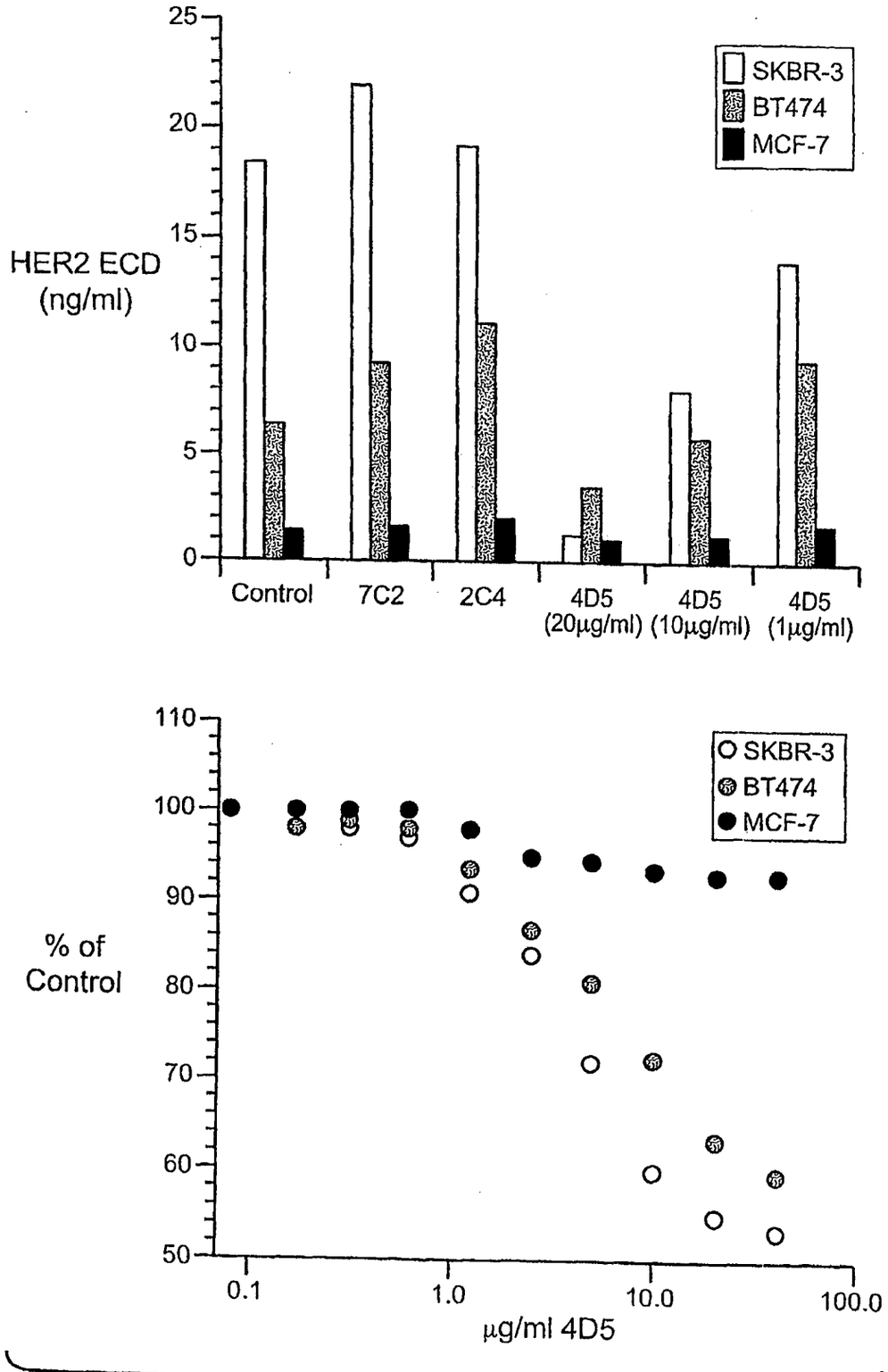
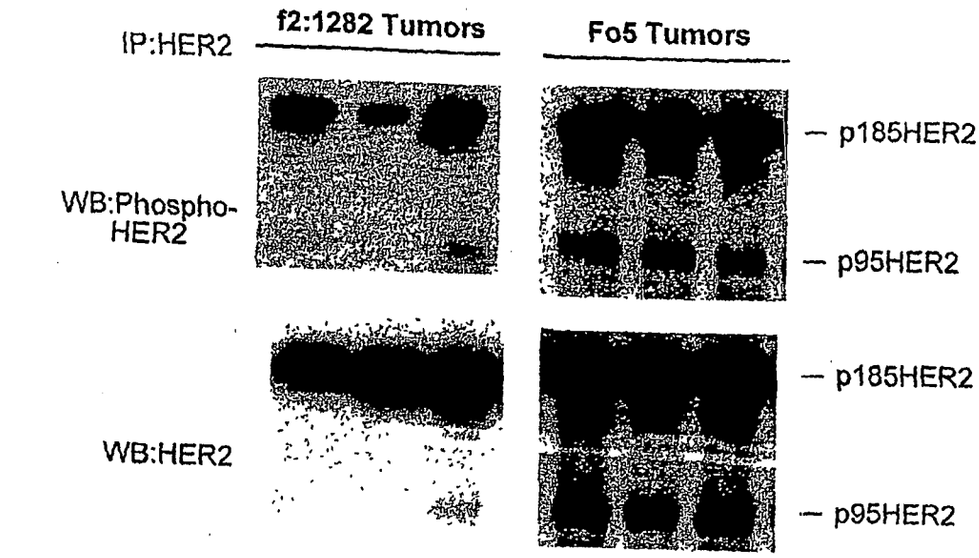

HER2 ECD (ng/ml)
25
20
15
10
5
0
SKBR-3
BT474
MCF-7
Control
7C2
2C4
4D5 (20µg/ml)
4D5 (10µg/ml)
4D5 (1µg/ml)
% of Control
110
100
90
80
70
60
50
0.1
1.0
10.0
100.0
µg/ml 4D5

FIG. 5


IP:HER2
f2:1282 Tumors
Fo5 Tumors
WB:Phospho-HER2
p185HER2
p95HER2
WB:HER2
p185HER2
p95HER2

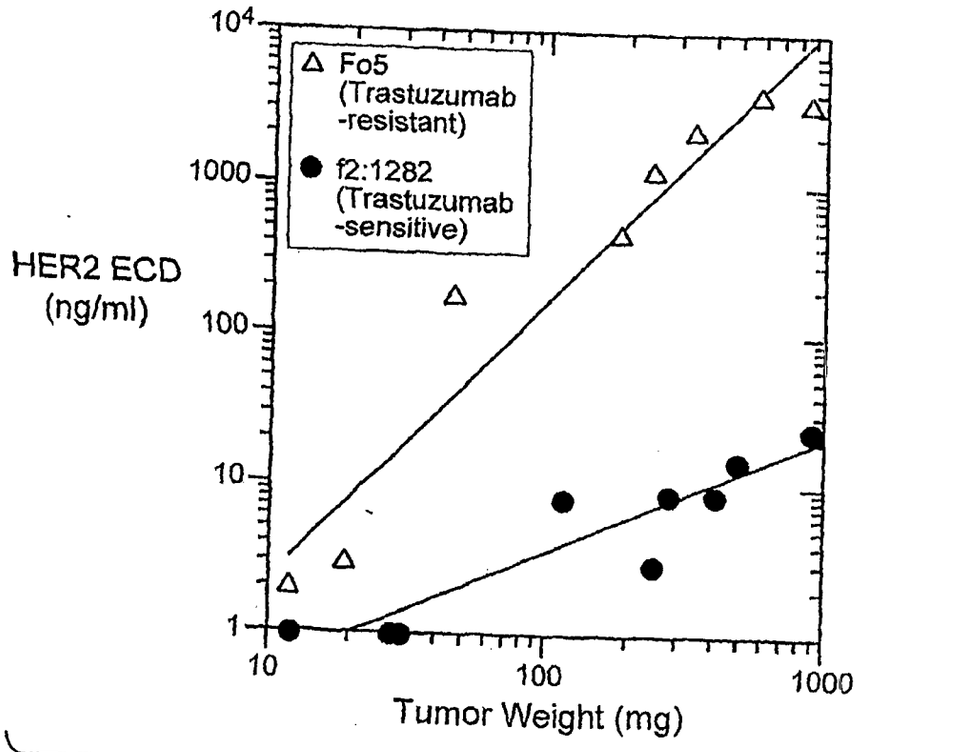
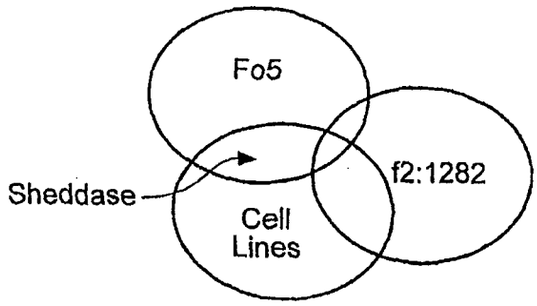

HER2 ECD (ng/ml)
Fo5 (Trastuzumab -resistant)
f2:1282 (Trastuzumab -sensitive)
10⁴
1000
100
10
1
10
100
1000
Tumor Weight (mg)

FIG. 6

Gene Chip Data and GENELOGIC™ Data



Conditions for Screen

Protease must be expressed in each subset
Upregulated in Fo5 tumors vs. f2:1282
Expressed in cell lines that shed HER2 ECD

FIG. 7

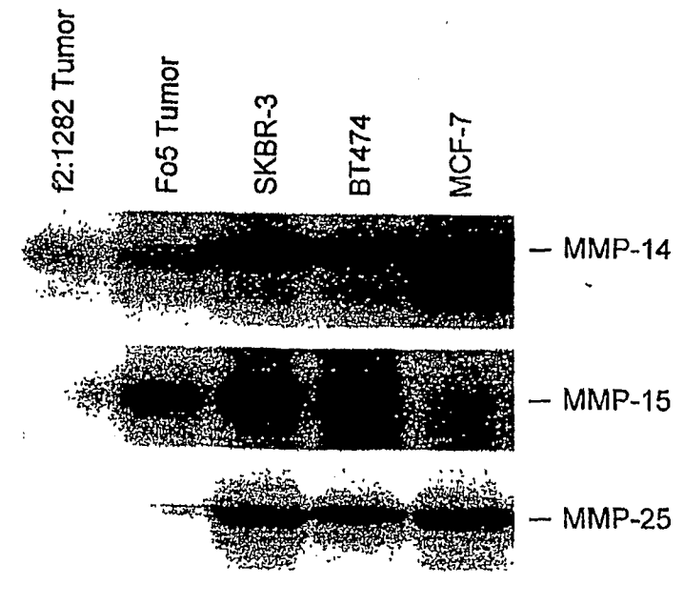
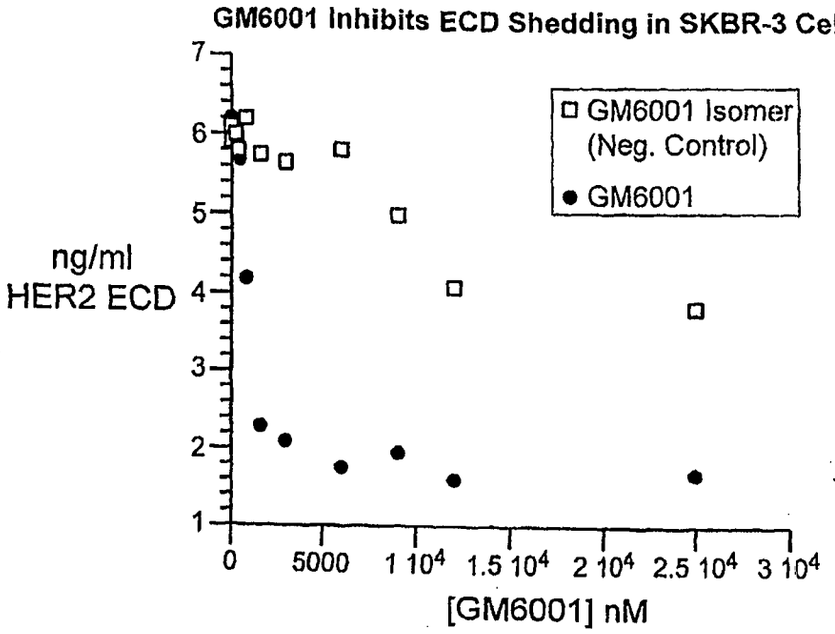
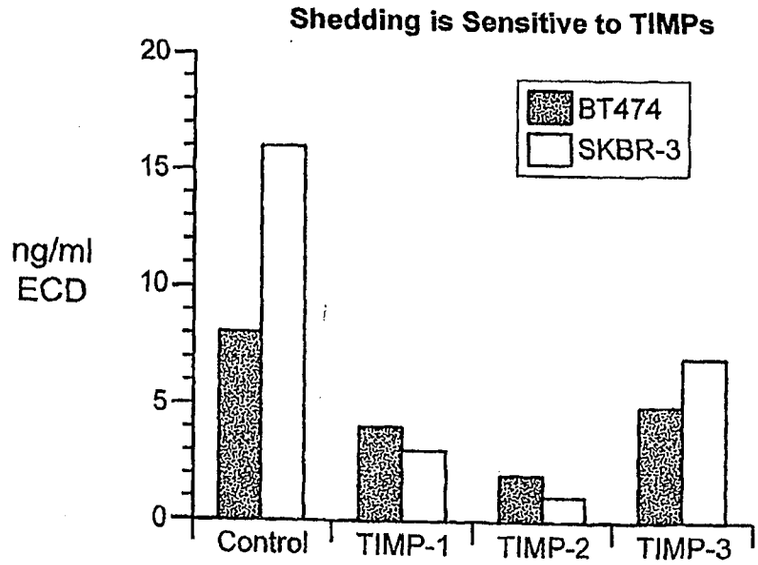


FIG. 9


GM6001 Inhibits ECD Shedding in SKBR-3 Cells
ng/ml HER2 ECD
GM6001 Isomer (Neg. Control)
GM6001
[GM6001] nM


Shedding is Sensitive to TIMPs
ng/ml ECD
BT474
SKBR-3
Control
TIMP-1
TIMP-2
TIMP-3


Potential Candidates from Screen:
MT2-MMP (MMP-15)
Control
Pervanadate
TIMP-1
TIMP-2
HER2ECD

FIG. 8

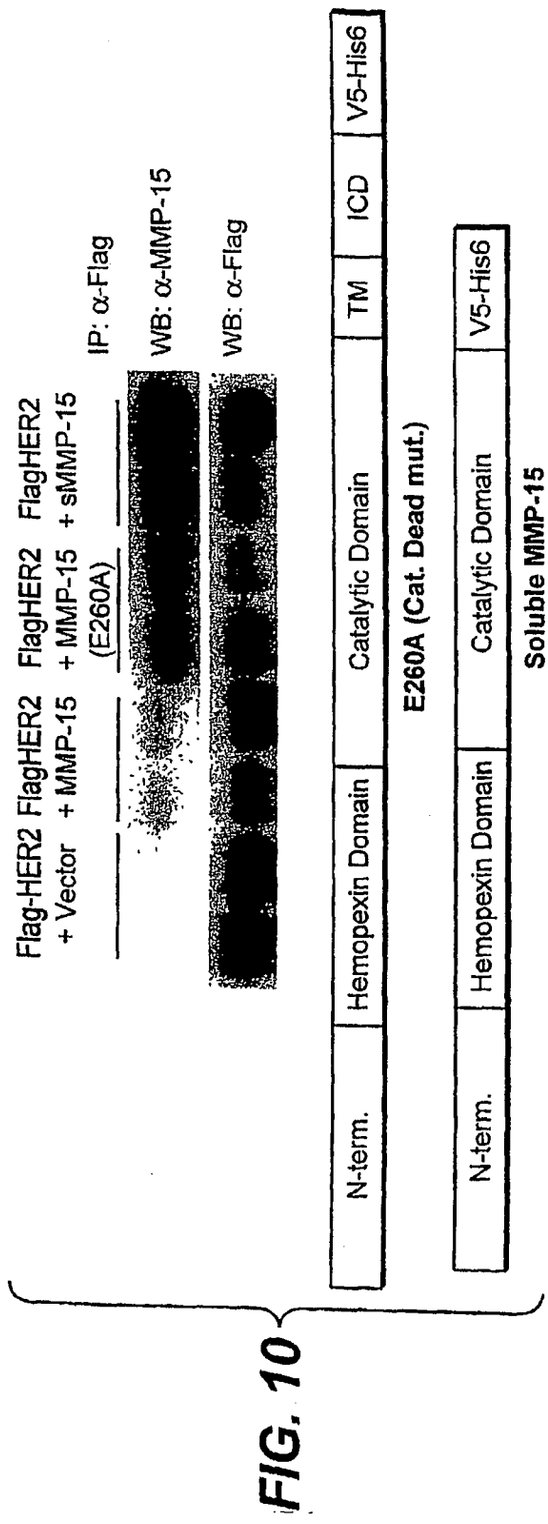

Flag-HER2 + Vector
FlagHER2 + MMP-15
FlagHER2 + MMP-15 (E260A)
FlagHER2 + sMMP-15
IP: α-Flag
WB: α-MMP-15
WB: α-Flag
N-term.
Hemopexin Domain
Catalytic Domain
TM
ICD
V5-His6
E260A (Cat. Dead mut.)
N-term.
Hemopexin Domain
Catalytic Domain
V5-His6
Soluble MMP-15

FIG. 10

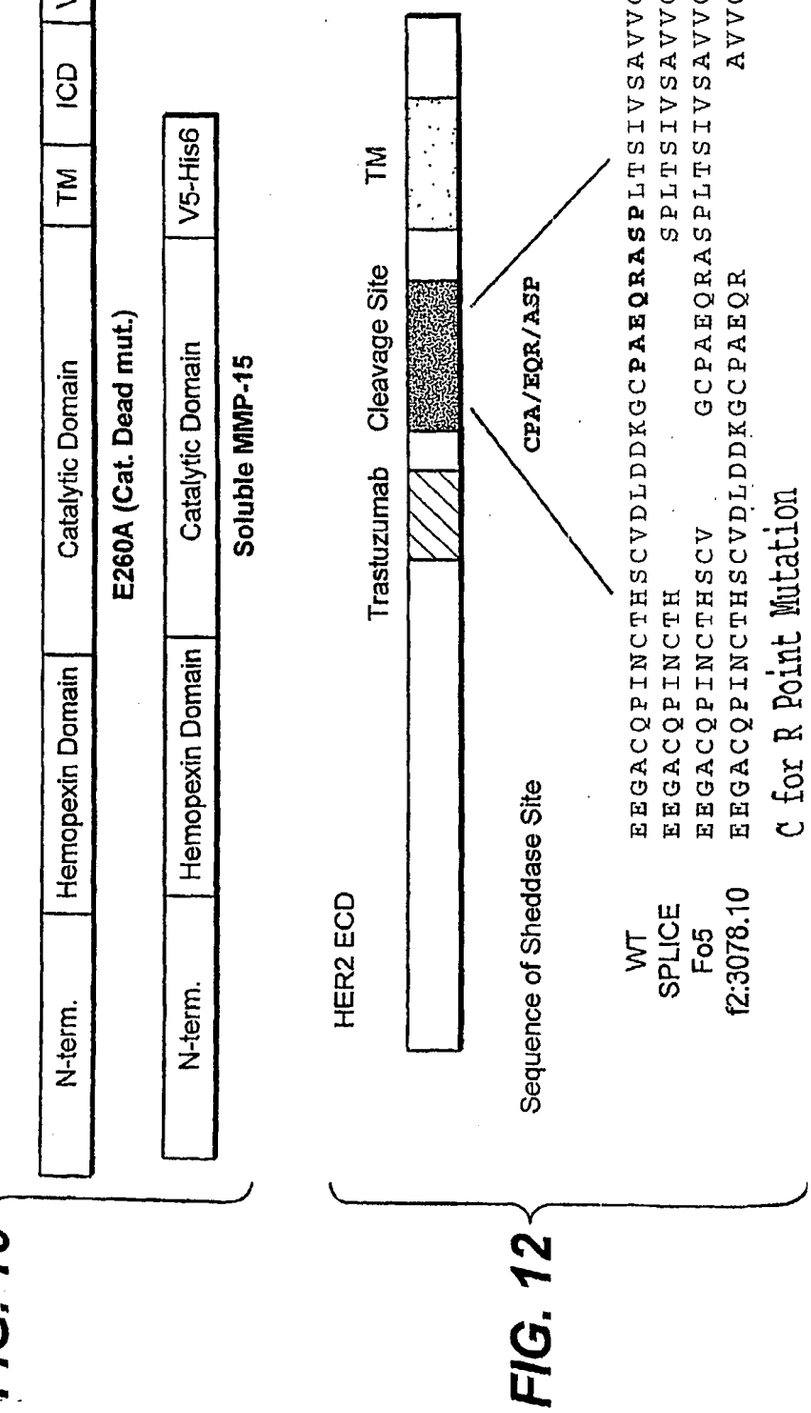

HER2 ECD
Trastuzumab
Cleavage Site
TM
Sequence of Sheddase Site
CPA/EQR/ASP
WT EEGACQPINCTHSCVDLDDKGCPAEQRASPLTSIVSAVVGIL
SPLICE Fo5 EEGACQPINCTH SPLTSIVSAVVGIL
f2:3078.10 EEGACQPINCTHSCV GCPAEQRASPLTSIVSAVVGIL
EEGACQPINCTHSCVDLDDKGCPAEQR AVVGIL
C for R Point Mutation

FIG. 12

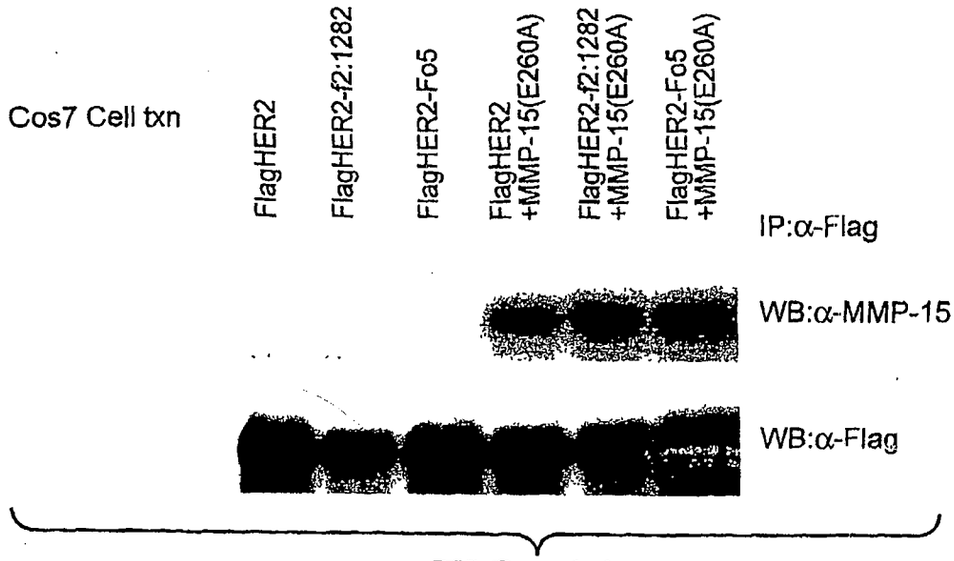
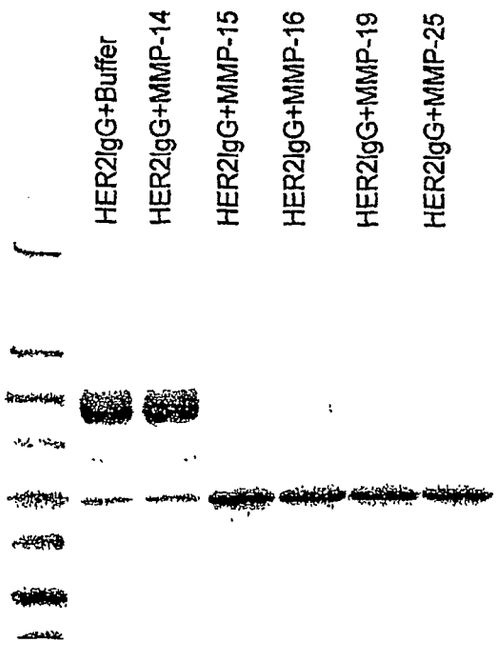


FIG. 11



SEQUENCE OF PROSTEASE DIGESTS
cleaved substrate is doublet of:

IgG

1) MMP-15: N-LTSIVSTRDKTHTCPP...
MMP-16: N-LTSIVSTRDKTHTCPP...
MMP-19: N-LTSIVSTRDKTHTCPP...
MMP-25: N-LTSIVSTRDKTHTCPP...

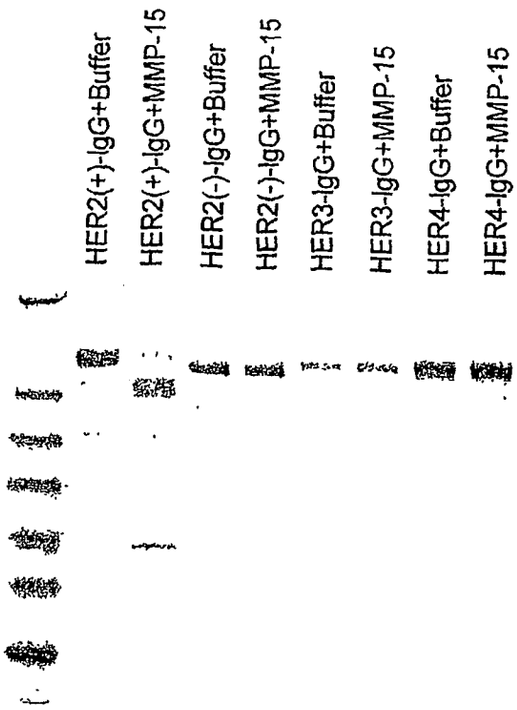
gD-tag

2) ALL Other: N-KYALADAS...

HER2ECD C-term Site TM

CPINCTHSCVDLDDKGCPA/EQR/ASPLTSIVSAVVGILLVVV

FIG. 13



Sequence Variation in HER2 Family Near TM Domain

TM

HER2: ...DLDDKGC**PAEQRASP**LTSIVSAVVGIL..
EGFR: PTNGPKIPS
HER3: CLGQTLVLIGKTLT
HER4(Jma): CIYYPWTGHSTLPQHART
HER4(Jmb): CIGLMDRT...

FIG. 14

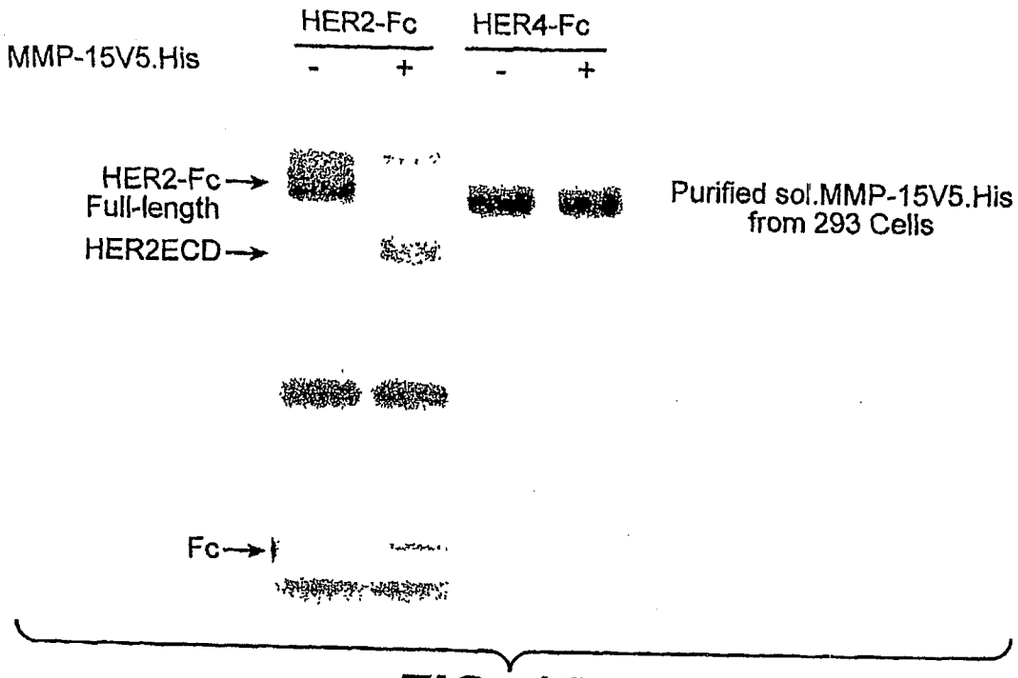

MMP-15V5.His
HER2-Fc
HER4-Fc
- + - +
HER2-Fc
Full-length
HER2ECD
Fc
Purified sol.MMP-15V5.His
from 293 Cells

FIG. 15

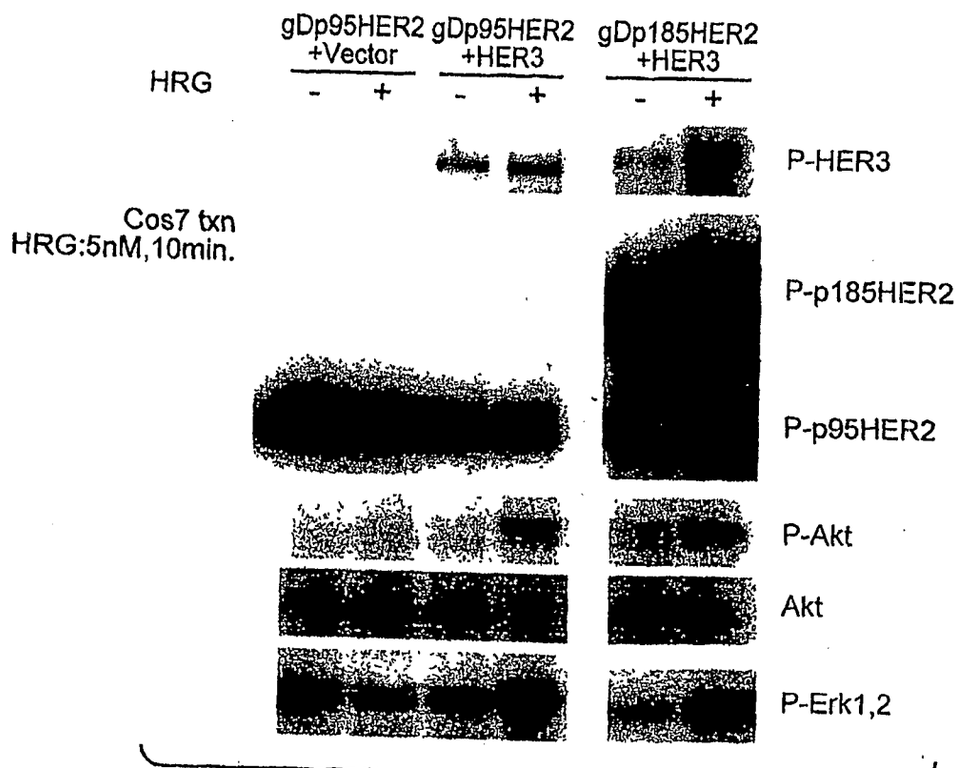

HRG
gDp95HER2
+Vector
gDp95HER2
+HER3
gDp185HER2
+HER3
- + - + - +
Cos7 txn
HRG:5nM,10min.
P-HER3
P-p185HER2
P-p95HER2
P-Akt
Akt
P-Erk1,2

FIG. 16

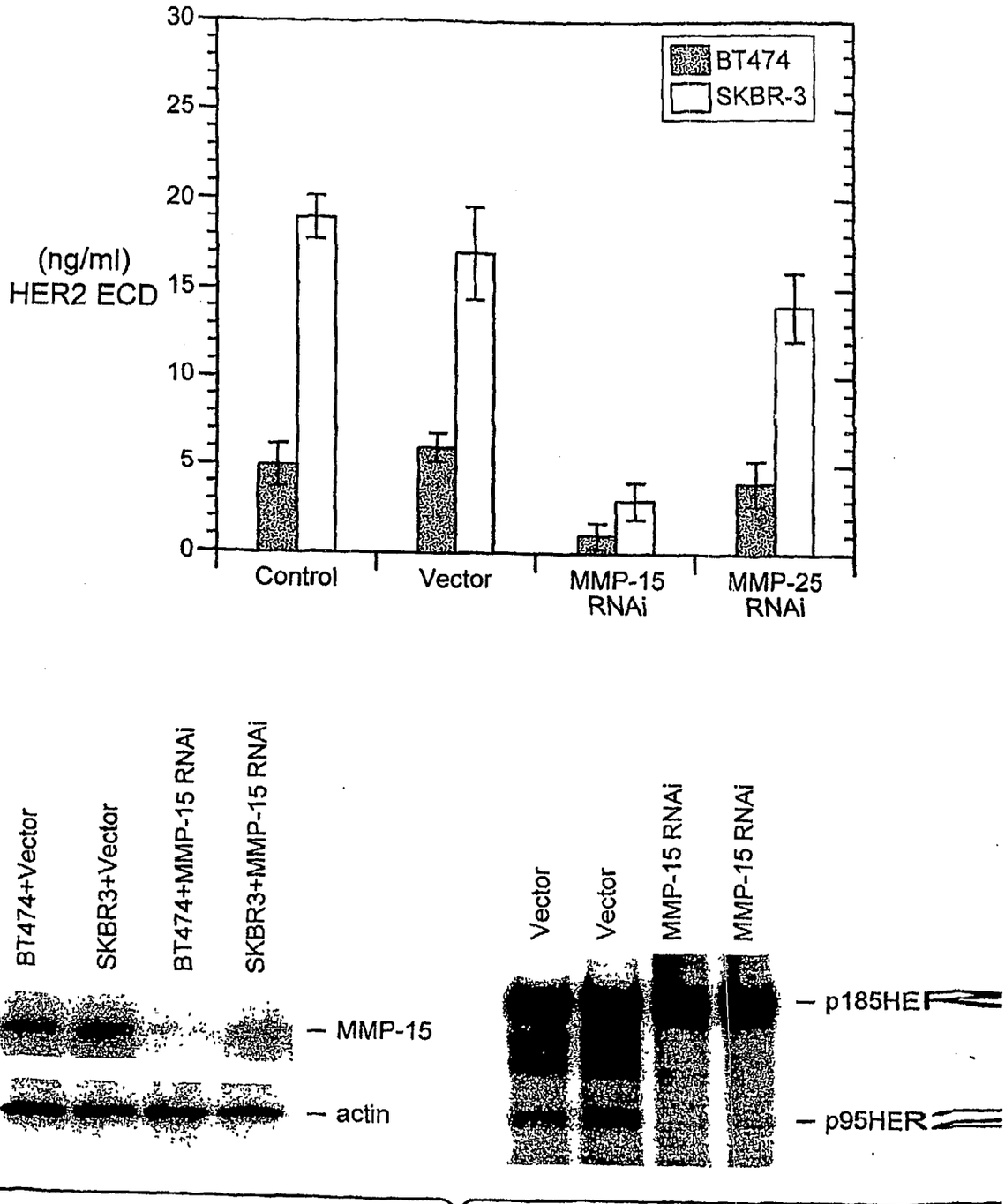
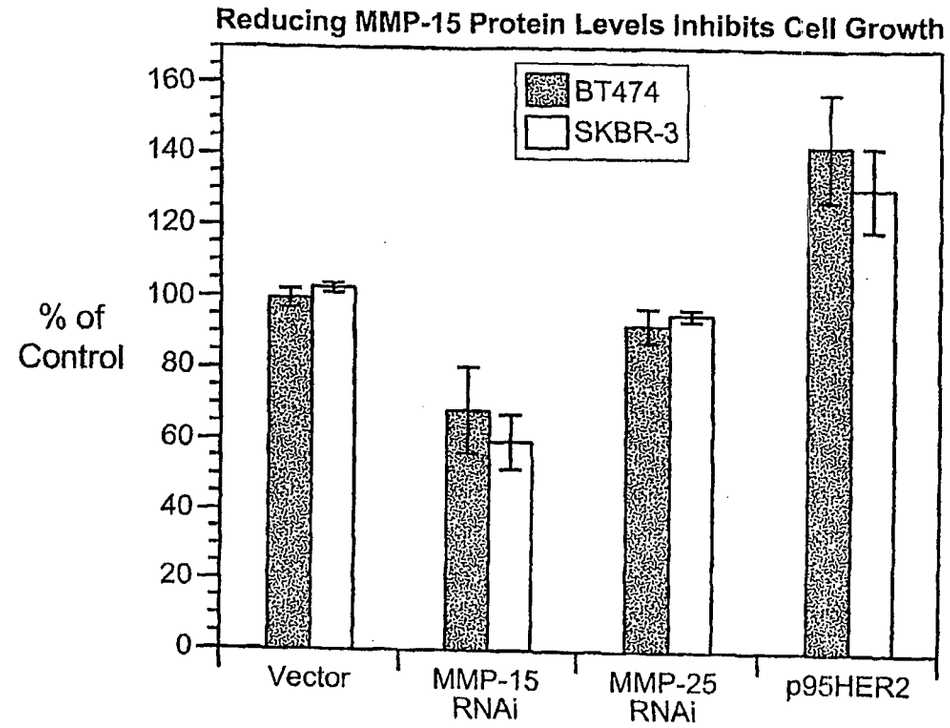

30
25
20
15
10
5
0
(ng/ml)
HER2 ECD
BT474
SKBR-3
Control
Vector
MMP-15 RNAi
MMP-25 RNAi
BT474+Vector
SKBR3+Vector
BT474+MMP-15 RNAi
SKBR3+MMP-15 RNAi
MMP-15
actin
Vector
Vector
MMP-15 RNAi
MMP-15 RNAi
p185HER
p95HER

FIG. 17


Reducing MMP-15 Protein Levels Inhibits Cell Growth
BT474
SKBR-3
% of Control
160
140
120
100
80
60
40
20
0
Vector
MMP-15 RNAi
MMP-25 RNAi
p95HER2

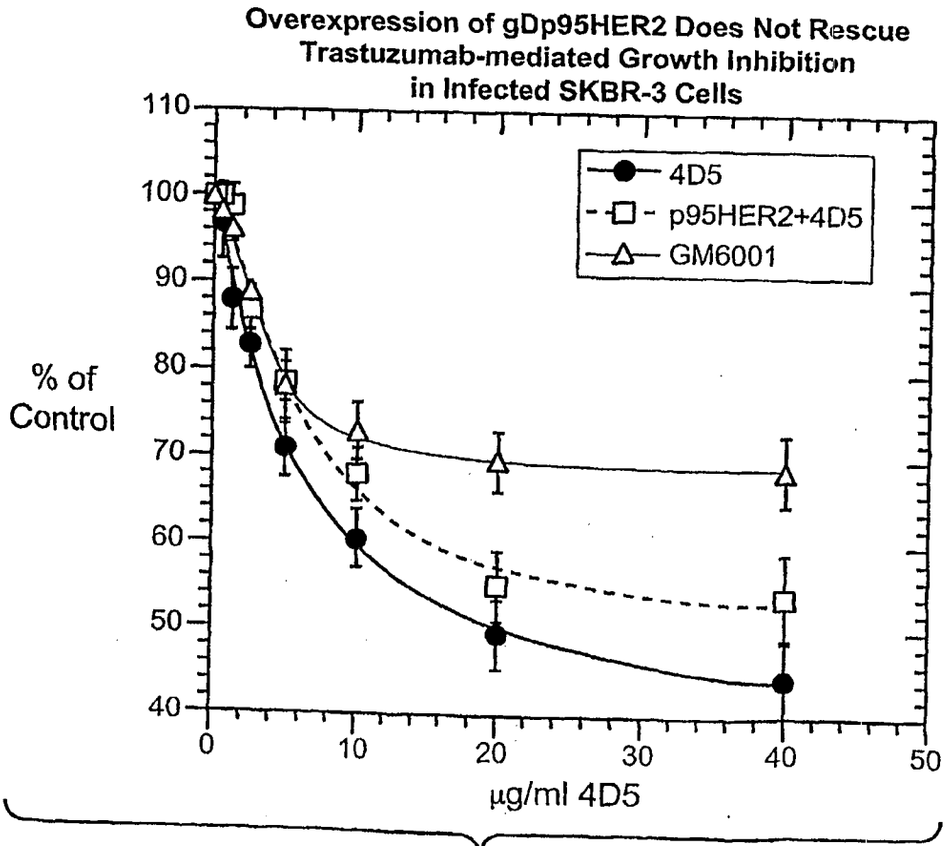
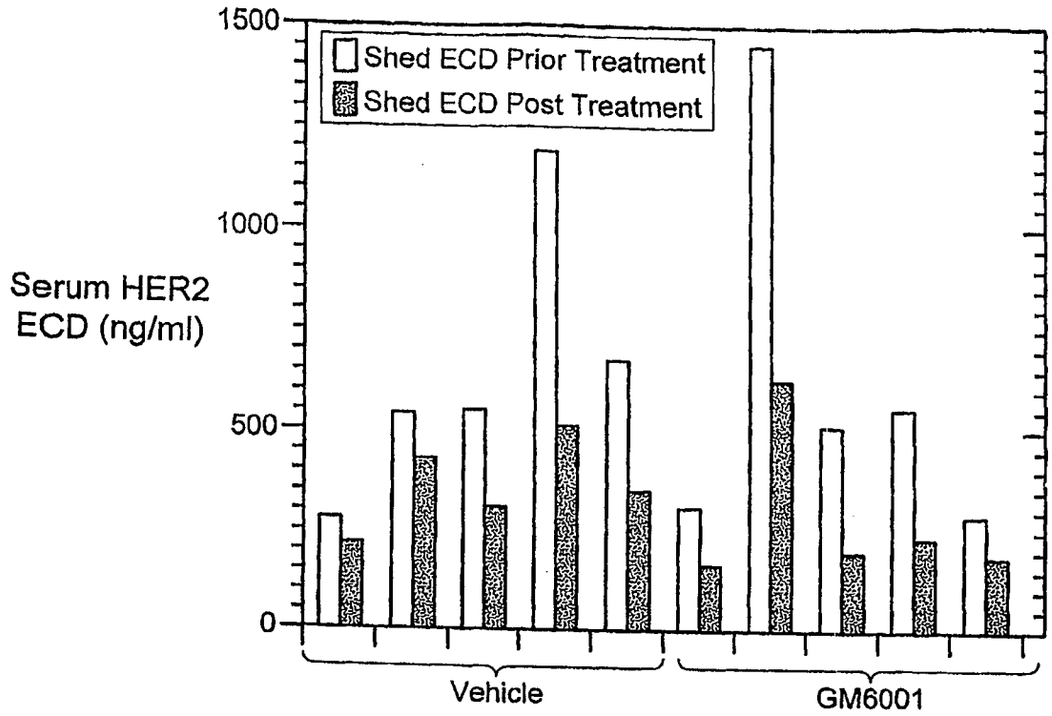

Overexpression of gDp95HER2 Does Not Rescue Trastuzumab-mediated Growth Inhibition in Infected SKBR-3 Cells
4D5
p95HER2+4D5
GM6001
% of Control
110
100
90
80
70
60
50
40
0
10
20
30
40
50
µg/ml 4D5

FIG. 18


1500
1000
500
0
Serum HER2 ECD (ng/ml)
Shed ECD Prior Treatment
Shed ECD Post Treatment
Vehicle
GM6001

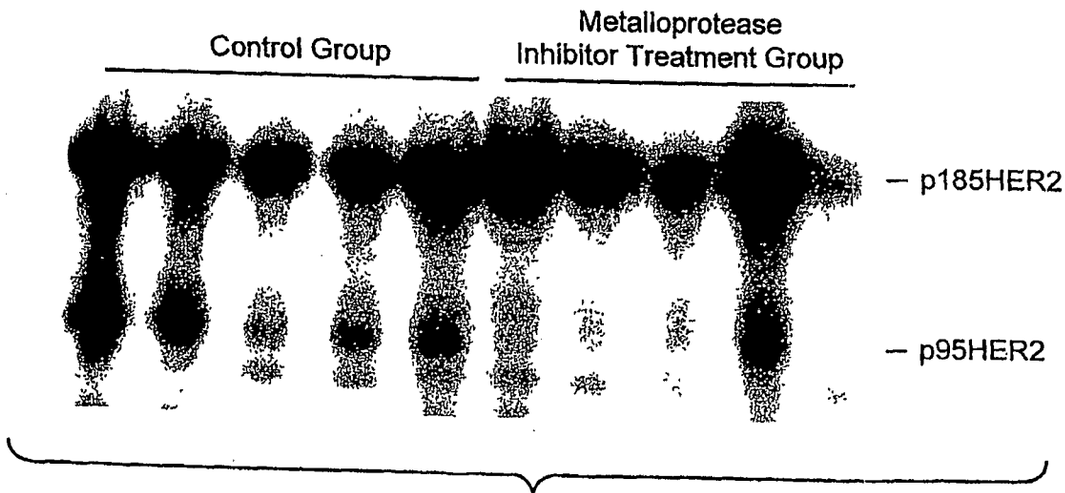

Control Group
Metalloprotease Inhibitor Treatment Group
p185HER2
p95HER2

FIG. 19

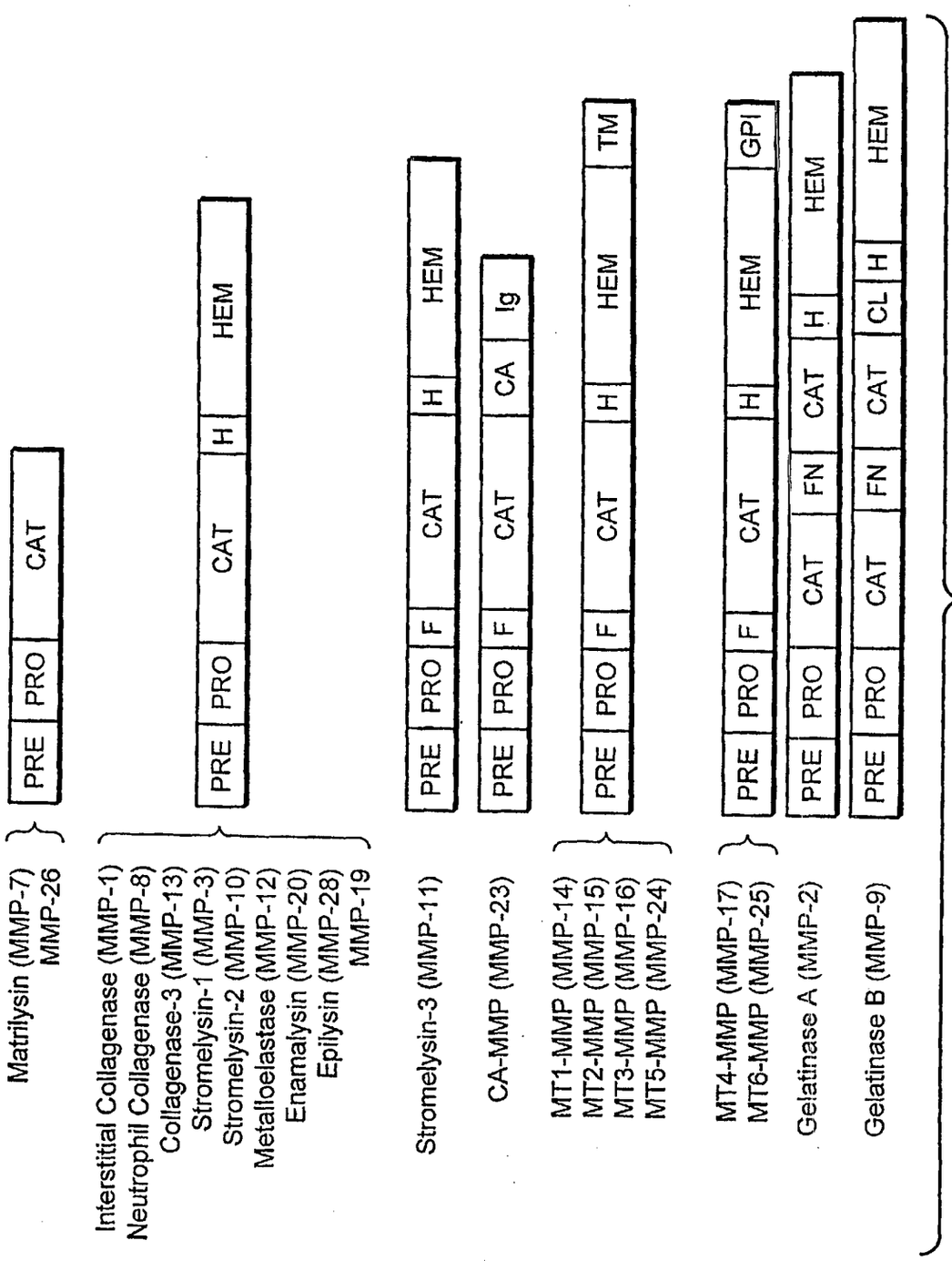

Matrilysin (MMP-7)
MMP-26
Interstitial Collagenase (MMP-1)
Neutrophil Collagenase (MMP-8)
Collagenase-3 (MMP-13)
Stromelysin-1 (MMP-3)
Stromelysin-2 (MMP-10)
Metalloelastase (MMP-12)
Enamalysin (MMP-20)
Epilysin (MMP-28)
MMP-19
Stromelysin-3 (MMP-11)
CA-MMP (MMP-23)
MT1-MMP (MMP-14)
MT2-MMP (MMP-15)
MT3-MMP (MMP-16)
MT5-MMP (MMP-24)
MT4-MMP (MMP-17)
MT6-MMP (MMP-25)
Gelatinase A (MMP-2)
Gelatinase B (MMP-9)
PRE
PRO
CAT
H
HEM
F
CA
Ig
TM
GPI
FN
CL

FIG. 20

Sequence Listing

<110> GENENTECH, INC.
CAREY, Kendall D.
SCHWALL, Ralph H.
SLIWKOWSKI, Mark X.

<120> INHIBITING HER2 SHEDDING WITH MATRIX METALLOPROTEASE ANTAGONISTS

<130> P2195R1

<150> US 60/651,348
<151> 2005-02-09

<160> 38

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<212> PRT
<213> Homo sapiens

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Thr	Gln	Val	Cys	Thr	Gly	Thr	Asp	Met	Lys	Leu	Arg	Leu	Pro	Ala
1				5				10						15
Ser	Pro	Glu	Thr	His	Leu	Asp	Met	Leu	Arg	His	Leu	Tyr	Gln	Gly
				20				25						30
Cys	Gln	Val	Val	Gln	Gly	Asn	Leu	Glu	Leu	Thr	Tyr	Leu	Pro	Thr
				35				40						45
Asn	Ala	Ser	Leu	Ser	Phe	Leu	Gln	Asp	Ile	Gln	Glu	Val	Gln	Gly
				50				55						60
Tyr	Val	Leu	Ile	Ala	His	Asn	Gln	Val	Arg	Gln	Val	Pro	Leu	Gln
				65				70						75
Arg	Leu	Arg	Ile	Val	Arg	Gly	Thr	Gln	Leu	Phe	Glu	Asp	Asn	Tyr
				80				85						90
Ala	Leu	Ala	Val	Leu	Asp	Asn	Gly	Asp	Pro	Leu	Asn	Asn	Thr	Thr
				95				100						105
Pro	Val	Thr	Gly	Ala	Ser	Pro	Gly	Gly	Leu	Arg	Glu	Leu	Gln	Leu
				110				115						120
Arg	Ser	Leu	Thr	Glu	Ile	Leu	Lys	Gly	Gly	Val	Leu	Ile	Gln	Arg
				125				130						135
Asn	Pro	Gln	Leu	Cys	Tyr	Gln	Asp	Thr	Ile	Leu	Trp	Lys	Asp	Ile
				140				145						150
Phe	His	Lys	Asn	Asn	Gln	Leu	Ala	Leu	Thr	Leu	Ile	Asp	Thr	Asn
				155				160						165
Arg	Ser	Arg	Ala	Cys	His	Pro	Cys	Ser	Pro	Met	Cys	Lys	Gly	Ser
				170				175						180
Arg	Cys	Trp	Gly	Glu	Ser	Ser	Glu	Asp	Cys	Gln	Ser	Leu	Thr	Arg
				185				190						195

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Thr Val Cys Ala Gly Gly Cys Ala Arg Cys Lys Gly Pro Leu Pro
1 5 10 15

Thr Asp Cys Cys His Glu Gln Cys Ala Ala Gly Cys Thr Gly Pro
20 25 30

Lys His Ser Asp Cys Leu Ala Cys Leu His Phe Asn His Ser Gly
35 40 45

Ile Cys Glu Leu His Cys Pro Ala Leu Val Thr Tyr Asn Thr Asp
50 55 60

Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg Tyr Thr Phe Gly
65 70 75

Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu Ser Thr Asp
80 85 90

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

Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys Pro
110 115 120

Cys Ala Arg Val

<210> 3
<211> 169
<212> PRT
<213> Homo sapiens

<400> 3
Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu Val Arg Ala Val
1 5 10 15

Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys Lys Ile Phe
20 25 30

Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp Pro Ala
35 40 45

Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe Glu
50 55 60

Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
65 70 75

Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile
80 85 90

Arg Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln
95 100 105

Gly Leu Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu
110 115 120

Gly Ser Gly Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe

125 130 135

Val His Thr Val Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln
140 145 150

Ala Leu Leu His Thr Ala Asn Arg Pro Glu Asp Glu Cys Val Gly
155 160 165

Glu Gly Leu Ala

<210> 4
<211> 142
<212> PRT
<213> Homo sapiens

<400> 4
Cys His Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro
1 5 10 15

Thr Gln Cys Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys
20 25 30

Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val
35 40 45

Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
50 55 60

Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val
65 70 75

Ala Cys Ala His Tyr Lys Asp Pro Pro Phe Cys Val Ala Arg Cys
80 85 90

Pro Ser Gly Val Lys Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys
95 100 105

Phe Pro Asp Glu Glu Gly Ala Cys Gln Pro Cys Pro Ile Asn Cys
110 115 120

Thr His Ser Cys Val Asp Leu Asp Asp Lys Gly Cys Pro Ala Glu
125 130 135

Gln Arg Ala Ser Pro Leu Thr
140

<210> 5
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<212> PRT
<213> Artificial sequence

<220>
<223> Sequence is synthesized

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1 5 10 15

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

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys

35 40 45

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

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

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

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

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
110 115 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
125 130 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
140 145 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
155 160 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
170 175 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
185 190 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
200 205 210

Arg Gly Glu Cys

<210> 6
<211> 449
<212> PRT
<213> Artificial sequence

<220>
<223> sequence is synthesized

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

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
20 25 30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
50 55 60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
65 70 75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp

80 85 90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
95 100 105

Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
110 115 120

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser
125 130 135

Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys
140 145 150

Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
155 160 165

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
170 175 180

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
185 190 195

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
200 205 210

Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
215 220 225

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
245 250 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
260 265 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
275 280 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
290 295 300

Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
305 310 315

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
320 325 330

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
335 340 345

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
350 355 360

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
365 370 375

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
380 385 390

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly

395 400 405

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
410 415 420

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
425 430 435

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
440 445

<210> 7
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<212> PRT
<213> Artificial sequence

<220>
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1 5 10 15

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

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

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
50 55 60

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

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

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

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
110 115 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
125 130 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
140 145 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
155 160 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
170 175 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
185 190 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
200 205 210

Arg Gly Glu Cys

<210> 8
<211> 448
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<220>
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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
             20                  25                  30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
             35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
             50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
             65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
             80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
             95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
            110                 115                 120

Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
            125                 130                 135

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp
            140                 145                 150

Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu
            155                 160                 165

Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly
            170                 175                 180

Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
            185                 190                 195

Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn
            200                 205                 210

Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr
            215                 220                 225

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
            230                 235                 240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
            245                 250                 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
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260 265 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
275 280 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
290 295 300

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp
305 310 315

Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
320 325 330

Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
335 340 345

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met
350 355 360

Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
365 370 375

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
380 385 390

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
395 400 405

Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
410 415 420

Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
425 430 435

Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
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<210> 9
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<210> 11
<211> 37
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<400> 11
gccgaagctt gccaccatgg gcagcgaccc gagcgcg 37

<210> 12

<211> 31
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<210> 13
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<400> 13
cgagtcgacc tcgaggccag ccctctgacg tcc 33

<210> 14
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<212> DNA
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<210> 15
<211> 27
<212> DNA
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<400> 15
ggcctcgagg gcctggcctg ccaccag 27

<210> 16
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ggcacgcgtc gtcagagggc tggctctctg 30

<210> 17
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<210> 18
<211> 21
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<400> 18
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<210> 19
<211> 66
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tggttttttg ctagcg 66

<210> 20
<211> 66
<212> DNA
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aggtcagatg gtggcg 66

<210> 21
<211> 32
<212> PRT
<213> Homo sapiens

<400> 21
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1 5 10 15

Cys Pro Ala Glu Gln Arg Ala Ser Pro Ala Ser Pro Leu Thr Ser
20 25 30

Ile Val

<210> 22
<211> 5
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<400> 22
Asp Leu Asp Asp Lys
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<210> 23
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<400> 23
Pro Ala Glu Gln Arg Ala Ser Pro
5

<210> 24
<211> 42
<212> PRT
<213> Homo sapiens

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1 5 10 15

Asp Leu Asp Asp Lys Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro
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Leu Thr Ser Ile Val Ser Ala Val Val Gly Ile Leu
35 40

<210> 25
<211> 26
<212> PRT
<213> Homo sapiens

<400> 25
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Thr Ser Ile Val Ser Ala Val Val Gly Ile Leu
20 25

<210> 26
<211> 37
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<213> Homo sapiens

<400> 26
Glu Glu Gly Ala Cys Gln Pro Ile Asn Cys Thr His Ser Cys Val
1 5 10 15

Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Val
20 25 30

Ser Ala Val Val Gly Ile Leu
35

<210> 27
<211> 33
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Asp Leu Asp Asp Lys Gly Cys Pro Ala Glu Gln Arg Ala Val Val
20 25 30

Gly Ile Leu

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<400> 28
Leu Thr Ser Ile Val Ser Thr Arg Asp Lys Thr His Thr Cys Pro
1 5 10 15

Pro

<210> 29
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<212> PRT
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<400> 29
Leu Thr Ser Ile Val Ser Thr Arg Asp Lys Thr His Thr Cys Pro
1 5 10 15

Pro

<210> 30
<211> 16
<212> PRT
<213> Homo sapiens

<400> 30
Leu Thr Ser Ile Val Ser Thr Arg Asp Lys Thr His Thr Cys Pro
1 5 10 15

Pro

<210> 31
<211> 16
<212> PRT
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<400> 31
Leu Thr Ser Ile Val Ser Thr Arg Asp Lys Thr His Thr Cys Pro
1 5 10 15

Pro

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<400> 32
Lys Tyr Ala Leu Ala Asp Ala Ser
5

<210> 33
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Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Val
20 25 30

Ser Ala Val Val Gly Ile Leu Leu Val Val Val
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(54) Título: INHIBICIÓN DE LA LIBERACIÓN DE HER2 (*"HER2 SHEDDING"*) CON ANTAGONISTAS DE METALOPROTEASAS DE MATRIZ

(57) Extracto: La presente solicitud describe el uso de antagonistas de metaloproteasas de matriz (MMP), especialmente de MMP-15, para inhibir la liberación de HER2 (***HER2 shedding***).

INHIBICIÓN DE LA LIBERACIÓN DE HER2 (*"HER2 SHEDDING"*) CON ANTAGONISTAS DE METALOPROTEASAS DE MATRIZ

Ésta es una solicitud no provisional que reivindica prioridad bajo 35 USC §119 a la solicitud provisional No. 60/651,348, presentada el 9 de febrero de, 2005, cuya divulgación total se incorpora por la presente por referencia.

Campo de la Invención

La presente invención tiene que ver con el uso de metaloproteasas de matriz (MMP), especialmente de MMP-15, para inhibir la liberación de HER2.

Antecedentes de la Invención

La familia HER de tirosina cinasas de receptor son importantes mediadores del crecimiento, diferenciación y supervivencia celular. La familia de receptores incluye cuatro miembros distintos incluido el receptor del factor de crecimiento epidérmico (EGFR, ErbB1, o HER1), HER2 (ErbB2 o $p185^{neu}$), HER3 (ErbB3) y HER4 (ErbB4 o tyro2).

EGFR, codificado por el gen *erb*B1, ha sido casualmente implicado en neoplasia maligna humana. En particular, se ha observado una expresión aumentada de EGFR en cáncer de mama, vejiga, pulmón, cabeza, cuello y estómago, así como en glioblastomas. La creciente expresión del receptor EGFR se asocia a menudo con una producción en aumento del ligando EGFR, factor α de crecimiento transformante (TGF-α), por las mismas células tumorales, lo cual se traduce en una activación del receptor por una ruta estimuladora autocrina. Baselga and Mendelsohn *Pharmac. Ther.* 64:127-154 (1994). Anticuerpos monoclonales dirigidos contra el EGFR o sus ligandos, TGF-α y EGF, han sido evaluados como agentes terapéuticos en el tratamiento de tales neoplasias malignas. Véase, por ejemplo, Baselga

and Mendelsohn., *supra*; Masui *et al. Cancer Research* 44:1002-1007 (1984); y Wu *et al. J. Clin. Invest.* 95: 1897-1905 (1995).

El segundo miembro de la familia HER, p185^{neu}, se identificó originalmente como el producto del gen transformante de neuroblastomas de ratas tratadas químicamente. La forma activada del protooncogén *neu* resulta de una mutación puntual (valina en ácido glutámico) en la región transmembrana de la proteína codificada. La amplificación del homólogo humano de *neu* se observa en neoplasias malignas de mama y ovario y se correlaciona con una prognosis deficiente (Slamon *et al.*, *Science*, 235: 177-182 (1987); Slamon *et al., Science*, 244:707-712 (1989); y Patente de los Estados Unidos No. 4,968,603). A la fecha, no se ha informado una mutación puntual análoga a la [detectada] en el protooncogén *neu* para tumores humanos. La sobreexpresión de HER2 (con frecuencia, pero no de manera uniforme, debida a amplificación génica) se ha observado igualmente en otros carcinomas, incluidos carcinomas del estómago, endometrio, glándula salival, pulmón, riñón, colon, tiroides, páncreas y vejiga. Véase, entre otros, King *et al., Science*, 229:91 A (1985); Yokota *et al., Lancet*: 1:765-767 (1986); Fukushige *et al., Mol Cell Biol*, 6:955-958 (1986); Guerin *et al., Oncogene Res.*, 3:21-31 (1988); Cohen *et al., Oncogene*, 4:81-88 (1989); Yonemura *et al., Cancer Res.*, 51: 1034 (1991); Borst *et al., Gynecol. Oncol.*, 38:364 (1990); Weiner *et al., Cancer Res.*, 50:421-425 (1990); Kern *et al., Cancer Res.*, 50:5184 (1990); Park *et al., Cancer Res.*, 49:66O5 (1989); Zhau *et al., Mol. Carcinog.*, 3:254-257 (1990); Aasland *et al., Br. J. Cancer* 57:358-363 (1988); Williams *et al., Pathobiology* 59:46-52 (1991); y McCann *et al., Cancer*, 65:88-92 (1990). HER2 puede sobreexpresarse en cancer de próstata (Gu *et al., Cancer Lett.* 99:185-9 (1996); Ross *et al., Hum. Pathol.* 28:827-33 (1997); Ross *et al., Cancer* 79:2162-70 (1997); y Sadasivan *et al., Urol.* 150:126-31 (1993)).

Se han descrito anticuerpos dirigidos contra los productos de proteína HER2 humana y 185^{neu} de rata.

Drebin y colegas han cultivado anticuerpos contra el producto génico *neu* de rata, p185^{neu}. Véase, por ejemplo, Drebin *et al., Cell* 41:695-706

(1985); Myers *et al., Meth. Enzym.* 198:277-290 (1991); y WO94/22478. Drebin *et al., Oncogene* 2:273-277 (1988) informan que mezclas de anticuerpos, reactivos con dos regiones distintas de p185^{neu}, resultan en efectos sinérgicos anti-tumorales sobre células NIH-3T3 transformadas con *neu*, implantadas en ratones atímicos. Véase también la Patente de los Estados Unidos 5,824,311 expedida el 20 de octubre de 1998.

Hudziak *et al., Mol. Cell. Biol.* 9(3): 1165-1172 (1989) describen la generación de panel de anticuerpos HER2, que se caracterizaron usando la línea celular SK-BR-3 de tumor de mama humano. La proliferación celular relativa de las células SK-BR-3, luego de la exposición a los anticuerpos, se determinó mediante tinción con cloruro de metilrosanilina (***"violet crystal"***) de las monocapas después de 72 horas. Usando este análisis, se obtuvo la máxima inhibición con el anticuerpo denominado 4D5, el cual inhibió la proliferación celular en 56%. Otros anticuerpos en el panel redujeron la proliferación celular en una magnitud menor en este análisis. Se encontró además que el anticuerpo 4D5 sensibilizaba las líneas celulares de tumor de mama, que sobreexpresaban HER2, a los efectos citotóxicos de TNF-α. Véase también la Patente de los Estados Unidos No. 5,677,171 expedida el 14 de octubre de 1997. Los anticuerpos HER2, discutidos en Hudziak *et al.*, se caracterizan igualmente en Fendly *et al., Cancer Research* 50:1550-1558 (1990); Kotts *et al., In Vitro* 26(3):59A (1990); Sarup *et al., Growth Regulation* 1:72-82 (1991); Shepard *et al., J. Clin. Immunol.* 11(3): 117-127 (1991); Kumar *et al., Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis *et al., Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras *et al., Oncogene* 9:1829-1838 (1994); Vitetta *et al., Cancer Research* 54:5301-5309 (1994); Sliwkowski *et al., J. Biol. Chem.* 269(20): 14661-14665 (1994); Scott *et al., J. Biol. Chem.* 266:14300-5 (1991); D'Souza *et al., Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis *et al., Cancer Research* 56:1457-1465 (1996); y Schaefer *et al., Oncogene* 15:1385-1394 (1997)..

Una versión humanizada recombinante del anticuerpo HER2 murino 4D5 (huMAb4-D5-8, rhuMAb HER2, Trastuzumab o HERCEPTIN®; Patente de los Estados Unidos No. 5,821,337) es clínicamente activa en

pacientes con neoplasias malignas de mama metastásicas, que sobreexpresan HER2, quienes han recibido terapia previa extensiva anti-neoplásica (Baselga *et al.*, *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab recibió aprobación de comercialización de la Dirección de Alimentos y Drogas de los Estados Unidos el 25 de septiembre de 1998 para el tratamiento de pacientes con cáncer metastático de mama, cuyos tumores sobreexpresan la proteína HER2.

Otros anticuerpos HER2 con varias propiedades se han descrito en Tagliabue *et al.*, *Int. J. Cancer* 47:933-937 (1991); McKenzie *et al.*, *Oncogene* 4:543-548 (1989); Maier *et al.*, *Cancer Res.* 51:5361-5369 (1991); Bacus *et al.*, *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski *et al.*, *PNAS (USA)* 88:8691-8695 (1991); Bacus *et al.*, *Cancer Research* 52:2580-2589 (1992); Xu *et al.*, *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk *et al.*, *Cancer Research* 52:2771-2776 (1992); Hancock *et al.*, *Cancer Res.* 51:4575-4580 (1991); Shawver *et al.*, *Cancer Res.* 54:1367-1373 (1994); Arteaga *et al.*, *Cancer Res.* 54:3758-3765 (1994); Harwerth *et al.*, *J. Biol. Chem.* 267:15160-15167 (1992); Patente de los Estados Unidos No. 5,783,186; y Klapper *et al.*, *Oncogene* 14:2099-2109 (1997).

La detección de homología ha resultado en la identificación de dos otros miembros de la familia del receptor HER; HER3 (Patentes de los Estados Unidos No. 5,183,884 y 5,480,968, así como Kraus *et al.*, *PNAS (USA)* 86:9193-9197 (1989)) y HER4 (Solicitud de Patente EP No. 599,274; Plowman *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); y Plowman *et al.*, *Nature,* 366:473-475 (1993)). Estos dos receptores despliegan una expresión cada vez mayor en por lo menos algunas líneas celulares de cáncer de mama.

Los receptores HER se encuentran generalmente en varias combinaciones en células, y se cree que la heterodimerización incrementa la diversidad de las respuestas celulares a una variedad de ligandos HER (Earp *et al.*, *Breast Cancer Research and Treatment* 35: 115-132 (1995)). EGFR se une por seis ligandos diferentes; factor de crecimiento epidérmico (EGF),

factor α de crecimiento transformante (TGF-α), anfirregulina, factor de crecimiento epidérmico de unión a heparina (HB-EGF), betacelulina y epirregulina (Groenen *et al., Growth Factors* 11:235-257 (1994)). Una familia de proteínas de herregulina, resultantes del corte y empalme alternativo de un solo gen, son ligandos para HER3 y HER4. La familia de herregulina incluye las herregulinas α, β y γ (Holmes *et al., Science*, 256:1205-1210 (1992); Patente de los Estados Unidos No. 5,641,869; y Schaefer *et al., Oncogene* 15:1385-1394 (1997)); factores de diferenciación *neu* (NDF), factores de crecimiento glial (GGF); actividad inductora del receptor de acetilcolina (ARIA); y factor derivado de neuronas motoras y sensoras (SMDF). Para una reseña, véase Groenen *et al., Growth Factors* 11:235-257 (1994); Lemke, G. *Molec. & Cell. Neurosci.* 7:247-262 (1996) y Lee *et al., Pharm. Rev.* 47:51-85 (1995). Recientemente se identificaron tres ligandos HER adicionales; neurregulina-2 (NRG-2), de la que se informa que se une a HER3 ó HER4 (Chang *et al., Nature* 387 509-512 (1997); y Carraway *et al., Nature* 387:512-516 (1997)); neurregulina-3 que une HER4 (Zhang *et al., PNAS (USA)* 94(18):9562-7 (1997)); y neurregulina-4 que une HER4 (Harari *et al., Oncogene* 18:2681-89 (1999)). HB-EGF, betacelulina y epirregulina se unen asimismo a HER4.

Aunque EGF y TGFα no unen HER2, EGF estimula a EGFR y a HER2 a formar un heterodímero, el cual activa EGFR y resulta en transfosforilación de HER2 en el heterodímero. La dimerización y/o transfosforilación parecen activar la tirosina cinasa HER2. Véase Earp *et al., supra.* Análogamente, cuando HER3 se co-expresa con HER2, se forma un complejo de señalización activo, y anticuerpos dirigidos contra HER2 son capaces de alterar este complejo (Sliwkowski *et al., J. Biol. Chem.,* 269(20): 14661-14665 (1994)). Adicionalmente, se incrementa la afinidad de HER3 para la herregulina (HRG) a un estado de mayor afinidad cuando se co-expresa con HER2. Véase también, Levi *et al., Journal of Neuroscience* 15: 1329-1340 (1995); Morrissey *et al., Proc. Natl. Acad. Sci. USA* 92: 1431-1435 (1995); y Lewis *et al., Cancer Res.,* 56: 1457-1465 (1996) con respecto al complejo de proteínas HER2-HER3. HER4, como

HER3, forma un complejo de señalización activa con HER2 (Carraway y Cantley, *Cell* 78:5-8 (1994)).

La red de señalización de HER se ilustra en Fig. 4.

Publicaciones de patente relacionadas con anticuerpos HER: US 5,677,171, US 5,720,937, US 5,720,954, US 5,725,856, US 5,770,195, US 5,772,997, US 6,165,464, US 6,387,371, US 6,399,063, US2002/0192211A1, US 6,015,567, US 6,333,169, US 4,968,603, US 5,821,337, US 6,054,297, US 6,407,213, US 6,719,971, US 6,800,738, US2004/0236078A1, US 5,648,237, US 6,267,958, US 6,685,940, US 6,821,515, WO98/17797, US 6,127,526, US 6,333,398, US 6,797,814, US 6,339,142, US 6,417,335, US 6,489,447, WO99/31140, US2003/0147884A1, US2003/0170234A1, US2005/0002928A1, US 6,573,043, US2003/0152987A1, WO99/48527, US2002/0141993A1, WO01/00245, US2003/0086924, US2004/0013667A1, WO00/69460, WO01/00238, WO01/15730, US 6,627,196B1, US6,632,979B1, WO01/00244, US2002/0090662A1, WO01/89566, US2002/0064785, US2003/0134344, WO 04/24866, US2004/0082047, US2003/0175845A1, WO03/087131, US2003/0228663, WO2004/008099A2, US2004/0106161, WO2004/048525, US2004/0258685A1, US 5,985,553, US 5,747,261, US 4,935,341, US 5,401,638, US 5,604,107, WO 87/07646, WO 89/10412, WO 91/05264, EP 412,116 B1, EP 494,135 B1, US 5,824,311, EP 444,181 B1, EP 1,006,194 A2, US 2002/0155527A1, WO 91/02062, US 5,571,894, US 5,939,531, EP 502,812 B1, WO 93/03741, EP 554,441 B1, EP 656,367 A1, US 5,288,477, US 5,514,554, US 5,587,458, WO 93/12220, WO 93/16185, US 5,877,305, WO 93/21319, WO 93/21232, US 5,856,089, WO 94/22478, US 5,910,486, US 6,028,059, WO 96/07321, US 5,804,396, US 5,846,749, EP 711,565, WO 96/16673, US 5,783,404, US 5,977,322, US 6,512,097, WO 97/00271, US 6,270,765, US 6,395,272, US 5,837,243, WO 96/40789, US 5,783,186, US 6,458,356, WO 97/20858, WO 97/38731, US 6,214,388, US 5,925,519, WO 98/02463, US 5,922,845, WO 98/18489, WO 98/33914, US 5,994,071, WO 98/45479, US 6,358,682 B1, US 2003/0059790, WO 99/55367, WO 01/20033, US 2002/0076695 A1, WO

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02/089842 y WO 03/86467.

El dominio extracelular (ECD) de HER2 se libera proteolíticame
de células de carcinoma de mama en cultivo (Petch *et al., Mol. Cell. B*
10:2973-2982 (1990); Scott *et al., Mol. Cell. Biol* 13:2247-2257 (1993);
Lee and Maihle, *Oncogene* 16:3243-3252(1998)), y se encuentra en el su
de algunos pacientes con cáncer (Leitzel *et al., J. Clin. Oncol.* 10:14
1443 (1992)). El ECD de HER2 puede ser un marcador sérico de cáncer
mama metastásico (Leitzel *et al., J. Clin. Oncol.* 10:1436-1443 (1992),
puede permitir que tumores que sobreexpresan HER2 evadan el cont
inmunológico (Baselga *et al., J. Clin. Oncol.* 14:737-744 (19
Brodowicz *et al., Int. J. Cancer* 73:875-879 (1997)). Niveles séricos
ECD de HER liberado representan un marcador independiente de
respuesta clínica deficiente (***"poor clinical outcome"***) en pacientes
cáncer de mama metastásico, que sobreexpresa HER2 (Ali *et al.,*
Chem. 48:1314-1320 (2002); Molina *et al., Clin. Cancer Res.* 8:347-
(2002)).

Un dominio extracelular truncado de HER2 es también el producto
un transcrito alternativo de 2.3 kb, generado por el uso de una señal
poliadenilación en un intrón (Scott *et al., Mol Cell. Biol.* 13:2247-2

(1993)). El transcrito alternativo se identificó por primera vez en la línea celular de carcinoma gástrico, MKN7 (Yamamoto *et al., Nature* 319:230-234 (1986); y Scott *et al., Mol Cell. Biol.* 13:2247-2257 (1993)), y el receptor truncado se localizó en el citoplasma, más bien que segregado de estas células tumorales (Scott *et al., Mol. Cell. Biol.* 13:2247-2257 (1993)).

Se ha identificado asimismo otro producto alternativamente empalmado de HER2, denominado "herstatina," (Doherty *et al., Proc. Natl. Acad. Sci.* 96:10869-10874 (1999); Azios *et al, Oncogene* 20:5199-5209 (2001); Justman and Clinton, *J. Biol. Chem.* 277:20618-20624 (2002)). Esta proteína consta de sub-dominios I y II del dominio extracelular, seguidos por una secuencia C-terminal única codificada por el intrón 8.

Otro mecanismo que puede explicar la respuesta clínica deficiente en tumores que sobreexpresan HER2 se sugiere por la observación de que, en algunas células tumorales que sobreexpresan HER2, el receptor se procesa por una metaloproteasa desconocida (o metaloproteinasa) para producir un receptor asociado con membrana, truncado (denominado algunas veces "***stub***" ("tocón, muñón") y también conocido como p95), y un dominio extracelular soluble (también conocido como ECD, ECD105, o p105).

Como en el caso de otros receptores HER, la pérdida del dominio de unión a ligando extracelular convierte al dominio asociado con membrana intracelular HER2 en una tirosina cinasa constitutivamente activa. Por lo tanto, se ha postulado que el procesamiento del ECD de HER2 crea un receptor constitutivamente activo que puede impartir directamente señales de crecimiento y supervivencia a la célula cancerosa. Véase, Patente de los Estados Unidos No. 6,541,214 (Clinton), y Solicitud de Patente de los Estados Unidos No. 2004/0247602A1 (Friedman *et al.*)

Saez *et al. Clin Cancer Res.* 12(2): 424-431 (enero de 2006) informan que pacientes cuyos tumores expresan niveles elevados de p95 tienen una respuesta significativamente peor que los pacientes que no. En la actualidad, el nivel de p95 puede determinarse únicamente mediante inmunotransferencia (***"Western blot"***).

Sumario de la Invención

En un primer aspecto, la invención concierne a un método para inhibir la liberación de HER2, que comprende tratar una célula, que expresa HER2, con un antagonista de metaloproteasa de matriz (MMP) en una cantidad efectiva para inhibir la liberación de HER2.

Adicionalmente, la invención provee un método para reducir el nivel sérico del dominio extracelular (ECD) de HER2 en un mamífero, que comprende administrar un antagonista de metaloproteasa de matriz (MMP) al mamífero en una cantidad efectiva para reducir el nivel sérico de ECD de HER2 en el mamífero.

En todavía otro aspecto, se provee un método para tratar cáncer en un mamífero, que comprende administrar un antagonista de metaloproteasa de matriz (MMP) al mamífero en una cantidad efectiva para tratar el cáncer.

Además, la invención se refiere a un método para tratar un cáncer resistente al inhibidor HER en un mamífero, que comprende administrar al mamífero un antagonista de metaloproteasa de matriz (MMP) en una cantidad efectiva para tratar el cáncer.

En todavía un aspecto adicional, la invención se relaciona con un método para reducir el nivel p95 HER2 en una célula, que comprende exponer la célula a un antagonista de metaloproteasa de matriz (MMP) en una cantidad efectiva para reducir el nivel p95 HER2.

La invención concierne asimismo a un método de diagnóstico (o pronóstico), que comprende evaluar MMP-15 (MT2-MMP) en una muestra de un paciente con cáncer, en donde un nivel de MMP-15 elevado o actividad indica que el paciente tiene un nivel sérico elevado de p95 HER2 y/o HER2 liberado, y/o tendrá una respuesta clínica deficiente. Preferiblemente, el nivel de MMP-15 (proteína o ácido nucleico) se evalúa en el método y se utiliza para identificar pacientes con un pronóstico deficiente, o que tendrá una respuesta clínica deficiente. Opcionalmente, el cáncer del paciente despliega además expresión HER, amplificación, o activación, muy preferiblemente la sobreexpresión de HER2 o amplificación.

Breve Descripción de los Dibujos

Figura 1 provee una representación esquemática de la estructura d proteína HER2 de longitud total, y las secuencias de aminoácidos para Dominios I-IV (SEQ ID NOs. 1-4, respectivamente) del dom extracelular de la misma.

Figuras 2A y 2B muestran las secuencias de aminoácidos d cadena ligera de trastuzumab (Fig. 2A; SEQ ID No. 5) y cadena pes (Fig. 2B; SEQ ID No. 6), respectivamente.

Figuras 3A y 3B muestran las secuencias de aminoácidos d cadena ligera de pertuzumab (Fig. 3A; SEQ ID NO. 7) y cadena pes (Fig. 3B; SEQ ID NO. 8). CDRs se muestran en negrilla. La m molecular calculada de la cadena ligera y la cadena pesada son 23,526 Da y 49,216.56 Da (cisteínas en forma reducida). La fracción carbohidr se une a Asn 299 de la cadena pesada.

Fig. 4 ilustra la red de señalización HER.

Fig. 5 ilustra la inhibición con trastuzumab de la liberación de E HER2 de líneas de células de cáncer de mama que sobreexpresan HE (SKBR3, MT474), comparadas con la línea de células de cáncer de ma que no lo sobreexpresantes (MCF-7).

Fig. 6 ilustra diferencias en los niveles de p95 HER2 y ECD HE liberado en tumores transgénicos MMTVHER2 (tumores f2:1282, sensib a trastuzumab; y tumores Fo5, resistentes a trastuzumab).

Fig. 7 describe la estrategia utilizada para identificar la metaloprot HER2 (***"HER2 sheddase"***).

Fig. 8 ilustra experimentos que demostraron que la sheddase te propiedades de una metaloproteasa.

Fig. 9 muestra la expresión de MMPs en tumores MMTV-HER líneas de células. MMP-15 es un candidato para explicar diferencias en tumores f2:1282 que son sensibles a trastuzumab, y tumores Fo5 que s resistentes a trastuzumab.

Fig. 10 refleja la interacción entre flag-HER2 y MMP-15.

Fig. 11 muestra la interacción entre flag-HER2-f2:1282 y flag-HER2-Fo5 y MMP-15. Las diferencias entre f2:1282 y Fo5 no son explicables por unión diferencial de de mutantes a MMP-15.

Fig. 12 ilustra mutaciones somáticas detectadas en ratones transgénicos MMTVHER2. Las secuencias son: sitio de sheddase (SEQ ID NO. 23), natural (WT) (SEQ ID NO. 24), empalme (SEQ ID NO. 25), Fo5 (SEQ ID NO. 26), y f2:3078.10 (SEQ ID NO. 27).

Fig. 13 describe los resultados del análisis de sheddase *in vitro*. gDHER29(DIV)-IgG es un sustrato para los dominios catalíticos de MMP-15, MMP-16, MMP-19, y MMP-25. Las secuencias para las degradaciones de proteasa (***"protease digests"***) son MMP-15 (SEQ ID NO. 28), MMP-16 (SEQ ID NO. 29), MMP-19 (SEQ ID NO. 30), MMP-25 (SEQ ID NO. 31), todos los demás (SEQ ID NO. 32); y para el sitio C-terminal ECD de HER2 (SEQ ID NO. 33).

Fig. 14 ilustra los resultados de un experimento que demuestran que MMP-15 no une (***"does not clip"***) otros receptores HER. Las secuencias que demuestran una variación de secuencia cerca del dominio de transmembrana son HER2 (SEQ ID NO. 34), EGFR (SEQ ID NO. 35), HER3 (SEQ ID NO. 36), HER4 (Jma) (SEQ ID NO. 37), y HER4 (Jmb) (SEQ ID NO. 38).

Fig. 15 muestra que MMP-15 "longitud total" une HER2(+)-IgG.

Fig. 16 demuestra un experimento que indicó que p95HER2 se fosforila constitutivamente, pero tiene que heterodimerizarse con HER3 para activar Akt.

Fig. 17 muestra que el ARN inhibidor (ARNi) de MMP-15 reduce la liberación ECD HER2 y los niveles p95 HER2 en células SKBR3 y BT474.

Fig. 18 ilustra cómo la inhibición del crecimiento mediada por trastuzumab en células SKBR3 es independiente de la inhibición de la liberación de HER2.

Fig. 19 demuestra que la inhibición de la actividad de metaloproteasa en tumores de xenoinjerto Fo5 reduce la liberación de HER2 e inhibe los niveles de p95HER2.

Fig. 20 describe miembros de la familia de metaloproteinasas de matriz (MMP). Los miembros de la familia MMP se agrupan de acuerdo con la estructura del dominio. Las abreviaturas utilizadas en esta Figura son: PRE, pre-dominio; PRO, pro-dominio; CAT, dominio catalítico; H, unión flexible (***"hinge"***); HEM, dominio de hemopexina; F, dominio de consenso de segmentación de furina; FN, dominio tipo fibronectina; GPI, ancla glucofosfatidil inositol; TM, dominio transmembrana; Ig, dominio tipo inmunoglobulina; CA, arreglo de cisteína; CL, dominio tipo colágeno.

Descripción Detallada de las Incorporaciones Preferidas

I. Definiciones

Los términos "metaloproteasa de matriz" o "MMP" en esta solicitud se refieren a una proteína que es un miembro de una superfamilia de metaloproteasas de matriz (MMP), dependiente de Zn o Ca para su actividad. MMP, en esta solicitud, incluye la preproproteína, proteína madura y formas variantes de la misma. Véase, además, Fig. 20 en esta solicitud con respecto a ejemplos de MMP con varios dominios. Las MMP se reseñan en Wagenaar-Miller *et al. Cancer and Metastasis Reviews* 23: 119-135 (2004).

Una "MMP anclada en membrana" o "MT-MMP", en esta solicitud, es una MMP como ya se definió, donde la MMP es capaz de adherirse a una membrana celular o bien mediante un dominio transmembrana (TM) o bien mediante un ancla glucofosfatidil inositol (GPI). Ejemplos de MT-MMP ancladas por un dominio transmembrana en esta solicitud incluyen MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP- 16), MT5-MMP (MMP-24). Ejemplos de MT-MMP ancladas por un ancla GPI incluyen MT4-MMP (MMP-17), y MT6-MMP (MMP-25). MMP-15 es la MT-MMP preferida en esta solicitud.

"MT2-MMP" y "MMP-15" son sinónimos en esta solicitud y describen la preproproteína NP_002415 en la base de datos NCB1, comprendiendo la proteína madura los aminoácidos 132-699 de la misma, así como formas variantes de la misma. Sustratos conocidos para MMP-15

incluyen colágeno, fibronectina, CD44, y complemento. MMP-15 se regula por aumento en algunas neoplasias malignas, y la sobreexpresión de esta proteasa aumenta la invasión del tumor y el crecimiento de las células tumorales.

Un "antagonista de MMP" es un agente que se une a y/o interfiere en alguna medida la actividad proteolítica de por lo menos una MMP. Preferiblemente, el antagonista de MMP se une selectivamente a, o inhibe, la MMP, sin unirse significativamente a, o inhibir, otras proteasas, tales como proteasas en la familia ADAM (una desintegrina y metaloproteasa). Ejemplos de antagonistas MMP en esta solicitud incluyen anticuerpos que se unen a MMP, inhibidores de molécula pequeña, pseudopéptidos que imitan sustratos MMP, moléculas no peptídicas que unen el zinc catalítico de MMPs, inhibidores aislados de tejido natural de MMPs (TIMPs), inhibidores de ácido nucleico, tal como ARN; o inhibidores complementarios, etc.

Un "antagonista MT-MMP" es un agente que se une a y/o interfiere en alguna medida la actividad proteolítica de por lo menos una MT-MMP. Preferiblemente, el antagonista MT-MMP se une selectivamente a, o inhibe, la MT-MMP, sin unirse significativamente a, o inhibir, otras proteasas (incluidas otras MMPs que no están ancladas a membrana). Ejemplos de antagonistas MT-MMP incluyen anticuerpos que se unen a MT-MMP, inhibidores de molécula pequeña, pseudopéptidos que imitan sustratos MT-MMP, moléculas no peptídicas que unen el zinc catalítico de MT-MMP, inhibidores aislados de tejido natural de MT-MMPs, inhibidores de ácido nucleico MT-MMP tales como ARN; o inhibidores complementarios, etc.

Un "antagonista MMP-15" es un agente que se une a y/o interfiere en alguna medida la actividad proteolítica de MMP-15. Preferiblemente, el antagonista MMP-15 se une selectivamente a, o inhibe, MMP-15, sin unirse significativamente a, o inhibir, otras proteasas (incluidas otras MMPs distintas de MMP-15). Ejemplos de antagonistas MMP-15 incluyen anticuerpos que se unen a MMP-15, inhibidores de molécula pequeña, pseudopéptidos que imitan sustratos MMP-15, moléculas no peptídicas que

unen el zinc catalítico de MMP-15, inhibidores aislados de tejido natural de MMP-15, inhibidores de ácido nucleico de MMP-15 tal como ARN; o inhibidores complementarios, etc.

Por "nivel MMP elevado" se da a entender una cantidad de MMP en una muestra biológica, tal como una muestra de un tumor, que excede la cantidad normal de la MMP, por ejemplo la cantidad en una muestra normal, o no tumoral, del mismo tipo de tejido. Tal "cantidad normal" de MMP (por ejemplo, de MMP-15) incluye una cantidad indetectable o ninguna cantidad de MMP-15. Niveles MMP elevados pueden determinarse de varias maneras, incluidas aquellas que miden la proteína MMP o el ácido nucleico MMP.

Un "receptor HER" es una proteína tirosina cinasa de receptor que pertenece a la familia de receptores HER e incluye los receptores EGFR, HER2, HER3 y HER4. El receptor HER incluye el receptor HER de secuencia natural, y variantes del mismo. Preferiblemente el receptor HER es un receptor HER humano de secuencia natural.

Un receptor HER de "longitud total" comprende un dominio extracelular, que puede unir un ligando HER y/o dimerizarse con otra molécula de receptor HER; un dominio de transmembrana lipófila; un dominio de tirosina cinasa intracelular; y un dominio de señalización carboxilo-terminal que alberga varios residuos de tirosina, los cuales pueden fosforilarse.

Los términos "ErbB1", "HER1", "receptor de factor de crecimiento epidérmico" y "EGFR" se emplean indistintamente en esta solicitud y se refieren a EGFR como se divulga, por ejemplo, en Carpenter *et al., Ann. Rev. Biochem.* 56:881-914 (1987), incluidas sus formas variantes (*por ejemplo*, un EGFR mutante de deleción como en Humphrey *et al., PNAS (USA)* 87:4207-4211 (1990)).

Las expresiones "ErbB2" y "HER2" se emplean indistintamente en esta solicitud y se refieren a la proteína HER2 humana, descrita por ejemplo en Semba *et al., PNAS (USA)*, 82:6497-6501 (1985) y Yamamoto *et al., Nature*, 319:230-234 (1986) (número de acceso a Genbank X03363), así

como a formas variantes del mismo, tales como formas alternativamente empalmadas (Siegel *et al. EMBO J.* 18(8):2149-2164 (1999)).

En esta solicitud, "dominio extracelular de HER2 " o "ECD de HER2" se refiere a un dominio de HER2 que está fuera de una célula, o bien anclado a una membrana de la célula, o en circulación, incluidos sus fragmentos. En una incorporación, el dominio extracelular de HER2 puede comprender cuatro dominios: "Dominio I" (residuos de aminoácidos desde alrededor de 1-195; SEQ ID NO:1), "Dominio II" (residuos de aminoácidos desde alrededor de 196-319; SEQ ID NO:2), "Dominio III" (residuos de aminoácidos desde alrededor de 320-488: SEQ ID NO:3), y "Dominio IV" (residuos de aminoácidos desde alrededor de 489-630; SEQ ID NO:4) (numeración de residuos sin péptido de señal). Véase Garrett *et al Mol. Cell.* 11: 495-505 (2003), Cho *et al. Nature* 421: 756-760 (2003), Franklin *et al. Cancer Cell* 5:317-328 (2004), and Plowman *et al Proc. Natl. Acad. Sci.* 90:1746-1750 (1993), así como Fig. 1 en esta solicitud.

En esta solicitud, "liberación de HER2" se refiere a la liberación de un fragmento de dominio extracelular (ECD) soluble de HER2 de la superficie celular de una célula, que expresa HER2. Tal liberación puede causarse por segmentación proteolítica de HER2 de superficie celular resultante en la liberación de un fragmento ECD de la superficie celular, o el ECD soluble o fragmento del mismo puede codificarse por un transcrito alternativo.

Por "nivel sérico de HER2 liberado" se da a entender la cantidad de ECD de HER2 presente en el suero o circulación de un mamífero. Tales niveles pueden evaluarse por varias técnicas, incluidas las descritas en: Ali *et al. Clin. Chem.* 48:1314-1320 (2002); Molina *et al. Clin. Cancer Res.* 8:347-353 (2002); Patente de los Estados Unidos No.4,933,294 expedida el 12 de junio de 1990; WO91/05264 publicada el 18 de abril de 1991; Patente de los Estados Unidos 5,401,638 expedida el 28 de marzo de 1995; o Sias *et al. J. Immunol. Methods* 132: 73-80 (1990).

En esta solicitud, "nivel sérico de HER2 liberado elevado" se refiere a una cantidad de HER2 liberado o ECD de HER2 en el suero de un

mamífero (por ejemplo, humano) que excede la cantidad presente en el suero de un mamífero normal (por ejemplo, un humano). Niveles séricos ECD de HER2 elevados pueden correlacionarse con un pronóstico deficiente y una capacidad de respuesta disminuida a la terapia endocrina y quimioterapia en pacientes con cáncer de mama avanzado.

La expresión "p95 HER2" en esta solicitud se refiere a una proteína HER2 truncada NH_2-terminal. Generalmente, p95 es un fragmento truncado unido a membrana (***"a membrane-bound stub fragment"***), que puede surgir de la segmentación de HER2 de longitud completa por una proteasa o sheddase (Yuan *et al. Protein Expression and Purification* 29: 217-222 (2003)). p95 puede tener un M_r de alrededor de 95.000 y puede fosforilarse (Molina *et al. Cancer Research* 4744-4749 (2001)). p95 se ha detectado en algunas muestras de cáncer de mama (Christianson *et al. Cancer Res.* 15:5123-5129 (1998)).

Por "nivel p95 elevado" se da a entender un nivel de p95 en una célula cancerosa que excede el nivel normal, por ejemplo el nivel en una célula normal o no cancerosa del mismo tipo de tejido que la célula cancerosa. Tal nivel p95 elevado puede resultar en una señalización constitutiva, y metástasis nodal (Molina *et al. Clin. Cancer Research* 8:347-353 (2002); Christianson *et al. Cancer Res.* 15:5123-5129 (1998)).

"Evaluar" un marcador, tal como MMP-15, tiene por objeto un análisis diagnóstico y/o pronóstico del mismo, incluido un análisis de la presencia o ausencia de ese marcador, cuantificación de la cantidad del mismo, y/o un análisis de actividad del mismo (*por ejemplo*, actividad incrementada).

Un paciente de cáncer con "una respuesta clínica deficiente" es uno con un pronóstico insuficiente, que tiene menos probabilidad de responder a la terapia contra el cáncer, tal como quimioterapia o terapia con un anticuerpo HER2, tal como trastuzumab. La respuesta clínica puede medirse por medios estándares, tales como supervivencia, incluida supervivencia libre de enfermedad, etc.

"ErbB3" y "HER3" se refieren al polipéptido receptor como se divulga, por ejemplo, en las Patentes de los Estados Unidos No. 5,183,884 y 5,480,968, así como Kraus *et al., PNAS (USA)* 86:9193-9197 (1989), incluidas formas variantes de los mismos.

Los términos "ErbB4" y "HER4" en esta solicitud se refieren al polipéptido receptor como se divulga, por ejemplo, en la Solicitud de Patente EP No. 599,274; Plowman *et al., Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); y Plowman *et al., Nature*, 366:473-475 (1993), incluidas formas variantes de los mismos, divulgadas en WO 99/19488, publicada el 22 de abril de 1999.

Por "ligando HER" se da a entender un polipéptido que se une a un receptor HER y/o lo activa. El ligando HER de particular interés en esta solicitud es un ligando HER humano de secuencia natural tal como el factor de crecimiento epidérmico (EGF) (Savage *et al., J. Biol. Chem.* 247:7612-7621 (1972)); factor α de crecimiento transformante (TGF-α) (Marquardt *et al., Science* 223:1079-1082 (1984)); anfirregulina también conocida como schwanoma o factor de crecimiento autocrino de queratinocitos (Shoyab *et al., Science* 243: 1074-1076 (1989); Kimura *et al., Nature* 348:257-260 (1990); y Cook *et al., Mol. Cell. Biol.* 11:2547-2557 (1991)); betacelulina (Shing *et al., Science* 259:1604-1607 (1993); y Sasada *et al., Biochem. Biophys. Res. Commun.* 190:1173 (1993)); factor de crecimiento epidérmico de unión a heparina (HB-EGF) (Higashiyama *et al., Science* 251:936-939 (1991)); epirregulina (Toyoda *et al., J. Biol. Chem.* 270:7495-7500 (1995); y Komurasaki *et al., Oncogene* 15:2841-2848 (1997)); una herregulina (véase más adelante); neurregulina-2 (NRG-2) (Carraway *et al., Nature* 387:512-516 (1997)); neurregulina-3 (NRG-3) (Zhang *et al., Proc. Natl. Acad. Sci.* 94:9562-9567 (1997)); neurregulina-4 (NRG-4) (Harari *et al., Oncogene* 18:2681-89 (1999)); y cripto (CR-1) (Kannan *et al., J. Biol. Chem.* 272(6):3330-3335 (1997)). Los ligandos HER que unen EGFR incluyen EGF, TGF-α, anfirregulina, betacelulina, HB-EGF y epirregulina. Los ligandos HER que unen HER3 incluyen herregulinas. Ligandos HER

capaces de unir HER4 incluyen betacelulina, epirregulina, HB-EGF, NRG-2, NRG-3, NRG-4 y herregulinas.

"Herregulina" (HRG), cuando se emplea en esta solicitud, se refiere a un polipéptido codificado por el producto génico herregulina como se divulga en la Patente de los Estados Unidos No. 5,641,869 o Marchionni *et al*, *Nature* 362:312-318 (1993). Ejemplos de herregulinas incluyen herregulina-α, herregulina-β1, herregulina-β2 y herregulina-β3 (Holmes *et al*, *Science* 256:1205-1210 (1992); y Patente de los Estados Unidos No.5,641,869); factor de diferenciación *neu* (NDF) (Peles *et al.*, *Cell* 69: 205-216 (1992)); actividad inductora de receptor acetilcolina (ARIA) (Falls *et al.*, *Cell* 72:801-815 (1993)); factores de crecimiento glial (GGFs) (Marchionni *et al.*, *Nature*, 362:312-318 (1993)); factor derivado de neuronas motoras y sensoriales (SMDF) (Ho *et al.*, *J. Biol. Chem.* 270:14523-14532 (1995)); γ-herregulina (Schaefer *et al.*, *Oncogene*, 15:1385-1394 (1997)).

Un "dímero HER" en esta solicitud es un dímero no covalentemente asociado, que comprende por lo menos dos diferentes receptores HER. Tales complejos pueden formarse cuando una célula, que expresa dos o más receptores HER, se expone a un ligando HER y puede aislarse por inmunoprecipitación y analizarse por SDS-PAGE como se describe en Sliwkowski *et al.*, *J. Biol. Chem.*, 269(20):14661-14665 (1994), por ejemplo. Ejemplos de tales dímeros HER incluyen los heterodímeros EGFR-HER2, HER2-HER3 y HER3-HER4. Más aún, el dímero HER puede comprender dos o más receptores HER2, combinados con un receptor HER diferente, tal como HER3, HER4 o EGFR. Otras proteínas, tales como la subunidad de receptor de citosina (por ejemplo, gp130) pueden asociarse con el dímero.

Una célula, cáncer, o muestra biológica que "despliega expresión, amplificación o activación HER" es una que, en una prueba diagnóstica, expresa (inclusive, sobreexpresa) HER, tiene el gen HER amplificado, y/o de otro modo demuestra activación o fosforilación de receptor(es) HER. Tal activación puede determinarse directa (*por ejemplo*, midiendo la

fosforilación de HER) o indirectamente (*por ejemplo*, perfilando la expresión génica o detectando heterodímeros HER).

Un cáncer o célula tumoral con "sobreexpresión o amplificación del receptor HER", es uno que tiene niveles significativamente más altos de una proteína HER2 o gen, comparado con una célula no cancerosa del mismo tipo de tejido. Tal sobreexpresión puede ser causada por amplificación génica o por transcripción o traducción incrementada. La sobreexpresión o amplificación de HER2 puede determinarse en un análisis diagnóstico o pronóstico evaluando niveles crecientes de la proteína HER2 presentes en la superficie de una célula (*por ejemplo*, mediante un análisis de inmunohistoquímica; IHC). Alternativa, o adicionalmente, se pueden medir niveles de ácido nucleico HER2 en la célula, *por ejemplo*, mediante hibridación *in situ* fluorescente (FISH; véase WO 98/45479 publicada en octubre de 1998), técnicas de ***Southern blotting***, o de reacción en cadena de polimerasa (PCR), tal como PCR cuantitativa en tiempo real (qRT-PCR). La sobreexpresión o amplificación de HER2 puede determinarse igualmente midiendo el HER2 liberado en un fluido biológico tal como suero (véase, *por ejemplo*, Patente de los Estados Unidos 4,933,294 expedida el 12 de junio de 1990; WO 91/05264 publicada el 18 de abril de 1991; Patente de los Estados Unidos 5,401,638 publicada el 28 de marzo de 1995; y Sias *et al. J. Immunol. Methods* 132: 73-80 (1990)). Aparte de los análisis anteriores, se encuentran a disposición del médico profesional varios análisis *in vivo*. Por ejemplo, se pueden exponer células dentro del organismo del paciente a un anticuerpo, que se marca opcionalmente con un marcador detectable, *por ejemplo*, un isótopo radioactivo, y puede evaluarse la unión del anticuerpo a células en el paciente, *por ejemplo*, mediante escaneo externo para detectar radioactividad, o analizando una biopsia tomada de un paciente previamente expuesto al anticuerpo.

Recíprocamente, un cáncer o célula tumoral que "no sobreexpresa o amplifica el receptor HER" es una que no tiene niveles más altos de los normales de la proteína de receptor HER o gen, comparada con una célula no cancerosa del mismo tipo de tejido. Anticuerpos que inhiben la

dimerización de HER, tales como pertuzumab, pueden utilizarse para tratar cáncer que no sobreexpresa o amplifica el receptor HER2.

Un "inhibidor HER" es un agente que interfiere la activación o función de HER. Ejemplos de inhibidores de HER incluyen anticuerpos HER (*por ejemplo*, anticuerpos EGFR, HER2, HER3, o HER4); inhibidores de dimerización HER; fármacos dirigidos a EGFR (***"EGFR-targeted drugs"***); antagonistas HER de molécula pequeña; inhibidores de tirosina cinasa HER; inhibidores duales de tirosina cinasa HER2 y EGFR tales como lapatinib/GW572016; moléculas complementarias (véase, por ejemplo, WO2004/87207); y/o agentes que se unen a, o interfieren la función de, moléculas señalizadoras corriente abajo, tales como MAPK o Akt. Preferiblemente, el inhibidor HER es un anticuerpo o molécula pequeña, que se une al receptor a HER. Ejemplos específicos de inhibidores de HER incluyen trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, y TAK165.

Un "inhibidor de dimerización de HER" es un agente que inhibe la formación de un dímero de HER. Preferiblemente, el inhibidor de la dimerización de HER es un anticuerpo, por ejemplo un anticuerpo que se une a HER2 en el sitio heterodimérico de unión (***"at the heterodimeric binding site"***) del mismo. El inhibidor de dimerización más preferido en esta solicitud es pertuzumab o el anticuerpo monoclonal 2C4 (MAb 2C4). Otros ejemplos de inhibidores de dimerización de HER incluyen anticuerpos que se unen a EGFR e inhiben su dimerización con uno o más de otros receptores HER (por ejemplo, el anticuerpo monoclonal EGFR 806, MAb 806, que se une a EGFR activado o "no anclado"; véase Johns *et al., J. Biol. Chem.* 279(29):30375- 30384 (2004)); anticuerpos que se unen a HER3 e inhiben su dimerización con uno o más de otros receptores HER; anticuerpos que se unen a HER4 e inhiben su dimerización con uno o más de otros receptores HER; inhibidores de dimerización peptídica (Patente de los Estados Unidos No. 6,417,168); inhibidores de dimerización complementaria; etc.

Un "anticuerpo HER" es un anticuerpo que se une a un receptor HER. Opcionalmente, el anticuerpo HER interfiere además la activación o función de HER. Preferiblemente, el anticuerpo HER se une al receptor HER2. Los anticuerpos HER2 de particular interés en esta solicitud son trastuzumab y pertuzumab. Ejemplos de anticuerpos EGFR incluyen cetuximab, ABX0303, EMD7200 y IMC-11F5.

"Activación de HER" se refiere a la activación, o fosforilación, de uno cualquiera o más receptores HER. Generalmente, la activación de HER resulta en transducción de señales (por ejemplo, la causada por un dominio de cinasa intracelular de un receptor HER, que fosforila residuos de tirosina en el receptor HER o un polipéptido sustrato). La activación de HER puede estar mediada por la unión de ligando HER a un dímero HER, que comprende el receptor HER de interés. La unión de ligando HER a un dímero HER puede activar un dominio de cinasa de uno o más de los receptores HER en el dímero y, por consiguiente, resulta en la fosforilación de residuos de tirosina en uno o más de los receptores HER y/o fosforilación de residuos de tirosina en polipéptido(s) sustrato(s) adicional(es), tales como cinasas intracelulares Akt o MAPK.

"Fosforilación" se refiere a la adición de uno o más grupo(s) fosfato a una proteína, tal como un receptor HER, o su sustrato.

Un anticuerpo que "inhibe la dimerización de HER" es un anticuerpo que inhibe, o interfiere, la formación de un dímero de HER. Preferiblemente, tal anticuerpo se une a HER2 en el sitio heterodimérico de unión del mismo. El anticuerpo inhibidor de dimerización más preferido en esta solicitud es pertuzumab o MAb 2C4. Otros ejemplos de anticuerpos que inhiben la dimerización de HER incluyen anticuerpos que se unen a EGFR e inhiben su dimerización con uno o más de otros receptores HER (por ejemplo el anticuerpo monoclonal EGFR 806, MAb 806, que se une a EGFR activado o "no anclado"; véase Johns *et al., J. Biol. Chem.* 279(29):30375-30384 (2004)); anticuerpos que se unen a HER3 e inhiben la dimerización del mismo con uno o más de otros receptores HER; y

anticuerpos que se unen a HER4 e inhiben la dimerización del mismo con uno o más de otros receptores HER.

Un "sitio heterodimérico de unión" en HER2, se refiere a una región en el dominio extracelular de HER2 que contacta, o interactúa con, una región en el dominio extracelular de EGFR, HER3 o HER4, luego de la formación de un dímero con ella. La región se encuentra en Dominio II de HER2, Franklin *et al., Cancer Cell* 5:317-328 (2004).

El anticuerpo HER2 puede ser uno que, como el trastuzumab, "inhibe la segmentación del ectodominio de HER2" (Molina *et al. Cancer Res.* 61:4744-4749(2001)) o puede ser uno que, como el pertuzumab, no inhibe significativamente la segmentación del ectodominio de HER2.

Un anticuerpo HER2 que "se une a un sitio heterodimérico de unión" de HER2, se une a residuos en dominio II (y, opcionalmente, se une también a residuos en otros de los dominios del dominio extracelular de HER2, tales como dominios I y III), y pueden impedir ampliamente, por lo menos en cierta medida, la formación de un heterodímero HER2-EGFR, HER2-HER3, o HER2-HER4. Franklin *et al. Cancer Cell* 5:317-328 (2004) caracterizan la estructura cristalina del HER2-pertuzumab, depositada con el RCSB Protein Data Bank (ID Code IS78), ilustrando un anticuerpo ejemplar que se une al sitio heterodimérico de unión de HER2.

Un anticuerpo que "se une al dominio II" de HER2 se une a residuos en dominio II y, opcionalmente, residuos en otro(s) dominio(s) de HER2, tales como dominios I y III. Preferiblemente, el anticuerpo que se une a dominio II se une al empalme entre dominios I, II y III de HER2.

Un polipéptido "de secuencia natural" es uno que tiene la misma secuencia de aminoácidos que un polipéptido (*por ejemplo*, receptor HER o ligando HER) derivado de la naturaleza. Tales polipéptidos de secuencia natural pueden aislarse de la naturaleza o pueden producirse por medios sintéticos o recombinantes. Por consiguiente, un polipéptido de secuencia natural puede tener la secuencia de aminoácidos de un polipéptido humano de ocurrencia natural, un polipéptido murino, o un polipéptido de cualquier otra especie mamífera.

El término "anticuerpo" en esta solicitud se emplea en el sentido más amplio y cubre específicamente anticuerpos monoclonales intactos, anticuerpos policlonales, anticuerpos multiespecíficos (*por ejemplo*, anticuerpos biespecíficos) formados, por lo menos, de dos anticuerpos intactos, y fragmentos de anticuerpo, siempre y cuando exhiban la actividad biológica deseada.

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 y/o unen el mismo epítopo(s), excepto por posibles variantes que pueden surgir durante la producción del anticuerpo monoclonal, estando tales variantes generalmente presentes en cantidades menores. Tal anticuerpo monoclonal incluye típicamente un anticuerpo, que comprende una secuencia polipeptídica que se une a una diana, en donde la secuencia polipeptídica de unión a una diana se obtuvo por un proceso que incluye la selección de una secuencia polipeptídica de unión a una diana única, de una pluralidad de secuencias polipeptídicas. Por ejemplo, el proceso de selección puede ser la selección de un clon único de una pluralidad de clones, tal como un ***pool*** de clones de hibridoma, clones de bacteriófagos o clones de ADN recombinante. Debe entenderse que la secuencia de unió a diana seleccionada puede alterarse adicionalmente, por ejemplo, para mejorar la afinidad por la diana, para humanizar la secuencia de unión a la diana, para intensificar su producción en un cultivo de células, para reducir su inmunogenicidad *in vivo*, para crear un anticuerpo multiespecífico, etc., y que un anticuerpo que comprenda la secuencia de unión a diana alterada es también un anticuerpo monoclonal de esta invención. En contraste con preparaciones de anticuerpos monoclonales, que típicamente incluyen diferentes anticuerpos dirigidos contra diferentes determinantes (epítopos), cada anticuerpo monoclonal se dirige contra un solo determinante en el antígeno. Además de su especificidad, las preparaciones de anticuerpos monoclonales son ventajosas en el sentido de que generalmente están incontaminadas por

otras inmunoglobulinas. El calificativo "monoclonal" indica el carácter del anticuerpo como obtenido de una población sustancialmente homogénea de anticuerpos, y no tiene que interpretarse como que requiere la producción del anticuerpo por algún método particular. Por ejemplo, los anticuerpos monoclonales a utilizarse de acuerdo con la presente invención pueden elaborarse por una variedad de técnicas, incluidas, por ejemplo, el método del hibridoma (*e.g.*, Kohler *et al., Nature*, 256:495 (1975); Harlow *et al., Antibodies*: *A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al*, in: Monoclonal Antibodies and T-Cell Hybridomas 563-681, (Elsevier, N. Y., 1981)), métodos de ADN recombinante (véase, *por ejemplo*, Patente de los Estados Unidos No. 4,816,567), tecnologías de exposición de proteínas sobre la superficie de bacteriófagos (***"phage display technologies"***) (véase, *por ejemplo*, Clackson *et al., Nature*, 352:624-628 (1991); Marks *et al, J. Mol. Biol*, 222:581-597 (1991); Sidhu *et al, J. Mol. Biol.* 338(2):299-310 (2004); Lee *et al, J. Mol. Biol.* 340(5):1073-1093 (2004); Fellouse, *Proc. Nat. Acad. Sci. USA* 101(34): 12467-12472 (2004); y Lee *et al. J. Immunol. Methods* 284(1-2): 119-132 (2004), y tecnologías para producir anticuerpos humanos o tipo humano en animales que tienen partes o la totalidad de los loci de inmunoglobulina humana o genes que codifican secuencias de inmunoglobulina humana (véase, *por ejemplo*, WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); *Bruggemann et al, Year in Immune.*, 7:33 (1993); Patentes de los Estados Unidos No. 5,545,806; 5,569,825; 5,591,669 (todas de GenPharm); Patente de los Estados Unidos No. 5,545,807; WO 1997/17852; Patentes de los Estados Unidos No. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; y 5,661,016; Marks et al., Bio/Technology, 10: 779-783 (1992); Lonberg *et al., Nature*, 368: 856-859 (1994); Morrison, *Nature*, 368: 812-813 (1994); Fishwild *et al., Nature Biotechnology*, 14: 845-851 (1996); Neuberger, *Nature Biotechnology*, 14: 826 (1996); y Lonberg and Huszar, *Intern. Rev. Immunol*, 13: 65-93 (1995).

Los anticuerpos monoclonales en esta solicitud incluyen específicamente 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)). Los anticuerpos quiméricos de interés en esta solicitud incluyen anticuerpos "primatizados", que comprenden secuencias de unión a antígeno de dominio variable, derivados de un primate no humano (*por ejemplo*, mono del Viejo Mundo, mono) y secuencias de región constante humanas.

"Fragmentos de anticuerpo" comprenden una porción de un anticuerpo intacto, que contiene preferiblemente su región de unión a antígeno. Ejemplos de fragmentos de anticuerpo incluyen fragmentos Fab, Fab', $F(ab')_2$ y Fv; diacuerpos; anticuerpos lineales; moléculas de anticuerpo de cadena única; y anticuerpos multiespecíficos formados de fragmento(s) de anticuerpo.

Un "anticuerpo intacto" en esta solicitud es uno que comprende dos regiones de unión a antígeno, y una región Fc. Preferiblemente, el anticuerpo intacto tiene una o más funciones efectoras.

Dependiendo de la secuencia de aminoácidos del dominio constante de sus cadenas pesadas, anticuerpos intactos pueden asignarse a diferentes "clases". Existen cinco clases principales de anticuerpos intactos: IgA, IgD, IgE, IgG, e IgM, y varias de éstas pueden subdividirse aún más en "subclases" (isotipos), por ejemplo, IgG1, IgG2, IgG3, IgG4, IgA, e IgA2. Los dominios constantes de cadena pesada que corresponden a las diferentes clases de anticuerpos se denominan α, δ, ε, γ, y μ,

respectivamente. Son bien conocidas las estructuras subunitarias y configuraciones tridimensionales de diferentes clases de inmunoglobulinas.

"Funciones efectoras" de anticuerpo se refieren a aquellas actividades biológicas atribuibles a la región Fc (una región Fc de secuencia natural o región FC variante de secuencia de aminoácidos) de un anticuerpo. Ejemplos de funciones efectoras de anticuerpo incluyen unión C1q; citotoxicidad dependiente de complemento; unión de receptor Fc; citotoxicidad celular dependiente de anticuerpo (ADCC); fagocitosis; regulación por disminución de receptores de superficie celular (por ejemplo, receptor de linfocitos B; BCR), etc.

"Citotoxicidad celular dependiente de anticuerpo" y "ADCC" se refieren a una reacción mediada por células, en la cual células citotóxicas no específicas, que expresan receptores Fc (FcR) (por ejemplo, linfocitos citolíticos naturales (NK), neutrófilos, y macrófagos), reconocen el anticuerpo unido en una célula diana y, posteriormente, causan lisis de la célula diana. Las células primarias para mediar ADCC, los linfocitos NK, expresan únicamente FcγRIII, mientras que los monocitos expresan FcγRI, FcγRII, y FcγRIII. La expresión de FcR en células hematopoyéticas se resume en la Tabla 3 en la página 464 de Ravetch *et al.*, *Annu. Rev. Immunol* 9:457-92 (1991). Para evaluar la actividad ADCC de una molécula de interés, puede realizarse un ensayo ADCC *in vitro*, tal como el descrito 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).

"Células efectoras humanas" son leucocitos que expresan uno o más FcR y realizan funciones efectoras. Preferiblemente, las células expresan por lo menos FcγRIII y realizan la función efectora ADCC. Ejemplos de leucocitos humanos, que median ADCC, incluyen células moonucleares de sangre periférica (PBMC), linfocitos citolíticos naturales (NK), monocitos,

linfocitos T citotóxicos y neutrófilos, prefiriéndose PBMC y linfocitos citolíticos naturales (NK). Las células efectoras pueden aislarse de una fuente natural de las mismas, por ejemplo, de sangre o PBMC como se describe en esta solicitud.

Los términos "receptor Fc" o "FcR" se usan para describir un receptor que se une a la región Fc de un anticuerpo. El FcR preferido es un FcR humano de secuencia natural. Más aún, un FcR preferido es uno que une un anticuerpo IgG (un receptor gamma) e incluye receptores de las subclases FcγRI, FcγRII, y FcγRIII, incluidas sus variantes alélicas y, alternativamente, formas empalmadas de estos receptores. Los receptores FcγRII incluyen FcγRIIA (un "receptor activador") y FcγRIIB (un "receptor inhibidor"), los cuales poseen secuencias de aminoácidos similares que difieren principalmente en sus dominios citoplásmicos. El receptor activador FcγRIIA contiene un motivo de activación a base de tirosina de inmunorreceptor (ITAM) en su dominio citoplásmico. El receptor inhibidor FcγRIIB contiene un motivo de inhibición a base de tirosina de inmunorreceptor (ITIM) en su dominio citoplásmico, (véase reseña en Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997). Los FcR se reseñan en Ravetch y Kinet, *Annu. Rev. Immunol.* 9: 457-92 (1991); Capel *et. al., Immunomethods* 4:25-34 (1994); y de Haas *et. al., J. Lab. Clin. Med.* 126: 330-41 (1995). Otros FcR, incluidos aquellos por identificar en el futuro, se abarcan por el término "FcR" en esta solicitud. El término comprende asimismo el receptor neonatal, FcRn, que es responsable de la transferencia de IgG maternas al feto. Guyer *et. al., J. Immunol.* 117: 587 (1976) y Kim *et. al., J. Immunol* 24:249 (1994)), y regula la homeostasis de inmunoglobulinas.

"Citotoxicidad dependiente de complemento" o "CDC" se refiere a la capacidad de una molécula de desintegrar una diana, en la presencia de complemento. La ruta de activación del complemento se inicia por la unión del primer componente del sistema complemento (C1q) a una molécula (*por ejemplo*, un anticuerpo) acomplejado con un antígeno afín. 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).

"Anticuerpos naturales" son usualmente glucoproteínas heterotetraméricas de alrededor de 150.000 daltons, compuestas de dos cadenas ligeras (L) idénticas y dos cadenas pesadas (H) idénticas. Cada cadena ligera se enlaza a una cadena pesada por un enlace disulfuro covalente, mientras que el número de enlazamientos disulfuro varía entre las cadenas pesadas de diferentes isotipos de inmunoglobulina. Cada cadena ligera y pesada posee también puentes disulfuro intracadena, regularmente espaciados. Cada cadena pesada tiene en un extremo un dominio variable (V_H), seguido por un número de dominios constantes. Cada cadena ligera tiene un dominio variable en un extremo (V_L) y un dominio constante en su otro extremo. El dominio constante de la cadena ligera se alinea con el primer dominio constante de la cadena pesada, y el dominio variable de la cadena ligera se alinea con el dominio variable de la cadena pesada. Se cree que residuos de aminoácidos particulares forman una interfase entre los dominios variables de cadena ligera y cadena pesada.

El término "variable" se refiere al hecho de que ciertas porciones de los dominios variables difieren extensivamente en secuencia entre anticuerpos y se utilizan en la unión y especificidad de cada anticuerpo particular para su antígeno particular. No obstante, la variabilidad no está uniformemente distribuida a través de los dominios variables de los anticuerpos. Ésta se concentra en tres segmentos denominados regiones hipervariables, tanto en los dominios variables de cadena pesada como en los de cadena ligera. Las porciones más altamente conservadas de los dominios variables se llaman las regiones de estructura alterna (FR) (***"the framework regions (FRs)"***). Los dominios variables de cadenas naturales pesada y ligera comprenden cada una cuatro FR que adoptan en gran medida una configuración de hoja β (***"β-sheet configuration"***), conectada por tres regiones hipervariables, que forman bucles que conectan y, en algunos casos, forman parte de la estructura de la hoja β. Las regiones hipervariables en cada cadena se mantienen juntas en estrecha proximidad

por las FR y, con las regiones hipervariables de la otra cadena, contribuyen a la formación del sitio de unión a antígeno de anticuerpos (véase Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Los dominios constantes no están implicados directamente en unir un anticuerpo a un antígeno, pero exhiben varias funciones efectoras, tales como participación del anticuerpo en citotoxicidad celular, dependiente de anticuerpo (ADCC).

El término "región hipervariable", cuando se emplea en esta solicitud, se refiere a los residuos de aminoácidos de un anticuerpo que son responsables de la unión a antígeno. La región hipervariable comprende generalmente residuos de aminoácidos de una "región determinante de complementariedad" o "CDR" (*por ejemplo*, residuos 24-34 (L1), 50-56 (L2) y 89-97 (L3) en el dominio variable de cadena ligera y 31-35 (H1), 50-65 (H2) y 95-102 (H3) en el dominio variable de cadena pesada; Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) y/o aquellos residuos de un "bucle hipervariable" (*por ejemplo*, residuos 26-32 (L1), 50-52 (L2) y 91-96 (L3) en el dominio variable de cadena ligera y 26-32 (H1), 53-55 (H2) y 96-101 (H3) en el dominio variable de cadena pesada; Chothia y Lesk *J. Mol. Biol.* 196:901-917 (1987)). Residuos de "región de estructura alterna" o "FR" son aquellos residuos de dominio variable distintos de los residuos de región hipervariable, como se definen en esta solicitud.

La digestión con papaína de anticuerpos produce dos fragmentos de unión a antígeno idénticos, llamados fragmentos "Fab", cada uno con un solo sitio de unión a antígeno, y un fragmento "Fc" residual, cuyo nombre refleja su capacidad de cristalizarse rápidamente. El tratamiento con pepsina proporciona un fragmento $F(ab')_2$ que posee dos sitios de unión a antígeno y es aún capaz de enlazar cruzadamente antígeno.

"Fv" es el fragmento de anticuerpo mínimo, que contiene un sitio completo de reconocimiento de antígeno y unión a antígeno. Esta región

consta de un dímero de un dominio variable de cadena pesada y cadena ligera en asociación estrecha, no covalente. Es en esta configuración que las tres regiones hipervariables de cada dominio variable interactúan para definir un sitio de unión a antígeno en la superficie del dímero V_H-V_L. Colectivamente, las seis regiones hipervariables confieren especificidad de unión a antígeno al anticuerpo. No obstante, incluso un dominio variable único (o la mitad de un Fv que comprende únicamente tres regiones hipervariables específicas para un antígeno) tiene la capacidad de reconocer y unir antígeno, si bien con una afinidad más baja que todo el sitio de unión.

El fragmento Fab contiene igualmente el dominio constante de la cadena ligera y el primer dominio constante (CH1) de la cadena pesada. Los fragmentos Fab' difieren de los fragmentos Fab por la adición de unos pocos residuos en el término carboxi del dominio CH1 de cadena pesada, incluyendo una o más cisteínas de la región de unión flexible del anticuerpo. Fab'-SH es la designación en esta solicitud para Fab', en el cual el residuo(s) de cisteína de los dominios constantes lleva por lo menos un grupo tiol libre. Fragmentos $F(ab')_2$ de anticuerpo se produjeron originalmente como pares de fragmentos Fab', que tienen cisteínas de unión flexible entre ellos. Otros acoplamientos químicos de fragmentos de anticuerpo son también conocidos.

Las "cadenas ligeras" de anticuerpos de cualquier especie vertebrada pueden asignarse a uno de dos tipos claramente distintos, llamados kappa (κ) y lambda (λ), basados en las secuencias de aminoácidos de sus dominios constantes.

Fragmentos de anticuerpo "Fv de cadena única" o "scFv" comprenden los dominios V_H y V_L del anticuerpo, en donde estos dominios están presentes en una sola cadena polipeptídica. Preferiblemente, el polipéptido Fv comprende además un conector polipeptídico entre los dominios V_H y V_L, que permite que el scFv forme la estructura deseada para unión a antígeno. Para una reseña del scFv, véase Plückthun en *The Pharmacology of Monoclonal Antibodies,* vol. 113, Rosenburg and Moore

eds., Springer-Verlag, New York, páginas 269-315(1994). Los fragmentos scFv del anticuerpo HER2 se describen en WO93/16185; Patente de los Estados Unidos No. 5,571,894; y Patente de los Estados Unidos No. 5,587,458.

El término "diacuerpos" se refiere a pequeños fragmentos de anticuerpo con dos sitios de unión a antígeno, los cuales fragmentos comprenden un dominio pesado variable (V_H) conectado a un dominio variable ligero (V_L) en la misma cadena polipeptídica (V_H–V_L). Usando un conector que es demasiado corto para permitir el apareamiento entre los dos dominios en la misma cadena, los dominios son forzados a emparejarse con los dominios complementarios de otra cadena y a crear dos sitios de unión a antígeno. Los diacuerpos se describen más completamente en, por ejemplo, EP 404,097; WO 93/11161; y Hollinger *et. al., Proc. Natl. Acad. Sci. USA*, 90: 6444-6448 (1993).

Formas "humanizadas" de anticuerpos no humanos (por ejemplo, de roedor) son anticuerpos quiméricos que contienen una secuencia mínima derivada de inmunoglobulina no humana. En su mayor parte, los anticuerpos humanizados son inmunoglobulinas humanas (anticuerpo recipiendario), en las cuales residuos de una región hipervariable del recipiendario 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 (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 recipiendario 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 bucles hipervariables 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).

Anticuerpos HER2 humanizados incluyen huMAb4D5-l, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 y huMAb4D5-8 o trastuzumab (HERCEPTIN®) como se describe en la Tabla 3 de la Patente de los Estados Unidos 5,821,337 expresamente incorporada en esta solicitud por referencia; los anticuerpos 520C9 humanizados (WO93/21319) y 2C4 humanizados, como se describe en esta solicitud.

Para los propósitos de esta solicitud, "trastuzumab," "HERCEPTIN®," y "huMAb4D5-8" se refieren a un anticuerpo que comprende las secuencias de aminoácidos de cadena ligera y pesada en SEQ ID NOS. 5 y 6, respectivamente.

En esta solicitud, "pertuzumab" y "OMNITARG™" se refieren a un anticuerpo que comprende las secuencias de aminoácidos ligeras variables y pesadas variables en SEQ ID Nos. 7 y 8, respectivamente.

Un "anticuerpo desnudo" es un anticuerpo (como se define en esta solicitud), que no se conjuga a una molécula heteróloga, tal como una fracción citotóxica o radiomarca.

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 incorporaciones preferidas, 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 no estará presente por lo menos un componente del entorno natural del anticuerpo. De ordinario, sin embargo, el antagonista aislado se preparará en por lo menos un paso de purificación.

Un anticuerpo "madurado por afinidad" es uno con una o más alteraciones en una o más regiones hipervariables 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 madurados por afinidad tendrán afinidades nanomolares o, incluso, picomolares para el antígeno diana. Anticuerpos madurados por afinidad se producen por procedimientos conocidos en el arte. Marks *et al.*, *Biotechnology*, 10:779-783 (1992) describe la maduración por afinidad por entremezcla de dominio (***"domain shuffling"***) VH y VL. Mutagénesis aleatoria de CDR y/o residuos de estructura alterna se describen en: Barbas, *et al.*, *Proc. Nat. Acad. Sci, USA* 91:3809-3813 (1994); Shier *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).

El término "anticuerpo de especie principal" en esta solicitud se refiere a la estructura del anticuerpo en una composición que es la molécula del anticuerpo cuantitativamente predominante en la composición.

Un anticuerpo de "variante de secuencia de aminoácidos" en esta solicitud es un anticuerpo con una secuencia de aminoácidos que difiere de un anticuerpo de especie principal. Ordinariamente, variantes de secuencia de aminoácidos poseerán por lo menos alrededor de 70% de homología con el anticuerpo de especie principal y, preferiblemente, serán por lo menos alrededor de 80%, más preferiblemente, por lo menos alrededor de 90% homólogas con el anticuerpo de especie principal. Las variantes de secuencia de aminoácidos poseen sustituciones, deleciones, y/o adiciones en

ciertas posiciones dentro de, o adyacentes a, la secuencia de aminoácidos del anticuerpo de especie principal.

Un anticuerpo de "variante de glucosilación" en esta solicitud es un anticuerpo con una o más fracciones carbohidrato unidas a él, que difieren de una o más fracciones carbohidrato unidas a un anticuerpo de especie principal.

Un anticuerpo "desamidado" es uno en el cual uno o más residuos asparagina del mismo se han derivado, por ejemplo, a un ácido aspártico, una succinimida, o un ácido iso-aspártico.

Los términos "cáncer" y "canceroso" se refieren a, o describen, la condición fisiológica en mamíferos, que se caracteriza típicamente por un crecimiento celular descontrolado. Ejemplos de cáncer incluyen, pero no se limitan a, carcinoma, linfoma, blastoma (incluidos, meduloblastoma y retinoblastoma), sarcoma (incluidos, liposarcoma y sarcoma de células sinoviales), tumores neuroendocrinos (incluidos, tumores carcinoides, gastrinoma e insulinoma - ***"islet cell cancer"***), mesotelioma, schwannoma (incluido neuroma acústico), meningioma, adenocarcinoma, melanoma y leucemia o neoplasias malignas linfoides. Ejemplos más particulares de tales neoplasias malignas incluyen carcinoma epidermoide – (***"squamous cell cancer"***) (*por ejemplo*, carcinoma epidermoide epitelial), cáncer de pulmón, incluidos cáncer de pulmón microcítico, cáncer de pulmón amicrocítico (NSCLC), adenocarcinoma del pulmón y carcinoma epidermoide del pulmón, cáncer del peritoneo, cáncer hepatocelular, cáncer gástrico o del estómago, incluido cáncer gastrointestinal, cáncer pancreático, glioblastoma, cáncer cervical, cáncer de ovarios, cáncer del hígado, cáncer de la vejiga, hepatoma, cáncer de mama (incluido cáncer de mama metastásico), cáncer de colon, cáncer rectal, cáncer colorrectal, cáncer del endometrio o carcinoma uterino, carcinoma de glándula salival, cáncer de riñón o renal, cáncer de próstata, cáncer de la vulva, cáncer de la tiroides, carcinoma hepático, carcinoma anal, carcinoma del pene, así como cáncer de cabeza y cuello.

Un mamífero con un "cáncer resistente al inhibidor HER" es uno que ha evolucionado a pesar de recibir terapia a base de inhibidor HER (es decir, el paciente "no responde al inhibidor HER"), o el mamífero ha evolucionado en un término de 12 meses (por ejemplo, en un término de 6 meses) luego de concluir un régimen terapéutico a base de inhibidor HER. La terapia a base de inhibidor HER incluye tratamiento con un inhibidor HER desnudo o conjugado, donde el inhibidor HER se administra como un agente único, o en combinación con otro(s) fármacos(s) anti-tumoral(es). El inhibidor HER puede ser trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, o TAK165, pero preferiblemente es trastuzumab.

Un "mamífero" para efectos de tratamiento se refiere a cualquier animal clasificado como un mamífero, incluidos humanos, animales domésticos y de granja, y animales de zoológico, deportivos, o mascotas, tales como perros, caballos, gatos, vacas, etc. Preferiblemente, el mamífero es humano.

Una "muestra de tumor" en esta solicitud es una muestra derivada, o que comprende células tumorales provenientes, de un tumor de un paciente. Ejemplos de muestras de tumor en esta solicitud incluyen, pero no se limitan a, biopsias de tumor, células tumorales en circulación, proteínas plasmáticas en circulación, fluido ascítico, cultivos de células primarias o líneas de células de tumores, o que exhiben propiedades tipo tumor, así como muestras preservadas de tumor, tales como muestras de tumor fijadas en formalina, incrustadas en parafina o muestras de tumor congeladas.

Una muestra de tumor "fijada" es una que se ha preservado histológicamente usando un fijador.

Una muestra de tumor "fijada en formalina" es una que se ha preservado usando formaldehído como el fijador.

Una muestra de tumor "incrustada" es una rodeada por un medio firme y generalmente duro tal como parafina, cera, celoidina, o una resina. La incrustación posibilita el corte de secciones finas para examen microscópico o para generación de microarreglos de tejido (TMAs).

Una muestra de tumor "incrustada en parafina" es una rodeada por una mezcla purificada de hidrocarburos sólidos derivados de petróleo.

En esta solicitud, una muestra de tumor "congelada" se refiere a una mezcla de tumor que está congelada, o se ha congelado.

Un "agente inhibidor de crecimiento", cuando se emplea en esta solicitud, se refiere a un compuesto o composición que inhibe el crecimiento de una célula, especialmente una célula cancerosa que expresa HER *in vitro* o *in vivo*. Por consiguiente, el agente inhibidor de crecimiento puede ser uno que reduce significativamente el porcentaje de células que expresan HER 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 de G1 y la detención de fase M. Bloqueadores clásicos de fase M incluyen las vincas (vincristina y vinblastina), taxanos e inhibidores topo II tales como doxorrubicina, epirrubicina, daunorrubicina, 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-fluorouracilo, 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.

Ejemplos de anticuerpos "inhibidores de crecimiento" son aquellos que se unen a HER2 e inhiben el crecimiento de células cancerosas, que sobreexpresan HER2. Anticuerpos HER2 inhibidores de crecimiento preferidos inhiben el crecimiento de células de tumor de mama SK-BR-3 en un cultivo de células en más de 20% y, preferiblemente, más de 50% (por ejemplo, desde alrededor de 50% a alrededor de 100%) con una concentración de anticuerpo de alrededor de 0.5 a 30 μg/ml, en donde la inhibición del crecimiento se determina seis días después de la exposición de las células SK-BR-3 al anticuerpo (véase Patente de los Estados Unidos No. 5,677,171 expedida el 14 de octubre de 1997). El análisis de inhibición

del crecimiento de las células SK-BR-3 se describe con más detalle en esa patente y más adelante en esta solicitud. El anticuerpo inhibidor de crecimiento preferido es una variante humanizada del anticuerpo monoclonal murino 4D5, por ejemplo, trastuzumab.

Un anticuerpo que "induce apoptosis" es uno que induce la muerte celular programada, determinada por la unión de anexina V, fragmentación de ADN, contracción celular (***"cell shrinkage"***), dilatación del retículo endoplasmático, fragmentación celular, y/o formación de vesículas de membrana (denominadas cuerpos apoptóticos). Usualmente la célula es una que sobreexpresa el receptor HER. Preferiblemente la célula es una célula tumoral, *por ejemplo*, una célula de mama, ovario, estómago, endometrio, glándula salival, pulmón, riñón, colon, tiroides, páncreas o vejiga. *In vitro*, la célula puede ser una célula SK-BR-3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 o SKOV3. Se dispone de varios métodos para evaluar los episodios celulares asociados con apoptosis. Por ejemplo, puede medirse la translocación de fosfatidil serina (PS) mediante unión de anexina; la fragmentación de ADN puede evaluarse a través de análisis de fragmentación del ADN en geles de agarosa ("***DNA laddering***"); y condensación nuclear/cromatina junto con fragmentación de ADN puede evaluarse por cualquier aumento en células hipodiploides. Preferiblemente, el anticuerpo que induce apoptosis es uno que resulta en alrededor de 2 a 50 veces, preferiblemente alrededor de 5 a 50 veces y, muy preferiblemente, alrededor de 10 a 50 veces, la inducción de unión de anexina, relativa a una célula no tratada, en un análisis de unión a anexina usando células BT474 (ver más adelante). Ejemplos de anticuerpos HER2 que inducen apoptosis son 7C2 y 7F3.

El "epítopo 2C4" es la región en el dominio extracelular de HER2 al cual se une el anticuerpo 2C4. Con el fin de detectar anticuerpos que se unen al epítopo 2C4, puede llevarse a cabo un análisis de bloqueo cruzado rutinario, tal como el descrito en *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988). Preferiblemente el anticuerpo bloquea la unión de 2C4 a HER2 en alrededor

de 50% o más. Alternativamente, puede realizarse mapeo de epítopos para evaluar si el anticuerpo se une al epítopo 2C4 de HER2. El epítopo 2C4 comprende residuos del dominio II en el dominio extracelular de HER2. 2C4 y pertuzumab se unen al dominio extracelular de HER2 en el empalme de los dominios I, II y III. Franklin *et al., Cancer Cell* 5:317-328 (2004).

El "epítopo 4D5" es la región en el dominio extracelular de HER2 a la cual se unen el anticuerpo 4D5 (ATCC CRL 10463) y trastuzumab. Este epítopo es cercano al dominio transmembrana de HER2, y está en el Dominio IV de HER2. Con el fin de detectar anticuerpos que se unen al epítopo 4D5, puede llevarse a cabo un rutinario análisis de bloqueo cruzado, tal como el descrito en *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988). Alternativamente, puede realizarse mapeo de epítopos para evaluar si el anticuerpo se une al epítopo 4D5 de HER2 (*por ejemplo*, uno cualquiera o más residuos en la región desde alrededor del residuo 529 a alrededor del residuo 625, inclusive, del ECD de HER2, numeración de residuos que incluye el péptido señal).

El "epítopo 7C2/7F3" es la región en el término N, en el Dominio I, del dominio extracelular de HER2, al cual se unen los anticuerpos 7C2 y/o 7F3 (cada uno depositado con la ATCC, véase más adelante). Con el fin de detectar anticuerpos que se unen al epítopo 7C2/7F3, puede llevarse a cabo un rutinario análisis de bloqueo cruzado, tal como el descrito en *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988). Alternativamente, puede realizarse mapeo de epítopos para evaluar si el anticuerpo se une al epítopo 7C2/7F3 en HER2 (*por ejemplo*, uno cualquiera o más residuos en la región desde alrededor del residuo 22 a alrededor del residuo 53 del ECD de HER2, numeración de residuos que incluye el péptido señal).

"Tratamiento" se refiere tanto a tratamiento terapéutico como a medidas profilácticas o preventivas. Las personas en necesidad de tratamiento incluyen aquellas que ya sufren la enfermedad, así como aquellas en las cuales tiene que prevenirse la enfermedad. Por consiguiente,

el paciente a tratar en esta solicitud puede haberse diagnosticado como portador de la enfermedad o puede estar predispuesto o ser susceptible a la enfermedad.

El término "cantidad efectiva" se refiere a una cantidad de un medicamento, efectiva para tratar cáncer en el paciente. La cantidad efectiva del medicamento puede disminuir el número de células cancerosas; reducir el tamaño del tumor; inhibir (*es decir*, retardar en alguna medida y, preferiblemente, detener) la infiltración de células cancerosas en órganos periféricos; inhibir (*es decir*, retrasar en alguna medida y, preferiblemente, detener) la metástasis del tumor; inhibir en alguna medida el crecimiento del tumor; y/o aliviar en alguna medida uno o más de los síntomas asociados con el cáncer. En la medida en que el medicamento puede prevenir el crecimiento y/o eliminar células cancerosas existentes, éste puede ser citostático y/o citotóxico. La cantidad efectiva puede ampliar la supervivencia libre de progresión, resultar en una respuesta objetiva (incluidas una respuesta parcial, PR, o respuesta completa, CR), incrementar el tiempo total de supervivencia, y/o mejorar uno o más síntomas de cáncer.

"Respuesta completa" o "remisión completa" tienen por objeto la desaparición de todos los signos de cáncer en respuesta al tratamiento. Esto no siempre significa que el cáncer se ha curado.

"Respuesta parcial" se refiere a una disminución en el tamaño de uno o más tumores o lesiones, o en la medida de cáncer en el organismo, en respuesta al tratamiento.

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, 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.

Un "agente quimioterapéutico" es un compuesto químico útil en el tratamiento de enfermedades como cáncer. Ejemplos de agentes quimioterapéuticos incluyen agentes alquilantes tales como tiotepa y ciclosfosfamida CYTOXAN®; alquilsulfonatos tales como busulfán, improsulfán y piposulfán; aziridinas tales como benzodopa, carbocuona, meturedopa, y uredopa; etileniminas y metilamelaminas incluidas altretamina, trietilenmelamina, trietilenfosforamida, trietilentiofosforamida y trimetilolomelamina; TKL 286 (TELCYTA™); acetogeninas (especialmente bulatacina y bulatacinona); delta-9-tetrahidrocannabinol (dronabinol, MARINOL®); beta-lapacona; lapacol; colquicinas; ácido betulínico; una camptotecina (incluido el análogo sintético topotecán (HYCAMTIN®), CPT-11 (irinotecán, CAMPTOSAR®), acetilcamptotecina, escopolectina, 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 sarcodictiina; espongistatina; mostazas nitrogenadas tales como clorambucil, clornafazina, colofosfamida, estramustina, ifosfamida, mecloretamina, clorhidrato de óxido de mecloretamina, melfalán, novobiocina, fenesterina, prednimustina, trofosfamida, mostaza de uracilo; nitrosureas tales como carmustina, clorozotocina, fotemustina, lomustina, nimustina, y ranimustina; biofosfonatos, tales como clodronato; antibióticos tales como los antibióticos de enediíno (*por ejemplo*, caliqueamicina, especialmente caliqueamicina gamma 1I y caliqueamicina omega I1 (véase, *por ejemplo*, Agnew, *Chem Intl. Ed. Engl.*, 33: 183-186 (1994)) y antraciclinas tales como annamicina, AD 32, alcarrubicina, daunorrubicin, dexrazoxano, DX-52-1, epirrubicina, GPX-100, idarrubicina, KRN5500, menogaril, dinemicina, incluida dinemicina A; una esperamicina, cromóforo de neocarzinostatina y cromóforos antibióticos relacionados de enediíno cromoproteína), aclacinomicinas, actinomicina, autramicina, azaserina,

bleomicinas, cactinomicina, carabicina, carminomicina, carzinofilina, cromomicinas, dactinomicina, daunorrubicina, detorrubicina, 6-diazo-5-oxo-L-norleucina, ADRIAMYCIN® doxorrubicina (incluidas, morfolino-doxorrubicina, cianomorfolino-doxorrubicina, 2-pirrolino-doxorrubicina, doxorrubicina liposómica, y deoxidoxorrubicina), esorrubicina, marcelomicina, mitomicinas tales como mitomicina C, ácido micofenólico, nogalamicina, olivomicinas, peplomicina, potfiromicina, puromicina, quelamicina, rodorrubicina, estreptonigrina, estreptozocina, tubercidina, ubenimex, zinostatina, y zorrubicina; análogos de ácido fólico tales como denopterina, pteropterina, y trimetrexato; análogos de purina tales como fludarabina, 6-mercaptopurina, tiamiprina, y tioguanina; análogos de pirimidina tales como ancitabina, azacitidina, 6-azauridina, carmofur, citarabina, dideoxiuridina, doxifluridina, enocitabina, y floxuridina; andrógenos tales como calusterona, propionato de dromostanolona, epitiostanol, mepitiostano, y testolactona; anti-adrenales tales como aminoglutetimida, mitotano, y trilostano; reabastecedor de ácido fólico tal como ácido folínico (leucovorina); aceglatona; agentes anti-neoplásicos anti-folato tales como ALIMTA®, LY231514 pemetrexed, inhibidores de dihidrofolato reductasa tales como metotrexato, antimetabolitos tales como 5-fluorouracil (5-FU) y sus profármacos tales como UFT, S-1 y capecitabina, e inhibidores de timidilato sintasa e inhibidores de glicinamida ribonucleótido formiltransferasa tales como raltitrexed (TOMUDEX^{RM}, TDX); inhibidores de dihidropirimidina dehidrogenasa tales como eniluracilo; glucósido de aldofosfamida; ácido aminolevulínico; amsacrina; bestrabucil; bisantreno; edatraxato; defofamina; demecolcina; diazicuona; elfornithine; acetato de eliptinio; una epotilona; etoglucid; nitrato de galio; hidroxiurea; lentinan; lonidainina; maitansinoides tales como maitansina y ansamitocinas; mitoguazona; mitoxantrona; mopidamol; nitracrina; pentostatina; phenamet; pirarrubicina; losoxantrona; 2-etilhidrazida; procarbazina; PSKL® complejo de polisacáridos (JHS Natural Products, Eugene, OR); razoxano; rizoxina; sizofirano; espirogermanio; ácido tenuazónico; triazicuona; 2,2',2"-triclorotrietilamina;

tricotecenos (especialmente T-2 toxina, verracurina A, roridina A y anguidina); uretano; vindesina (ELDISINE®, FILDESIN®); dacarbazina; mannomustina; mitobronitol; mitolactol; pipobroman; gacitosina; arabinósido ("Ara-C"); ciclofosfamida; tiotepa; taxoides y taxanos, *por ejemplo*, paclitaxel TAXOL® (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ líbre de cremóforo, formulación de nanopartículas diseñadas con albúmina de paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), y TAXOTERE® docetaxel (Rhône-Poulenc Rorer, Antony, France); clorambucil; agente quimioterapéutico antimetabolito tales como gemcitabina (GEMZAR®), 5-fluorouracil (5-FU), capecitabina (XELODA™), 6-mercaptopurina, metotrexato, 6-tioguanina, pemetrexed, raltitrexed, arabinosilcitosina ARA-C citarabina (CYTOSAR-U®), dacarbazina (DTIC-DOME®), azocitosina, deoxicitosina, piridmideno, fludarabina (FLUDARA®), cladrabina, y 2-deoxi-D-glucosa; 6-tioguanina; mercaptopurina; agentes quimioterapéuticos a base de platino tales como carboplatino, cisplatino, u oxaliplatino; vinblastina (VELBAN®), etopósido (VP-16); ifosfamida; mitoxantrona; vincristina (ONCOVIN®); vinca alcaloide; vinorelbina (NAVELBINE®); novantrona; edatrexato; daunomicina; aminopterina; xeloda; ibandronato; inhibidor de topoisomerasa RFS 2000; difluorometilornitina (DMFO); retinoides tales como ácido retinoico, sales farmacéuticamente aceptables, ácidos o derivados de cualquiera de los anteriores; así como combinaciones de dos o más de los anteriores tales como CHOP, una abreviatura de una terapia combinada de ciclofosfamida, doxorrubicina, vincristina, y prednisolona, y FOLFOX, una abreviatura para 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 o inhibir la acción de las hormonas en tumores, tales como anti-estrógenos y moduladores selectivos de receptor de estrógeno (SERM), incluidos, por ejemplo, tamoxifeno (incluido tamoxifeno NOLVADEX®), raloxifeno, droloxifeno, 4-hidroxitamoxifeno, trioxifeno, keoxifeno, LY117018, onapristona, y toremifeno FARESTON®;

inhibidores de aromatasa que inhiben la enzima aromatasa, la cual regula la producción de estrógenos en las glándulas suprarrenales, tales como, por ejemplo, 4(5)-imidazoles, aminoglutetimida, acetato de megestrol MEGASE®, exemestano AROMASIN®, formestanie, fadrozol, RIVISOR® vorozol, letrozol FEMARA®, y anastrozol ARIMIDEX®; y anti-andrógenos tales como flutamida, nilutamida, bicalutamida, leuprolida, y goserelina; así como troxacitabina (un análogo de 1,3-dioxolano nucleósido citosina); oligonucleótidos complementarios, particularmente los que inhiben la expresión de genes en vías señalizadoras implicadas en proliferación celular aberrante, tal como, por ejemplo, PKC-alpha, Raf, H-Ras, y receptor de factor de crecimiento epidérmico (EGF-R); vacunas tales como vacunas de terapia génica, por ejemplo, vacuna ALLOVECTIN®, vacuna LEUVECTIN®, y vacuna VAXID®; PROLEUKIN® rIL-2; inhibidor de topoisomerasa 1 LURTOTECAN®; ABARELIX® rmRH; y sus sales, ácidos o derivados farmacéuticamente aceptables de cualquiera de los anteriores.

Un "agente anti-angiogénico" se refiere a un compuesto que bloquea, o interfiere en algún grado, el desarrollo de vasos sanguíneos. El factor anti-angiogénico puede ser, por ejemplo, una molécula pequeña o anticuerpo que se une a un factor de crecimiento o receptor de factor de crecimiento, involucrado en promover angiogénesis. El factor anti-angiogénico preferido en esta solicitud es un anticuerpo que se une al Factor de Crecimiento Endotelial Vascular (VEGF), tal como bevacizumab (AVASTIN®).

El término "citocina" es un término genérico para proteínas liberadas por una población de células que actúan sobre otra célula como mediadores intercelulares. Ejemplos de tales citocinas son linfocinas, monocinas, y hormonas polipeptídicas tradicionales. Incluidas entre las citocinas se encuentran somatotropina tal como somatotropina humana, somatotropina humana N-metionilo, y somatotropina bobina; hormona paratiroide; tiroxina; insulina; proinsulina; relaxina; prorrelaxina; hormonas de glucoproteína tales como folitropina (FSH), tirotropina (TSH), y lutropina (LH); factor de crecimiento hepático; factor de crecimiento de fibroblastos;

prolactina; lactógeno placentario; factor de necrosis tumoral α y β; sustancia inhibidora de Müller; péptido asociado con gonadotropina de ratón; inhibina; activina; factor de crecimiento endotelial vascular; integrina; trombopoyetina (TPO); factores de crecimiento neurales; tales como NGF-β; factor de crecimiento plaquetario; factores de crecimiento tansformantes (TGF) tales como TGF-α y TGF-β; factor de crecimiento insulinoide tipo I y II; eritropoyetina (EPO); factores osteoinductores; interferones tales como interferón α, β, y γ; factores estimulantes de colonias (CSF) tales como CSF de macrófago (M-CSF); CSF de granulocito-macrófago (GM-CSF); y CSF de granulocito (G-CSF); interleucinas (IL) tales como IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; un factor de necrosis tumoral tal como TNF-α o TNF-β; y otros factores polipeptídicos incluido LIF y ligando de kit (KL). Tal como se emplea en esta solicitud, el término citocina incluye proteínas de fuentes naturales o de cultivo de células recombinantes y equivalentes biológicamente activos de las citocinas de secuencia natural.

Tal como se emplea en esta solicitud, el término "fármaco dirigido a EGFR" se refiere a un agente terapéutico que se une a EGFR y, opcionalmente, inhibe la activación de EGFR. Ejemplos de tales agentes incluyen anticuerpos y pequeñas moléculas que se unen a EGFR. Ejemplos de anticuerpos que se unen a EGFR incluyen MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (véase, Patente de los Estados Unidos No. 4,943, 533, Mendelsohn *et al.*) y sus variantes, tales como 225 quimerizado (C225 o Cetuximab; ERBUTIX®) y 225 humano reconfigurado (H225) (véase, WO 96/40210, Imclone Systems Inc.); IMC-11F8, un anticuerpo dirigido a EGFR, completamente humano (Imclone), anticuerpos que unen EGFR mutante tipo II (Patente de los Estados Unidos No. 5,212,290); anticuerpos humanizados y quiméricos que unen EGFR, como se describe en Patente de los Estados Unidos No. 5,891,996; y anticuerpos humanos que unen EGFR, tales como ABX-EGF (véase WO98/50433, Abgenix); EMD 55900 (Stragliotto *et al. Eur. J. Cancer* 32A:636-640 (1996));

EMD7200 (matuzumab) un anticuerpo EGFR humanizado dirigido contra EGFR que compite con los dos EGF y TGF-α por la unión EGFR; y mAb 806 o mAb 806 humanizado (Johns *et al, J. Biol. Chem.* 279(29):30375-30384 (2004)). El anticuerpo anti-EGFR puede conjugarse con un agente citotóxico, generando en esa forma un inmunoconjugado (véase, por ejemplo, EP659,439A2, Merck Patent GmbH). Ejemplos de moléculas pequeñas que se unen a EGFR incluyen ZD 1839 o Gefitinib (IRESSA™; Astra Zeneca), CP-358774 o Erlotinib (TARCEVA™; Genentech/OSI) y AG1478, AG1571 (SU 5271; Sugen); EMD-7200.

Un "inhibidor de tirosina cinasa" es una molécula que inhibe la actividad de una tirosina cinasa tal como un receptor HER. Ejemplos de tales inhibidores incluyen los fármacos dirigidos a EGFR anotados en el párrafo precedente, el inhibidor de tirosina cinasa HER2 de molécula pequeña tal como TAK165 disponible de Takeda, CP-724,714, un inhibidor selectivo oral de la tirosina cinasa de receptor ErbB2 (Pfizer y OSI); inhibidores duales HER tales como EKB-569 (disponible de Wyeth), el cual se une preferencialmente a EGFR pero inhibe las células que sobreexpresan a los dos HER2 yEGFR, GW572016 (disponible de Glaxo) un inhibidor de tirosina cinasa HER2 y EGFR oral; PKI-166 (disponible de Novartis); inhibidores pan-HER tales como canertinib (CI-1033; Pharmacia); inhibidores de Raf-1 tales como el agente complementario ISIS-5132, disponible de ISIS Pharmaceuticals, que inhibe la señalización Raf-1; inhibidores de TK no dirigidos a HER tales como mesilato de Imatinib (Gleevac™) disponible de Glaxo; inhibidor de cinasa I regulada por MAPK extracelular CI-1040 (disponible de Pharmacia); quinazolinas, tales como PD 153035, 4-(3-cloroanilino)-quinazolina; piridopirimidinas; pirimidopirimidinas; pirrolopirimidinas, tales como CGP 59326, CGP 60261 y CGP 62706; pirazolopirimidinas, 4-(fenilamina)-7H-pirrolo[2,3-d]pirimidinas; curcumina (diferuloil metano, 4,5-bis (4-fluoroanilino) ftalimida); tirfostinas que contienen fracciones nitrotiofeno; PD-0183805 (Warner-Lambert); moléculas complementarias (por ejemplo, aquellas que se unen a ácido nucleico que codifica HER); quinoxalinas (Patente de los

Estados Unidos No. 5,804,396); trifostinas (Patente de los Estados Unidos No. 5,804,396); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); inhibidores de pan-HER tales como CI-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); mesilato de Imatinib (Gleevac; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); CI-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Sugen); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-IC11 (Imclone); o como se describe en cualquiera de las siguientes publicaciones de patente: Patente de los Estados Unidos No. 5,804,396; WO99/09016 (American Cyanamid); WO98/43960 (American Cyanamid); WO97/38983 (Warner Lambert); WO99/06378 (Warner Lambert); WO99/06396 (Warner Lambert); WO96/30347 (Pfizer, Inc); WO96/33978 (Zeneca); WO96/3397 (Zeneca); y WO96/33980 (Zeneca.

II. Inhición de la Liberación de HER2

La presente solicitud concierne a un método para inhibir la liberación de HER2, que comprende tratar, o exponer, una célula que expresa HER2 con o a un antagonista de metaloproteasa de matriz (MMP) en una cantidad efectiva para inhibir la liberación de HER2. Preferiblemente, el antagonista de MMP es un antagonista de MMP anclada en membrana (MT-MMP), tales como MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT5-MMP (MMP-24), MT4-MMP (MMP-17), o MT6-MMP (MMP-25). Muy preferiblemente, la MT-MMP es MMP-15 y, deseablemente, el antagonista se une selectiva o preferencialmente a MMP-15, sin unir significativamente otras proteasas o MMP distintas de MMP-15, y/o el antagonista interfiere la función proteolítica de MMP-15 sin interferir significativamente la función de otras proteasas o MMPs distintas de MMP-15.

En la incorporación preferida, la célula tratada exhibe expresión, amplificación o activación de HER y/o MMP. Por ejemplo, la célula puede exhibir sobreexpresión o amplificación de HER2 y/o MMP-15.

La actividad del antagonista de MMP puede aumentarse combinándolo con otro medicamento anti-neoplásico, inhibidor HER, o

anticuerpo HER2 (tales como trastuzumab o pertuzumab). Ejemplos de inhibidores HER, que pueden combinarse con el antagonista de MMP, incluyen trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, y TAK165.

La invención se refiere asimismo a un método para reducir el nivel sérico del dominio extracelular (ECD) de HER2 en un mamífero, que comprende administrar un antagonista de metaloproteasa de matriz (MMP) al mamífero en una cantidad efectiva para reducir el nivel sérico ECD de HER2 en el mamífero. Opcionalmente, el mamífero tiene un elevado nivel de MMP.

La invención provee igualmente un método de tratar cáncer en un mamífero, que comprende administrar un antagonista de metaloproteasa de matriz (MMP) al mamífero en una cantidad efectiva para tratar cáncer. El cáncer puede exhibir expresión, amplificación o activación de HER y/o MMP. Por ejemplo, el cáncer puede exhibir sobreexpresión o amplificación de HER2 y/o MMP-15. En una incorporación, el mamífero tratado tiene un nivel sérico elevado de HER2 liberado o un nivel p95 HER2 elevado.

Esta invención se relaciona también con un método para tratar un cáncer resistente al inhibidor HER en un mamífero, que comprende administrar al mamífero un antagonista de metaloproteasa de matriz (MMP) en una cantidad efectiva para tratar el cáncer. Por ejemplo, el mamífero puede ser resistente a un anticuerpo HER2, tal como trastuzumab.

Se provee en igual forma un método para reducir el nivel p95 HER2 en una célula, que comprende exponer la célula a un antagonista de metaloproteasa de matriz (MMP) en una cantidad efectiva para reducir el nivel p95 HER2.

Pueden utilizarse varios antagonistas MMP, pero preferiblemente el antagonista MMP es un inhibidor de molécula pequeña o un anticuerpo. A continuación se describen métodos para elaborar anticuerpos.

III. Producción de Anticuerpos

Sigue una descripción con respecto a técnicas ejemplares para la producción de anticuerpos, utilizadas de acuerdo con la presente invención.

El antígeno a usarse para la producción de anticuerpos puede ser, *por ejemplo*, una forma soluble del antígeno o una porción del mismo, que contiene el epítopo deseado. Alternativamente, células que expresan el antígeno en su superficie celular (*por ejemplo*, células NIH-3T3 transformadas para sobreexpresar HER2; o una línea de célula de carcinoma tal como células SK- BR-3, véase Stancovski *et al. PNAS* (USA) 88:8691-8695 (1991)) puede emplearse para generar anticuerpos. Otras formas de antígeno útil para generar anticuerpos serán evidentes a los expertos en el arte.

(i) Anticuerpos Policlonales

Los anticuerpos policlonales se producen preferiblemente en animales por inyecciones múltiples subcutáneas (sc) o intraperitoneales (ip) del antígeno relevante y un coadyuvante. Puede ser útil conjugar el antígeno relevante a una proteína que sea inmunógena en la especie a inmunizar, *por ejemplo*, hemocianina de lapa, seroalbúmina, tiroglobulina bovina, o inhibidor de tripsina de soya, usando un agente bi-funcional o derivatizante, *por ejemplo*, éster de maleimidobenzoil sulfosuccinimida (conjugación mediante residuos de cisteína), N-hidroxisuccinimida (mediante residuos de lisina), glutaraldehído, anhídrido succínico, $SOCl_2$, o $R^1N=C=NR$, donde R y R^1 son diferentes grupos alquilo.

Los animales se inmunizan contra el antígeno, conjugados inmunógenos, o derivados, combinando, *por ejemplo,* 100 µg ó 5 µg de la proteína o conjugado (para conejos o ratones, respectivamente) con 3 volúmenes de coadyuvante completo de Freund e inyectando la solución intradérmicamente en múltiples sitios. Un mes después, los animales se revacunaron con 1/5 a 1/10 la cantidad original de péptido o conjugado en coadyuvante completo de Freund por inyección subcutánea en múltiples sitios. Siete a catorce días después, los animales se desangran, y el suero se analiza para establecer la concentración de anticuerpos. Los animales se revacunan hasta que se nivela la concentración. Preferiblemente, el animal se revacuna con el conjugado del mismo antígeno, pero conjugado a una proteína diferente y/o a través de un reactivo diferente de enlazamiento

cruzado. Asimismo pueden elaborarse conjugados en cultivo de células recombinantes como fusiones de proteína. Además, agentes agregantes tales como sulfato de aluminio son apropiados para intensificar la respuesta inmunitaria.

(ii) Anticuerpos Monoclonales

Varios métodos para preparar anticuerpos monoclonales en esta solicitud se encuentran disponibles en el arte. Por ejemplo, los anticuerpos monoclonales pueden elaborarse usando el método del hibridoma, descrito por primera vez por Kohler *et al., Nature*, 256:495 (1975), por métodos de ADN recombinante (Patente de los Estados Unidos No. 4,816,567).

En el método del hibridoma, se inmuniza un ratón u otro animal hospedero apropiado, como un hámster, en la forma descrita más arriba en el texto, para provocar linfocitos que produzcan o sean capaces de producir anticuerpos, que se unirán específicamente a la proteína usada para inmunización. Como alternativa, los linfocitos se pueden inmunizar *in vitro*. En seguida, los linfocitos se fusionan con células de mieloma usando un agente fusionante apropiado, tal como polietilen glicol, para formar una célula de hibridoma (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

Las células de hibridoma así preparadas se siembran y cultivan en un medio de cultivo adecuado, que preferiblemente contiene una o más sustancias que inhiben el crecimiento o supervivencia de las células de mieloma progenitoras no fusionadas. Por ejemplo, si las células de mieloma progenitoras carecen de la enzima hipoxantina guanina fosforribosil transferasa (HGPRT o HPRT), el medio de cultivo para los hibridomas incluirá típicamente hipoxantina, aminopterina, y timidina (medio HAT), sustancias que impiden el crecimiento de células deficientes en HGPRT.

Células preferidas de mieloma son aquellas que se fusionan eficientemente, favorecen una producción estable de alto nivel de anticuerpo por las células seleccionadas productoras de anticuerpo, y son sensibles a un medio tal como HAT. Entre éstas, líneas celulares de

mieloma preferidas son líneas de mieloma murino, tales como las derivadas de tumores de ratón MOPC-21 y MPC-11 disponibles del Salk Institute Cell Distribution Center, San Diego, California USA, y células SP-2 ó X63-Ag8-653 disponibles de la American Type Culture Collection, Rockville, Maryland, USA. También se han descrito líneas celulares de mieloma humano y heteromieloma ratón-humano para la producción de anticuerpos monoclonales humanos (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, páginas 51-63 (Marcel Dekker, Inc., New York, 1987)).

El medio de cultivo en el cual crecen las células del hibridoma se analiza para determinar la producción de anticuerpos monoclonales dirigidos contra el antígeno. Preferiblemente, la especificidad de unión de anticuerpos monoclonales producidos por células del hibridoma se determina por inmunoprecipitación o por un análisis de unión *in vitro*, tal como radioinmunoanálisis (RIA) o enzimoinmunoanálisis absorbente (ELISA).

Por ejemplo, la afinidad de unión del anticuerpo monoclonal puede determinarse mediante el análisis Scatchard de Munson *et al., Anal. Biochem.*, 107:220 (1980).

Una vez identificadas las células del hibridoma que producen anticuerpos de la especificidad, afinidad y/o actividad deseada, los clones pueden subclonarse limitando los procesos de dilución y cultivarse por métodos estándar (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986). Medios apropiados de cultivo para este propósito incluyen, por ejemplo, medio D-MEM o RPMI-1640. Adicionalmente, las células de hibridoma pueden cultivarse *in vivo* como tumores ascíticos en un animal.

Los anticuerpos monoclonales segregados por los subclones se separan convenientemente del medio de cultivo, fluido ascítico, o suero mediante procedimientos convencionales de purificación de inmunoglobulina tales como, por ejemplo, proteína A-sefarosa,

cromatografía en hidroxilapatita, electroforesis en gel, diálisis, o cromatografía de afinidad.

El ADN que codifica los anticuerpos monoclonales se aísla fácilmente y se secuencia usando procedimientos convencionales (*por ejemplo*, usando sondas de oligonucléotidos que son capaces de unirse específicamente a genes que codifican las cadenas pesadas y ligeras de anticuerpos murinos). Las células de hibridoma sirven como fuente preferida de tal ADN. Una vez aislado, el ADN puede ponerse en vectores de expresión, los cuales se transfectan luego en células hospederas tales como células de *E. coli*, células COS de simio, células derivadas de ovario de hámster chino (CHO), o células de mieloma, que no producen de otro modo proteína de anticuerpo, para obtener la síntesis de anticuerpos monoclonales en las células hospederas recombinantes. Artículos de reseña sobre la expresión recombinante en bacterias de ADN que codifican el anticuerpo incluyen Skerra *et al., Curr. Opinion in Immunol.*, 5:256-262 (1993) y Plückthun, *Immunol. Revs.* 130:151-188(1992).

En una incorporación adicional, los anticuerpos monoclonales o fragmentos de anticuerpo pueden aislarse de bibliotecas de bacteriófagos de anticuerpos, generadas usando las técnicas descritas en McCafferty *et al., Nature*, 348:552-554 (1990). Clackson *et al., Nature*, 352:624-628 (1991) y Marks *et al., J. Mol. Biol.*, 222:581-597 (1991) describen el aislamiento de anticuerpos humanos y murinos, respectivamente, usando bibliotecas de bacteriófagos. Publicaciones subsiguientes describen la producción de anticuerpos humanos de alta afinidad (rango nM) mediante entremezcla de cadenas (Marks *et al., Bio/Technology*, 10:779-783 (1992)), así como infección combinatoria y recombinación *in vivo* como una estrategia para construir bibliotecas muy grandes de bacteriófagos (Waterhouse *et al., Nuc. Acids Res.*, 21:2265-2266 (1993)). Por consiguiente, estas técnicas son alternativas viables a las técnicas tradicionales de hibridoma de anticuerpo monoclonal para el aislamiento de anticuerpos monoclonales.

El ADN puede modificarse además, por ejemplo, sustituyendo la secuencia codificante para dominios constantes de cadenas ligeras y

pesadas humanas en vez de las secuencias murinas homólogas (Patente de los Estados Unidos No. 4,816,567; Morrison *et al., Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), o empalmando covalentemente a la secuencia codificante de inmunoglobulina toda, o parte, de la secuencia codificante para un polipéptido distinto de inmunoglobulina.

Típicamente tales polipéptidos distintos de inmunoglobulina se sustituyen por los dominios constantes de un anticuerpo, o se sustituyen por los dominios variables de un sitio combinante de antígeno de un anticuerpo, para crear un anticuerpo bivalente, quimérico, que comprende un sitio combinante de antígeno, con especificidad para un antígeno, y otro sitio combinante de antígeno con especificidad para un antígeno diferente.

(*iii*) *Anticuerpos Humanizados*

Métodos para humanizar anticuerpos no humanos se han descrito en el arte. Preferiblemente, un anticuerpo humanizado tiene 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 "importados", los cuales se toman típicamente de un dominio variable "importado". La humanización puede realizarse esencialmente siguiendo el método de Winter y colaboradores (Jones *et al., Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-327 (1988); Verhoeyen *et al., Science* 239:1534-1536 (1988), 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, ligeros 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 humanas de dominio variable conocidas. La secuencia humana más próxima a la del roedor se acepta entonces como la región de estructura alterna (FR) humana para el anticuerpo humanizado (Sims *et al., J. Immunol.*, 151:2296 (1993); Chothia *et al., J. Mol. Biol*, 196:901 (1987). Otro método usa una región de estructura alterna particular derivada de la secuencia consenso de todos los anticuerpos humanos de un subgrupo particular de cadenas ligeras y pesadas. La misma estructura alterna puede utilizarse para varios anticuerpos humanizados diferentes (Carter *et al., Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al., J. Immnol*, 151:2623 (1993)).

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 preferido, anticuerpos humanizados se preparan por un proceso de análisis de las secuencias progenitoras y varios productos humanizados conceptuales usando modelos tri-dimensionales de las secuencias progenitoras y humanizadas. Comúnmente se cuenta con modelos tri-dimensionales de inmunoglobulina y son familiares a los expertos en el arte. Se dispone de programas de computador, los cuales ilustran y exponen estructuras probables de conformación tri-dimensional de secuencias seleccionadas de la inmunoglobulina candidata. La inspección de estas exposiciones 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 inmunoglulina candidata de unir su antígeno. De esta forma, pueden seleccionarse residuos FR y combinarse de las secuencias del recipiendario e importadas, de modo que se obtenga la característica del anticuerpo deseada, tal como una 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.

(iv) Anticuerpos Humanos

Como una alternativa a la humanización, pueden generarse anticuerpos humanos. Por ejemplo, actualmente es posible producir animales transgénicos (*por ejemplo*, ratones) que, luego de la inmunización, son capaces de producir un completo repertorio de anticuerpos humanos en la ausencia de producción de inmunoglobulina endógena. Por ejemplo, se ha descrito que la deleción homocigótica del gen de la región de unión a la cadena pesada (J_H) del anticuerpo en ratones mutantes quiméricos y de línea germinativa resulta en la completa inhibición de la producción endógena de anticuerpos. La transferencia del arreglo de genes de inmunoglobulina de línea germinativa humana en tales ratones mutantes de línea germinativa resultará en la producción de anticuerpos humanos luego de la exposición al antígeno. Véase, *por ejemplo*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); Patentes de los Estados Unidos No. 5,591,669, 5,589,369 y 5,545,807.

Alternativamente, puede usarse tecnología exposición en bacteriófagos (McCafferty *et al., Nature* 348:552-553 (1990)) para producir anticuerpos humanos y fragmentos de anticuerpo *in vitro*, a partir de repertorios de genes de dominio variable (V) de inmunoglobulina de donantes no inmunizados. De acuerdo con esta técnica, genes de dominio V de anticuerpo se clonan sin cambio en la fase de lectura (***"are cloned in-frame"***) en un gen de cápside mayor o menor de un bacteriófago filamentoso, tal como M13 ó fd, y se exponen como fragmentos funcionales de anticuerpo en la superficie de la partícula del bacteriófago. Debido a que la partícula filamentosa contiene una copia de ADN monocatenario del genoma del bacteriófago, las selecciones basadas en las propiedades funcionales del anticuerpo resultan igualmente en la selección del gen codificante del anticuerpo que exhibe estas propiedades. Por consiguiente, el bacteriófago imita algunas de las propiedades del linfocito B. La exposición en bacteriófagos puede realizarse en una variedad de formatos; para su reseña véase, *por ejemplo*, Johnson, Kevin S. y Chiswell, David J.,

Current Opinion in Structural Biology 3:564-571 (1993). Para la exposición en bacteriófagos pueden emplearse varias fuentes de segmentos de gen V. Clackson *et al., Nature* 352:624-628 (1991) aislaron un arreglo diverso de anticuerpos de anti-oxazolona de una pequeña biblioteca combinatoria aleatoria de genes V, derivados de los bazos de ratones inmunizados. Puede construirse un repertorio de genes V de donantes no inmunizados humanos, y pueden aislarse anticuerpos contra un arreglo diverso de antígenos (incluidos auto-antígenos), siguiendo esencialmente las técnicas descritas por Marks *et al, J. Mol. Biol.* 222:581-597 (1991), o Griffith *et al., EMBO J.* 12:725-734 (1993). Véase también las Patentes de los Estados Unidos No. 5,565,332 y 5,573,905.

Como ya se discutió, pueden generarse asimismo anticuerpos humanos mediante linfocitos B activados *in vitro* (véase las Patentes de los Estados Unidos 5,567,610 y 5,229,275).

Anticuerpos HER2 humanos se describen en la Patente de los Estados Unidos No. 5,772,997 expedida el 30 de junio de 1998 y WO 97/00271 publicada el 3 de enero de 1997.

(v) Fragmentos de Anticuerpo

Se han desarrollado varias técnicas para la producción de fragmentos de anticuerpo, que comprenden una o más regiones de unión a antígeno. Tradicionalmente, estos fragmentos se derivaron por digestión proteolítica de anticuerpos intactos (véase, *por ejemplo*, Morimoto *et al., Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); y Brennan *et al., Science 229*:81 (1985)). Sin embargo, estos fragmentos pueden producirse ahora directamente por células hospederas recombinantes. Por ejemplo, los fragmentos de anticuerpo pueden aislarse de las bibliotecas de bacteriófagos de anticuerpos, discutidas anteriormente. Alternativamente, fragmentos Fab'-SH pueden recuperarse directamente de *E. coli* y acoplarse químicamente para formar fragmentos $F(ab')_2$ (Carter *et al., Bio/Technology* 10:163-167 (1992)). De acuerdo con otra metodología, fragmentos $F(ab')_2$ pueden aislarse directamente de cultivo de células hospederas recombinantes. Otras técnicas para la producción de fragmentos de

anticuerpo serán evidentes al profesional experto. En otras incorporaciones, el anticuerpo de elección es un fragmento Fv de cadena única (scFv). Véase WO 93/16185; Patente de los Estados Unidos No. 5,571,894 y Patente de los Estados Unidos No. 5,587,458. El fragmento de anticuerpo puede ser igualmente un "anticuerpo lineal", *por ejemplo*, como se describe en la Patente de los Estados Unidos 5,641,870. Tales fragmentos de anticuerpo lineal pueden ser monoespecíficos o bi-específicos.

(vi) Anticuerpos Biespecíficos

Anticuerpos biespecíficos son anticuerpos que tienen especificidades de unión para por lo menos dos epítopos diferentes. Anticuerpos biespecíficos ejemplares pueden unirse a dos epítopos diferentes de la proteína HER2. Otros anticuerpos similares pueden combinar un sitio de unión HER2 con sitio(s) de unión para EGFR, HER3 y/o HER4. Alternativamente, un brazo HER2 puede combinarse con un brazo que se fije a una molécula desencadenante en un leucocito, tal como una molécula de receptor de leucocito T (*por ejemplo,* CD2 ó CD3), o receptores Fc para IgG (FcγR), tales como FcγRI (CD64), FcγRII (CD32) y FcγRIII (CD 16), de modo que se enfoquen mecanismos de defensa celular a la célula que expresa HER2. Anticuerpos biespecíficos pueden usarse asimismo para localizar agentes citotóxicos contra células que expresan HER2. Estos anticuerpos poseen un brazo de unión HER2 y un brazo que une el agente citotóxico (*por ejemplo*, saporina, anti-interferón-α, vinca alcaloide, cadena de ricina A, metotrexato o el isótopo radioactivo hapteno). Los anticuerpos biespecíficos pueden prepararse como anticuerpos de longitud total o fragmentos de anticuerpo (por ejemplo, anticuerpos biespecíficos $F(ab')_2$).

WO 96/16673 describe un anticuerpo HER2/FcγRIH biespecífico, y la Patente de los Estados Unidos No. 5,837,234 divulga un anticuerpo HER2/FcγRI biespecífico IDM1 (Osidem). Un anticuerpo HER2/Fcα biespecífico se muestra en WO98/02463. La Patente de los Estados Unidos No. 5,821,337 enseña una anticuerpo HER2/CD3 biespecífico. MDX-210 es un HER2-FcγRIII Ab biespecífico.

Métodos para elaborar anticuerpos biespecíficos son conocidos en el arte. La producción tradicional de anticuerpos biespecíficos de longitud total se basa en la co-expresión de dos parejas de cadena ligera-cadena pesada de inmunoglobulina, donde las dos cadenas tienen especificidades diferentes (Millstein *et al., Nature*, 305:537-539 (1983)). Debido a la clasificación aleatoria de cadenas ligeras y pesadas de inmunoglobulina, estos hibridomas (cuadromas) producen una mezcla potencial de 10 diferentes moléculas de anticuerpo, de las cuales sólo una posee la estructura biespecífica correcta. La purificación de la molécula correcta, lo cual se hace usualmente por los pasos de cromatografía de afinidad, es más bien difícil, y los rendimientos del producto son bajos. Procedimientos similares se divulgan en WO 93/08829, y en Traunecker *et al., EMBO J.*, 10:3655-3659 (1991).

De acuerdo con un enfoque diferente, dominios variables de anticuerpo con las especificidades de unión deseadas (sitios de combinación anticuerpo-antígeno) se fusionan a secuencias de dominio constante de inmunoglobulina. La fusión es preferiblemente con un dominio constante de cadena pesada de inmunoglobulina, que comprende por lo menos parte de las regiones de unión flexible, CH2 y CH3. Se prefiere que la primera región constante de cadena pesada (CH1) contenga el sitio necesario para unión de la cadena ligera, presente en por lo menos una de las fusiones. Los ADN que codifican las fusiones de cadena pesada de inmunoglobulina y, si se desea, la cadena ligera de inmunoglobulina, se insertan en factores de expresión separados, y se co-transfectan en un organismo hospedero apropiado. Esto facilita una gran flexibilidad en ajustar las proporciones mutuas de los tres fragmentos del polipéptido en incorporaciones, cuando proporciones desiguales de las tres cadenas polipeptídicas usadas en la construcción proporcionan los rendimientos óptimos. No obstante, es posible insertar las secuencias codificantes para las dos o todas las tres cadenas polipeptídicas en un vector de expresión, cuando la expresión de por lo menos dos cadenas polipeptídicas en proporciones iguales resulta en

altos rendimientos, o cuando las proporciones no son de significación particular.

En una incorporación preferida de este enfoque, los anticuerpos biespecíficos se componen de una cadena pesada de inmunoglobulina híbrida con una primera especificidad de unión en un brazo, y una pareja de cadena ligera-cadena pesada de inmunoglobulina híbrida (que proporciona una segunda especificidad de unión) en el otro brazo. Se encontró que esta estructura asimétrica facilita la separación del compuesto biespecífico deseado de combinaciones no deseadas de cadena de inmunoglobulina, en la medida en que la presencia de una cadena ligera de inmunoglobulina en sólo una mitad de las moléculas biespecíficas ofrece un modo fácil de separación. Esta metodología se divulga en WO 94/04690. Para mayores detalles de generar anticuerpos biespecíficos, véase, por ejemplo, Suresh *et al., Methods in Enzymology* 121:210 (1986).

De acuerdo con otro enfoque descrito en la Patente de los Estados Unidos 5,731,168, la interfase entre una pareja de moléculas de anticuerpo puede manipularse genéticamente para maximizar el porcentaje de heterodímeros que se recuperan del cultivo de células recombinantes. La interfase preferida comprende por lo menos una parte del dominio C_H3 de un dominio constante de anticuerpo. En este método, una o más cadenas laterales pequeñas de aminoácidos de la interfase de la primera molécula de anticuerpo se reemplazan con cadenas laterales más grandes (*por ejemplo,* tirosina o triptófano). "Cavidades" compensatorias de tamaño idéntico o similar a la cadena(s) lateral(es) grande(s) se crea(n) en la interfase de la segunda molécula de anticuerpo, reemplazando cadenas laterales de aminoácidos grandes con más pequeñas (*por ejemplo,* alanina o treonina). Esto proporciona un mecanismo para aumentar el rendimiento del heterodímero sobre otros productos finales indeseados tales como homodímeros.

Anticuerpos biespecíficos incluyen anticuerpos enlazados cruzadamente o "heteroconjugados". Por ejemplo, uno de los anticuerpos en el heteroconjugado puede acoplarse a avidina, el otro a biotina. Tales

anticuerpos se han propuesto, por ejemplo, dirigir células del sistema inmunitario contra células no deseadas (Patente de los Estados Unidos No. 4,676,980), y para tratamiento de la infección por VIH (WO 91/00360, WO 92/200373, y EP 03089). Los anticuerpos heteroconjugados pueden elaborarse usando cualesquiera métodos convenientes de enlazamiento cruzado. Agentes apropiados de enlazamiento cruzado son bien conocidos en el arte, y se divulgan en la Patente de los Estados Unidos No. 4,676,980, junto con un número de técnicas de enlazamiento cruzado.

De igual manera, en la literatura se han descrito técnicas para generar anticuerpos biespecíficos de fragmentos de anticuerpo. Por ejemplo, anticuerpos biespecíficos pueden prepararse usando enlazamiento químico. Brennan *et al., Science* 229: 81 (1985) describen un procedimiento en donde anticuerpos intactos se segmentan proteolíticamente para generar fragmentos $F(ab')_2$. Estos fragmentos se reducen en la presencia del agente acomplejante ditiol arsenita de sodio para estabilizar ditioles vecinos e impedir la formación de disulfuro intermolecular. Los fragmentos Fab' generados se convierten luego en derivados de tionitrobenzoato (TNB). Uno de los derivados Fab'-TNB se reconvierte entonces en el Fab'-tiol por reducción con mercaptoetilamina y se mezcla con una cantidad equimolar del otro derivado Fab'-TNB para formar el anticuerpo biespecífico. Los anticuerpos biespecíficos producidos pueden usarse como agentes para la inmovilización selectiva de enzimas.

El avance reciente ha facilitado la recuperación directa de fragmentos Fab'-SH de *E. coli*, los cuales pueden acoplarse químicamente para formar anticuerpos biespecíficos. Shalaby *et al., J. Exp. Med.*, 175: 217-225 (1992) describen la producción de una molécula de anticuerpo biespecífico $F(ab')_2$ totalmente humanizado. Cada fragmento Fab' se segregó separadamente de *E. coli* y se sometió a acoplamiento químico dirigido *in vitro* para formar el anticuerpo biespecífico. El anticuerpo biespecífico así formado fue capaz de unirse a células que sobreexpresaban el receptor HER2 y linfocitos T humanos normales, así como de desencadenar la actividad lítica de linfocitos citotóxicos humanos contra dianas de tumor de mama humano.

Se han descrito asimismo varias técnicas para elaborar y aislar fragmentos de anticuerpo biespecífico, directamente del cultivo de células recombinantes. Por ejemplo, se han producido anticuerpos biespecíficos usando cremalleras de leucina. Kostelny *et al., J. Immunol.*, 148(5): 1547-1553 (1992). Los péptidos de cremallera de leucina de las proteínas Fos y Jun se enlazaron a las porciones Fab' de dos anticuerpos diferentes mediante fusión de genes. Los homodímeros de anticuerpo se redujeron en la región de unión flexible para formar monómeros y luego se re-oxidaron para formar heterodímeros del anticuerpo. Este método puede utilizarse igualmente para la producción de homodímeros de anticuerpo. La tecnología de "diacuerpos" descrita por Hollinger *et al., Proc. Natl. Acad Sci. USA*, 90: 6444-6448 (1993) ha proporcionado un mecanismo alternativo para elaborar fragmentos de anticuerpos biespecíficos. Los fragmentos comprenden un dominio variable de cadena pesada (V_H), conectado a un dominio variable de cadena ligera (V_L) por un conector que es demasiado corto para permitir el emparejamiento entre los dos dominios en la misma cadena. Por consiguiente, los dominios V_H y V_L de un fragmento son forzados a emparejarse con los dominios V_L y V_H complementarios del otro fragmento, formando así dos sitios de unión a antígeno. En la misma forma se ha informado de otra estrategia para elaborar fragmentos de anticuerpos biespecíficos mediante el uso de dímeros Fv de cadena única (sFv). Véase Gruber *et al., J. Immunol.*, 152:5368 (1994).

Se contemplan anticuerpos con más de dos valencias. Por ejemplo, pueden prepararse anticuerpos triespecíficos. Tutt *y colaboradores, J. Immunol.* 147: 60 (1991).

(vii) Otras modificaciones de secuencias de aminoácidos

Se contemplan modificaciones de secuencias 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 nucleotídicos apropiados en el ácido

nucleico del anticuerpo, o por síntesis peptídica. Tales modificaciones incluyen, por ejemplo, deleciones, y/o inserciones en y/o sustituciones, de residuos en las secuencias de aminoácidos del anticuerpo. Cualquier combinación de deleción, inserción, y sustitución se realiza para arribar al constructo final, siempre que el constructo final posea las características deseadas. Los cambios de aminoácidos pueden alterar igualmente el proceso pos-translacional del anticuerpo, tal como modificando el número o posición de los sitios de glucosilación.

Un método útil para la identificación de ciertos residuos o regiones del anticuerpo que son localizaciones preferidas para mutagénesis se denomina "mutagénesis por rastreo de alanina" como se describe por Cunningham and Wells *Science*, 244:1081-1085 (1989). Aquí, se identifican 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 el antígeno. Aquellas localizaciones de aminoácidos que demuestren sensibilidad funcional a las sustituciones se refinan entonces introduciendo variantes adicionales u otras en, o por, los sitios de substitución. En consecuencia, aunque el sitio para introducir una variación en la secuencia de aminoácidos está predeterminado, la naturaleza de la mutación *per se* no necesita estar predeterminada. Por ejemplo, para analizar el desempeño de una mutación en un sitio dado, se conduce mutagénesis aleatoria o por rastreo de alanina en el codón o región diana, y se detectan las variantes del anticuerpo expresadas con respecto a la actividad deseada.

Las inserciones de secuencias de aminoácidos incluyen fusiones amino- y/o carboxilo-terminales que van en longitud desde un residuo a polipéptidos que contienen cien o más residuos, así como inserciones intrasecuencia de residuos simples o múltiples de aminoácidos. Ejemplos de inserciones terminales incluyen un anticuerpo con un residuo metionilo N-terminal o el anticuerpo fusionado a un polipéptido citotóxico. Otras variantes de inserción de la molécula de anticuerpo incluyen la fusión al N-

o C-término del anticuerpo a una enzima (por ejemplo, para ADEPT) o un polipéptido que aumenta la semivida sérica 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 de anticuerpo reemplazado por un residuo diferente. Los sitios de máximo interés para mutagénesis de sustitución incluyen las regiones hipervariables, pero pueden contemplarse asimismo alteraciones de la región FR.

Puede sustituirse además cualquier residuo de cisteína no involucrado en mantener la conformación apropiada del anticuerpo, generalmente con serina, para mejorar la estabilidad oxidativa de la molécula y prevenir entrelazamiento cruzado aberrante. Recíprocamente, enlace(s) de cisteína puede(n) agregarse al anticuerpo para mejorar su estabilidad (particularmente donde el anticuerpo es un fragmento de anticuerpo tal como un fragmento Fv).

Un tipo particularmente preferido de variante de sustitución involucra sustituir uno o más residuos de región hipervariable de un anticuerpo progenitor (*por ejemplo*, un anticuerpo humano o humanizado). Generalmente, la(s) variante(s) resultante(s) para el desarrollo posterior tendrá(n) propiedades biológicas mejoradas relativas al anticuerpo progenitor, del cual se generan. Un modo conveniente para generar tales variantes de sustitución es maduración por afinidad usando la técnica de exposición en bacteriófagos. 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. Las variantes de anticuerpo así generadas se exponen en una manera monovalente de partículas de bacteriófago filamentoso como fusiones al producto del gen III de M13 empaquetado con cada partícula. Las variantes expuestas en bactriófago se detectan entonces 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 por rastreo de alanina, para

identificar residuos de región hipervariable que contribuyen significativamente a la unión al 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 HER2 humano. 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 detección, como se describe en esta solicitud, y pueden seleccionarse para desarrollo posterior anticuerpos con propiedades superiores en uno o más análisis relevantes.

Otro tipo de variante de aminoácidos del anticuerpo altera el patrón de glucosilación original del anticuerpo. Por alterar se da a entender eliminar una o más fracciones carbohidrato halladas en el anticuerpo, y/o agregar uno o más sitios de glucosilación que no están presentes en el anticuerpo.

La glucosilación de anticuerpos es típicamente N- u O-enlazada. N-enlazada se refiere a la unión de la fracción carbohidrato a la cadena lateral de un residuo de asparagina. Las secuencias tripeptídicas asparagina-X-serina y asparagina-X-treonina, donde X es cualquier aminoácido excepto prolina, son las secuencias de reconocimiento para unión enzimática de la fracción carbohidrato a la cadena lateral de asparagina. Por consiguiente, la presencia de cualquiera de estas secuencias tripeptídicas en un polipéptido crea un sitio potencial de glucosilación. Glucosilación O-enlazada se refiere a la unión de uno de los azúcares N-acetilgalactosamina, galactosa, o xilosa a un hidroxiaminoácido, muy comúnmente serina o treonina, si bien pueden usarse igualmente 5-hidroxiprolina o 5-hidroxilisina.

La adición de sitios de glucosilación al anticuerpo se lleva convenientemente a cabo alterando la secuencia de aminoácidos, de manera que contenga una o más de las secuencias tripeptídicas ya descritas (para sitios de glucosilación N-enlazados). La alteración puede hacerse además por la adición de, o sustitución por, uno o más residuos de serina o treonina

a la secuencia del anticuerpo original (para sitios de glucosilación O-enlazados).

Donde el anticuerpo comprende una región Fc, el carbohidrato unido a ella puede alterarse. Por ejemplo, anticuerpos con una estructura carbohidrato madura, que carece de la fucosa unida a una región Fc del anticuerpo, se describen en la Solicitud de Patente de los Estados Unidos No. US 2003/0157108 Al, Presta, L. Véase también US 2004/0093621 Al (Kyowa Hakko Kogyo Co., Ltd). Anticuerpos con una N-acetilglucosamina bisectante (GlcNAc) en el carbohidrato unido a una región Fc del anticuerpo se referencian en WO03/011878, Jean-Mairet *et al.*, y la Patente de los Estados Unidos No. 6,602,684, Umana *et al.* Anticuerpos con por lo menos un residuo de galactosa en el oligosacárido unido a una región Fc del anticuerpo se divulgan en WO97/30087, Patel *et al.* Véase, además, WO98/58964 (Raju, S.) y WO99/22764 (Raju, S.) en lo concerniente a anticuerpos con carbohidrato alterado unido a la región Fc de los mismos.

Puede ser deseable modificar el anticuerpo de la invención con respecto a la función efectora, *por ejemplo*, con el fin de intensificar la citotoxicidad celular dependiente de antígeno (ADCC) y/o la citotoxicidad dependiente de complemento (CDC) del anticuerpo. Esto puede lograrse introduciendo una o más sustituciones de aminoácidos en una región Fc del anticuerpo. Alternativa, o adicionalmente, puede(n) introducirse en la región Fc un residuo(s) de cisteína, permitiendo así la formación de enlaces disulfuro intracadena en esta región. El anticuerpo homodimérico así generado puede tener una capacidad de internalización mejorada y/o creciente eliminación celular mediada por complemento y citotoxicidad celular dependiente del anticuerpo (ADCC). Véase Caron *et al., J. Exp. Med.* 176:1191-1195 (1992) y Shopes, *J. Immunol.* 148: 2918-2922 (1992). Anticuerpos homodiméricos con actividad anti-tumoral mejorada pueden prepararse también usando entrecruzadores heterobifuncionales como se describe en Wolff *et al., Cancer Research* 53: 2560-2565 (1993). Alternativamente, puede diseñarse un anticuerpo que tenga regiones Fc duales y pueda, por ello, tener lisis de complemento mejorada y

posibilidades ADCC. Véase Stevenson *et al., Anti-Cancer Drug Design* 3:219-230 (1989).

WO00/42072 (Presta, L.) describe anticuerpos con función ADCC mejorada en la presencia de células efectoras humanas, donde los anticuerpos comprenden sustituciones de aminoácidos en su región Fc. Preferiblemente, el anticuerpo con ADCC mejorada comprende sustituciones en las posiciones 298, 333, y/o 334 de la región Fc. Preferiblemente la región Fc alterada es una región Fc de IgG1 humana, que comprende o consta de sustituciones en una, dos o tres de estas posiciones.

Anticuerpos con unión C1q alterada y/o citotoxicidad dependiente de complemento (CDC) se describen en WO99/51642, Patente de los Estados Unidos No. 6,194,551Bl, Patente de los Estados Unidos No. 6,242,195Bl, Patente de los Estados Unidos No. 6,528,624Bl y Patente de los Estados Unidos No. 6,538,124 (Idusogie *et al.*). Los anticuerpos comprenden una sustitución de aminoácido en una o más de las posiciones de aminoácidos 270, 322, 326, 327, 329, 313, 333 y/o 334 de la región Fc de los mismos (usando numeración de residuos de región Fc como en Kabat).

Para incrementar la semivida sérica del anticuerpo, se puede incorporar un epítopo de unión a receptor de salvamento al anticuerpo (especialmente un fragmento de anticuerpo), como se describe en la Patente de los Estados Unidos 5,739,277, por ejemplo. Tal como se emplea en esta solicitud, el término "epítopo de unión a receptor de salvamento" se refiere a un epítopo de la región Fc de una molécula IgG (*por ejemplo*, IgG_1, IgG_2, IgG_3, o IgG_4) que es responsable de aumentar la semivida sérica *in vivo* de la molécula IgG.

Anticuerpos con unión mejorada al receptor Fc neonatal (FcRn), y semividas incrementadas, se describen en WO00/42072 (Presta, L.) y US2005/0014934A1 (Hinton *et al.*). Estos anticuerpos comprenden una región Fc con una o más sustituciones en ella, las cuales refuerzan la unión de la región Fc a FcRn. Por ejemplo, la región Fc puede tener sustituciones en una o más de las posiciones 238, 250, 256, 265, 272, 286, 303, 305, 307, 311, 312, 314, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424, 428 o

434 de la misma (usando numeración de residuos de región Fc como en Kabat). La variante preferida de anticuerpo, que incluye la región Fc, con unión FcRn mejorada FcRn comprende sustituciones de aminoácidos en una, dos o tres de las posiciones 307, 380 y 434 de la región Fc del mismo.

También se contemplan anticuerpos genéticamente manipulados con tres o más (preferiblemente, cuatro) sitios de unión funcional a antígeno (Solicitud de los Estados Unidos No. US2002/0004587 A1, Miller *et al.*).

Moléculas de ácido nucleico que codifican variantes de secuencia de aminoácidos del anticuerpo se preparan mediante 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 oligonucleótidos (o dirigida al sito), mutagénesis PCR, y mutagénesis de casete de una variante previamente preparada o una versión no variante del anticuerpo.

(viii) Inmunoconjugados

La invención concierne además a inmunoconjugados que comprenden un anticuerpo conjugado a un agente citotóxico tal como un agente quimioterapéutico, 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).

Agentes quimioterapéuticos útiles en la generación de tales inmunoconjugados se han descrito más arriba. Conjugados de un anticuerpo y una o más toxinas de molécula pequeña, tales como una caliqueamicina, una maitansina (Patente de los Estados Unidos No. 5,208,020), un tricoteno, y CC1065 se contemplan igualmente en esta solicitud.

En una incorporación preferida de la invención, el anticuerpo se conjuga con una o más moléculas de maitansina (*por ejemplo*, alrededor de 1 a alrededor de 10 moléculas de maitansina por molécula de anticuerpo). La maitansina puede, por ejemplo, convertirse en May-SS-Me, el cual puede reducirse a May-SH3 y hacerse reaccionar con anticuerpo

modificado (Chari *et al. Cancer Research* 52: 127-131 (1992)), para generar un inmunoconjugado maitansinoide-anticuerpo.

Otro inmunoconjugado de interés comprende un anticuerpo conjugado con una o más moléculas de caliqueamicina. La familia caliqueamicina de antibióticos es capaz de producir cortes de ADN bicatenario en concentraciones sub-picomolares. Análogos estructurales de caliqueamicina que pueden usarse incluyen, pero no se limitan a, γ_1^I α_2^I, α_3^I, N-acetil- γ_1^I, PSAG y θ_1^I (Hinman *et al., Cancer Research* 53: 3336-3342 (1993) y Lode *et al., Cancer Research* 58: 2925-2928 (1998)). Véase, también, las Patentes de los Estados Unidos No. 5,714,586; 5,712,374; 5,264,586; y 5,773,001 expresamente incorporadas en esta solicitud por referencia.

Toxinas enzimáticamente activas y sus fragmentos, que pueden usarse, incluyen la cadena A de la difteria, fragmentos activos de no unión de toxina de la difteria, la cadena de exotoxina A (de *Pseudomonas aeruginosa)*, la cadena de ricina A, la cadena de abrina A, la cadena de modeccina A, 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 un inmunoconjugado formado entre un anticuerpo y un compuesto con actividad nucleolítica (*por ejemplo*, una ribonucleasa o una ADN endonucleasa tal como una deoxiribonucleasa; DNasa).

Una variedad de isótopos radioactivos se encuentra disponible para la producción de anticuerpos HER2 radioconjugados. Los ejemplos incluyen At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} e isótopos radioactivos de Lu.

Conjugados del anticuerpo y agente citotóxico pueden elaborarse 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 dimetil adipimidato), ésteres activos (tales como disuccinimidil suberato), aldehídos (tales como glutareldehído), compuestos bis-azido (tales como bis (p-azidobenzoil)hexanodiamina), derivados de bis-diazonio (tales como bis-(p-diazoniobenzoil)-etilendiamina), diisocianatos (tales como tolien 2,6-diisocianato), y compuestos de flúor bis-activo (tales como 1,5-difluoro-2,4-dinitrobenceno). Por ejemplo, puede prepararse una inmunotoxina de ricina como se describe en Vitetta *et al., 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 enlazante puede ser un "enlazante segmentable" que facilite la liberación del medicamento citotóxico en la célula. Por ejemplo, pueden usarse un enlazante lábil a ácidos, un enlazante sensible a peptidasa, un enlazante dimetílico o un enlazante que contenga disulfuro (Chari *et al., Cancer Res.* 52:127-131 (1992)).

Alternativamente, puede elaborarse una proteína de fusión que comprenda el anticuerpo y un agente citotóxico, *por ejemplo*, mediante técnicas recombinantes o síntesis peptídica.

En esta solicitud se contemplan otros inmunoconjugados. Por ejemplo, el anticuerpo puede enlazarse a uno de una variedad de polímeros no proteináceos, *por ejemplo*, polietilen glicol, polipropilen glicol, polioxialquilenos, o copolímeros de polietilen glicol y polipropilen glicol. En igual forma, el anticuerpo puede 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 administración de medicamentos (por ejemplo, liposomas, microesferas de albúmina, microemulsiones, nanopartículas y nanocápsulas), o en macroemulsiones. Tales técnicas y otras formulaciones

apropiadas se divulgan en *Remington's Pharmaceutical Sciences*, 16th edition, Oslo, A., Ed., (1980).

Los anticuerpos divulgados en esta solicitud pueden formularse además como inmunoliposomas. Los liposomas que contienen el anticuerpo se preparan por métodos conocidos en el arte, tales como los descritos en Epstein *et al., Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang *et al., Proc. Natl. Acad. Sci. USA*, 77: 4030 (1980); Patente de los Estados Unidos No. 4,485,045 y 4,544,545; y WO 1997/38731 publicada el 23 de octubre de 1997. Liposomas con tiempo de circulación mejorado se divulgan en la Patente de los Estados Unidos No. 5,013,556.

Liposomas particularmente útiles pueden generarse por el método de evaporación de fase reversa con una composición lipídica que comprenda fosfatidilcolina, colesterol, y fosfatidiletanolamina derivada de PEG (PEG-PE). Los liposomas se extruyen a través de filtros de tamaño de poro definido para proporcionar liposomas con el diámetro deseado. Fragmentos Fab' del anticuerpo de la presente invención pueden conjugarse a los liposomas como se describe en Martin *et al., J. Biol. Chem.*, 257:286-288 (1982) mediante una reacción de intercambio de disulfuro. Un agente quimioterapéutico se encuentra contenido opcionalmente en el liposoma. Véase Gabizon *et al., J. National Cancer Inst.* 81(19):1484(1989).

IV. Formulaciones Farmacéuticas

Formulaciones terapéuticas de los antagonistas de MMP utilizados de acuerdo con la presente invención se preparan mezclando un antagonista de MMP, que tiene el grado apropiado de pureza, con vehículos, excipientes, o estabilizantes opcionales (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. ed. (1980)), en la forma de formulaciones liofilizadas o soluciones acuosas. Vehículos, diluyentes, excipientes y estabilizantes aceptables son preferiblemente no tóxicos a los recipiendarios en las dosis y concentraciones empleadas, e incluyen amortiguadores tales como fosfato, citrato, 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 como glicina, glutamina, asparagina, histidina, arginina, o lisina; monosacáridos, disacáridos, y otros carbohidratos incluidos glucosa, mannosa, o dextrinas; agentes quelantes tales como EDTA; azúcares tales como sacarosa, mannitol, trehalosa o sorbitol; iones contrarios formadores de sales tales como sodio; complejos de metal (*por ejemplo*, complejos de Zn-proteína); y/o surfactantes no iónicos tales como TWEEN™, PLURONICS™ o polietilen glicol (PEG). Formulaciones liofilizadas de anticuerpo se describe en WO 97/04801, incorporada expresamente en esta solicitud por referencia.

La formulación en esta solicitud puede contener asimismo más de un compuesto activo, según sea necesario, para la indicación particular que se está tratando, preferiblemente aquellos con actividades complementarias que no se afectan negativamente entre sí. Varios medicamentos que pueden combinarse con el antagonista de MMP se describen en la siguiente sección Métodos de Tratamiento. Tales moléculas están convenientemente presentes en combinación, en cantidades que son efectivas para el propósito previsto.

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 administración de medicamentos (por ejemplo, liposomas, microesferas de albúmina, microemulsiones, nano-partículas y nanocápsulas) o en macroemulsiones. Tales técnicas se divulgan en *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Pueden prepararse preparaciones de liberación sostenida. Ejemplos apropiados de preparaciones de liberación sostenida incluyen matrices semipermeables de polímeros hidrófobos sólidos, que contienen el antagonista, 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)), polilácticos (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 LUPRON DEPOT™ (microesferas inyectables compuestas de copolímero de ácido láctico-ácido glicólico y acetato de leuprolida), y ácido poli-D-(-)-3-hidroxibutírico.

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.

V. Tratamiento

Ejemplos de varias neoplasias malignas que pueden tratarse con el antagonista MMP se enumeran en la anterior sección Definiciones. La administración del antagonista MMP resultará en una mejoría en los signos o síntomas del cáncer.

El antagonista MMP se administra a un paciente humano de acuerdo con métodos conocidos, tales como administración intravenosa, *por ejemplo*, como una inyección intravenosa rápida o por infusión continua durante un período de tiempo, por vías intramuscular, intraperitoneal, intracerobrospinal, subcutánea, intra-articular, intrasinovial, intratecal, oral, tópica, o inhalación.

Para la prevención o tratamiento de la enfermedad, la dosis del antagonista MMP dependerá del tipo de cáncer a tratarse, como ya se definió, la gravedad y evolución del cáncer, si el anticuerpo se administra para efectos preventivos o terapéuticos, terapia previa, la historia clínica del

paciente y la respuesta al anticuerpo, y la discreción del médico que atiende al paciente.

Aunque el antagonista MMP puede ser el único medicamento anti-tumoral administrado, el paciente se trata opcionalmente con una combinación del antagonista MMP, y uno o más de otro(s) agente(s) anti-tumoral(es). La administración combinada incluye coadministración o administración concurrente, usando formulaciones separadas o una sola formulación farmacéutica, y administración consecutiva en cualquier orden, en donde preferiblemente existe un período de tiempo durante el cual los dos (o todos) los agentes activos ejercen simultáneamente sus actividades biológicas. Por lo tanto, el otro agente anti-tumoral puede administrarse antes, o después, de la administración del antagonista MMP. En esta incorporación, el momento adecuado – ***timing*** – entre por lo menos una administración del antagonista MMP y por lo menos una administración del otro agente anti-tumoral es preferiblemente aproximadamente 1 mes o menos y, muy preferiblemente aproximadamente 2 semanas o menos. Alternativamente, el antagonista MMP y el otro agente anti-tumoral se administran concurrentemente al paciente, en una sola formulación o en formulaciones separadas. El tratamiento con la combinación del antagonista MMP y el otro agente anti-tumoral puede traducirse en un beneficio terapéutico sinérgico, o más que aditivo, al paciente.

Ejemplos de segundos agentes anti-tumorales que pueden combinarse con el antagonista MMP incluyen: uno o más agente(s) quimioterapéutico; un inhibidor HER (*por ejemplo*, trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, TAK165, etc); inhibidor Raf y/o (véase, por ejemplo, WO 2003/86467); un anticuerpo HER2 inhibidor de crecimiento tal como trastuzumab; un inhibidor de dimerización HER tal como Pertuzumab; un anticuerpo HER2 que induce apoptosis de una célula que sobreexpresa HER2, tal como 7C2, 7F3 o variantes humanizadas del mismo; un anticuerpo dirigido contra un antígeno asociado con un tumor, tal como EGFR, HER3, HER4; un compuesto anti-hormonal, *por ejemplo*, un

compuesto anti-estrógeno tal como tamoxifeno, o un inhibidor de aromatasa; un cardioprotector (para prevenir o reducir cualquier disfunción del miocardio, asociada con la terapia); una citocina; un medicamento dirigido a EGFR (tal como TARCEVA®, IRESSA® o Cetuximab); un agente anti-angiogénico (especialmente bevacizumab comercializado por Genentech bajo la marca registrada AVASTIN™); un inhibidor de tirosina cinasa; un inhibidor COX (por ejemplo un inhibidor COX-1 ó COX-2); un medicamento anti-inflamatorio non esteroide, Celecoxib (CELEBREX®); un inhibidor de farnesil transferasa (por ejemplo, Tipifarnib/ZARNESTRA® R115777 disponible de Johnson and Johnson o Lonafarnib SCH66336 disponible de Schering-Plough); un anticuerpo que une la proteína oncofetal CA 125 tal como Oregovomab (MoAb B43.13); una vacuna HER2 (tal como la vacuna HER2 Auto Vac de Pharmexia, o la vacuna de proteína APC8024 de Dendreon, o la vacuna de péptido HER2 de GSK/Corixa); inyección de liposomas de HCl de doxorrubicina (DOXIL®); un inhibidor de topoisomerasa I tal como topotecán; taxano; un inhibidor de tirosina ciansa dual HER2 y EGFR tal como lapatinib/GW572016; TLK286 (TELCYTA®); EMD-7200; un medicamento para bajar la temperatura tal como acetaminofén, difenhidramina, o meperidina; un factor de crecimiento hematopoyético, etc.

Posologías apropiadas para cualquiera de los anteriores agentes coadministrados son aquellos utilizados actualmente y pueden disminuirse debido a la acción combinada (sinergia) del agente y el antagonista MMP.

Además de los anteriores regímenes terapéuticos, el paciente puede someterse a resección quirúrgica de las células cancerosas y/o a terapia de radiación.

Aparte de la administración de antagonistas MMP proteína al paciente, la presente solicitud contempla la administración de antagonistas MMP mediante terapia génica. Véase, por ejemplo, WO 1996/07321 publicada el 14 de marzo de 1996 concerniente al empleo de terapia génica para generar anticuerpos intracelulares.

Existen dos estrategias principales para introducir el ácido nucleico (contenido opcionalmente en un vector) en las células del paciente: *in vivo* y *ex vivo*. Para tratamiento *in vivo*, el ácido nucleico se inyecta directamente en el paciente, usualmente en el sitio donde se requiere el anticuerpo. Para tratamiento *ex vivo*, se retiran las células del paciente, se introduce el ácido nucleico en estas células aisladas, y las células modificadas se administran al paciente ya sea directamente o ya sea, por ejemplo, encapsuladas en membranas porosas que se implantan en el paciente (véase, *por ejemplo*, las Patentes de los Estados Unidos No. 4,892,538 y 5,283,187). Hay una variedad de técnicas disponibles para introducir ácidos nucleicos en células viables. Las técnicas varían dependiendo de si el ácido nucleico se transfiere a células cultivadas *in vitro*, o *in vivo* en las células del hospedero previsto. Técnicas apropiadas para la transferencia de ácido nucleico en células mamíferas *in vitro* incluyen el uso de liposomas, electroporación, microinyección, fusión celular, DEAE-dextrina, el método de precipitación con fosfato cálcico, *etc.* Un vector usado comúnmente para la transferencia *ex vivo* del gen es un retrovirus.

Las técnicas de transferencia de ácido nucleico *in vivo* actualmente preferidas incluyen transfección con factores víricos (tales como adenovirus, virus Herpes simplex I, o virus adeno-asociado) y sistemas a base de lípidos (lípidos útiles para transferencia mediada por lípidos del gen son DOTMA, DOPE y DC-Chol, por ejemplo). En algunas situaciones es deseable proveer la fuente de ácido nucleico con un agente que focalice las células diana, tal como un anticuerpo específico para una proteína de membrana de superficie celular o la célula diana, un ligando para un receptor en la célula diana, etc. Donde se emplean liposomas, proteínas que se fijan a una proteína de membrana de superficie celular, asociadas con endocitosis pueden utilizarse para focalizar y/o facilitar la captación, *por ejemplo*, cápsides proteicas o sus fragmentos, trópicos para un tipo de célula particular, anticuerpos para proteínas que experimentan internalización durante el ciclo (***"in cycling"***), y proteínas que reconocen la localización intracelular e incrementan la semivida intracelular. La técnica de

endocitosis mediada por receptor se describe, por ejemplo, por Wu *et al., J. Biol. Chem.* 262:4429-4432(1987); y Wagner *et al. Proc. Natl. Acad. Sci. USA* 87: 3410-3414 (1990). Para una reseña de los protocolos actualmente conocidos de terapia génica y marcación de genes, véase Anderson *et al., Science* 256:808-813(1992). Véase también WO 1993/25673 y las referencias citadas allí.

VI. Depósito de Materiales

Las siguientes líneas de células de hibridoma se depositaron en la American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA (ATCC):

Designación Anticuerpo	ATCC No.	Fecha Depósito
7C2	ATCC HB-12215	Octubre 17, 1996
7F3	ATCC HB-12216	Octubre 17, 1996
4D5	ATCC CRL 10463	Mayo 24, 1990
2C4	ATCC HB-12697	Abril 8, 1999

Detalles adicionales de la invención se ilustran por los siguientes Ejemplos no limitantes. Las divulgaciones de todas las citas en esta especificación se incorporan expresamente en esta solicitud por referencia.

EJEMPLO 1

El siguiente ejemplo investigó el papel de metaloproteasas de matriz (MMP) en la liberación de HER2 (***"HER2 shedding"***).

MATERIALES Y MÉTODOS

Cultivo de Células y Transfecciones

Todas la líneas de células en este estudio se obtuvieron de la American Type Tissue Culture Collection (ATCC, Manassas, VA). Células BT 474, MCF-7, y SKBR-3 se mantuvieron en DMEM:Ham's F12 con alto contenido de glucosa (50:50), suplementado con 10% FBS termo-inactivado y 2mM L-glutamina. Células Cos-7 se mantuvieron en DMEM con alto contenido de glucosa, suplementado con 10% FBS termo-

inactivado y 2mM L-glutamina. Las líneas de células se mantuvieron a 37°C en un incubador humidificado, provisto con 5% CO_2. Células BT 474 y SKBR-3 se transfectaron transitoriamente con constructos, como se describe, mediante electroporación usando un kit V, de acuerdo con las instrucciones del fabricante (AMAXA™). Células Cos-7 se transfectaron, como se describe, usando LIPOFECTAMINE™ 2000 (Invitrogen) de acuerdo con las recomendaciones del fabricante.

Líneas de Tumor de Xenoinjerto de Ratón Fo5 y f2: 1282

Las líneas Fo5 y f2:1282 se han descrito previamente (Finkle *et al., Clin. Cancer Res.* 10: 2499- 2511 (2004)). Estas líneas se derivaron de tumores primarios de un ratón transgénico MMTV HER2 y se insertaron en el relleno graso mamario (***"the mammary fat pad"***) de ratones FVB.

Inyecciones GM6001

Tumores Fo5 se transplantaron en ratones FVB y se dejaron crecer a un tamaño medio de 400-600 mm^3 antes de iniciar el estudio. GM6001 (3-[N-hidroxicarbonil]-[2R]-isobutilpropionil-L-triptófano metilamida) se obtuvo de Calbiochem y se reconstituyó en una mezcla de 4% carboximetilcelulosa/0.9% PBS y se administró diariamente por inyección intraperitoneal a 100mg/kg de peso corporal durante 3 días. Veinticuatro horas después del tercer día de inyección, los animales fueron sacrificados, y se recogió el suero mediante punción cardiaca. Se midió el tamaño de los tumores el día 0 y el día 4. Se extirparon los tumores y se congelaron rápidamente para análisis posterior.

Preparación de ARN y arreglos AFFYMETRIX™

ARN total se aisló de muestras de tumores congelados rápidamente utilizando un kit RNAEASY™ (Qiagen, Chatsworth, CA). Se retiró el ADN genómico residual mediante tratamiento con DNAsa I (Roche Molecular Biochemicals). Se realizaron experimentos de microarray, y se analizó como se describe previamente (Jin, H *et al., Circulation* 103:736-

742 (2001)). Las muestras se hibridaron al arreglo único de genoma de ratón AEFYMETRIX™ (MOE430P) y arreglo de genes conocidos (MOE430A) (AFFYMETRIX™, Inc., Santa Clara, CA). Los experimentos se hicieron en tres réplicas para tres muestras de tumor Fo5 y tres muestras de tumor f2:1282. Se llevó a cabo una comparación pareada Mann-Whitney, y aquellos genes con un incremento doble en expresión, que tenían una concordancia mayor de 80% se consideraron significativos. La Base de Datos GeneLogic Bioexpress (GeneLogic, Gaithersburg, MD), una colección de datos de expresión génica de análisis microarray AFFYMETRIX™ de líneas de células y tejidos, se utilizó para examinar la expresión de proteasas identificadas de la detección/cribado diferencial de tumores Fo5-f2:1282 respecto a la expresión en líneas de células SKBR-3 y BT 474.

ELISA de ECD de HER2

Se recogió suero de ratones que albergaban tumores de xenoinjerto Fo5 ó f2:1282 mediante punción cardiaca, y se detectaron los niveles de ECD de HER2 usando un ELISA HER2 previamente descrito (Finkle et al. Clin. Cancer Res. 10: 2499-2511 (2004)). Se diluyó el suero 1:50 usando amortiguador de análisis (PBS/0.5% BSA, 0.05% TWEEN® 20/10 ppm PROCLIN® 300/0.2% BGG/0.25% CHAPS/0.15 M NaCl/5mM EDTA (pH 7.4), seguido por diluciones adicionales 1:2 en serie. Se capturó el HER2 liberado usando un anticuerpo policlonal anti-HER2 en cabra (Genentech) sobre placas NUNC® Maxisorp y se detectó usando un anticuerpo policlonal anti-HER2 en conejo, conjugado a biotina (Genentech), seguido por un anticuerpo AMDEX™-estrepavidina- peroxidasa de rábano (Amersham Pharmacia Biotech), aplicando un procedimiento descrito previamente (Sias *et al., J. Immunol. Meth.* 109:219-27 (1990)). Para experimentos de cultivo de células, las células se trataron como se indica, y se recogió medio acondicionado y se diluyó en amortiguador de análisis en serie 1:2. La concentración de HER2 liberado en el suero o en medio acondicionado se determinó de un ajuste de 4 parámetros de una curva

estándar, usando proteína ECD de HER2 recombinante purificada (Genentech) como estándar.

Constructos de ADN y Purificación de Proteína

MMP-15 humana se clonó en pRK5 mediante PCR usando un clon de origen como plantilla. Se generó una versión de longitud total, marcada C-terminalmente con V5His mediante PCR utilizando cebadores 5'-GCCGAAGCTTGCCACCATGGGCAGCGACCCGAGCGCG-3' (SEQ ID NO. 9) y 5'-GCTGTCTAGAGTTCACCGTCCGTGCCAGTGCCACCTCCTCC-3' (SEQ ID NO. 10) y se clonó en pcDNA3.1V5His.

Una versión soluble de MMP-15, que carece del dominio transmembrana, se generó mediante PCR utilizando cebadores 5'-GCCGAAGCTTGCCACCATGGGCAGCGACCCGAGCGCG-3' (SEQ ID NO. 11) y 5'-GCTGTCTAGAGACCCACTCCTGCAGCGAGCG-3' (SEQ ID NO. 12).

Un mutante catalíticamente muerto de MMP-15 se generó sustituyendo un residuo de alanina por glutamina en el aminoácido 260 de la proteína madura mediante mutagénesis dirigida al sitio (***"site-directed mutagenesis"***).

pRK5.gDHER2-Fc (pRK5.gDHER2-IgG) se generó mediante PCR usando cebadores 5'-CGAGTCGACCTCGAGGCCAGCCCTCTGACGTCC-3' (SEQ ID NO. 13) y 5'-GGCACGCGTCGTCAGAGGGCTGGCTCTCTG-3' (SEQ ID NO. 14) y se clonó en el pRK5.gDHER2-Fc digerido con Xhol-Mlu (Fitzpatrick et. al, FEBBS Lett. 431(l):102-6 (1998)). Éste se introdujo en el punto putativo de segmentación de HER2 identificado de ECD de HER2 purificado, aislado de medio acondicionado SKBR-3 (Yuan *et al, Protein Expression and Purification* 29:217-222 (2003)).

Se generó asimismo una versión truncada de gDHER2-Fc que sólo contenía dominio IV de HER2 usando cebadores PCR 5'-GGCCTCGAGGGCCTGGCCTGCCACCAG-3' (SEQ ID NO. 15) y 5'-GGCACGCGTCGTCAGAGGGCTGGCTCTCTG-3' (SEQ ID NO. 16).

pRK5.HER3-Fc y pRK5.HER4-Fc se han descrito previamente (Fitzpatrick *et al., FEBBS Lett.* 431(1): 102-6 (1998)). Se introdujo un

epítopo FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; (SEQ ID NO. 17)) en el N-término de pRK5. Proteínas de fusión HER-Fc se transfectaron en células 293 y se purificaron de medio acondicionado libre de suero como se describe previamente (Fitzpatrick *et. al, FEBBS Lett*, 431(l):102-6 (1998)). Se generó proteína MMP-15V5His y sMMP-15V5His(E260A) soluble de medio acondicionado con células 293 transitoriamente transfectadas y se purificó utilizando agarosa Ni-NTA, de acuerdo con las recomendaciones del fabricante (Qiagen).

Inactivación shRNA de MMP-15

Se evaluaron diferentes shRNA con respecto a la capacidad de reducir niveles de proteína MMP-15 cuando se introducían en células. Se descubrió que la shRNA más efectiva dirigida contra MMP-15 humana reconocía bp 5'-CCACCATCTGACCTTTAGCTT-3' (SEQ ID NO. 18) correspondiente a nt 1396-1414 (GENBANK™ número de acceso NM_002428). Se introdujo el RNAi para MMP-15 se introdujo en los sitios BamHl-EcoRI vector pSIREN (BD Biosciences) como oligos:

5'-
GATCCGCCACCATCTGACCTTTAGCTTCAAGAGAGCTAAAGGTCAGATGGTG
GTTTTTTGCTAGCG- 3' (SEQ ID NO. 19) y

5'-
AATTCGCTAGCAAAAAACCACCATCTGACCTTTAGCTCTCTTGAAGCTAAAG
GTCAGATGGTGGCG -3' (SEQ ID NO. 20) que contienen una horquilla interna para formar un shRNA. Un shRNA para MMP-25 se adquirió de OPENBIOSYSTEMS™. Los constructos shRNA pSIREN) o el vector solo se transfectaron transitoriamente en células SKBR-3 y BT 474 usando el kit V AMAXA™, siguiendo las recomendaciones del fabricante. Luego de 48 horas, se recogió medio acondicionado y se analizó con respecto a HER2 liberado mediante ELISA HER2, y las células se desintegraron directamente en la placa usando amortiguador de muestra 2x. Se detectaron proteínas MMP-15, HER2 de longitud total, y p95 HER2 mediante inmunotransferencia ***Western blotting*** utilizando un anticuerpo anti-MMP-

15 policlonal en conejo (Labvision Cat. No. RB-1546-P) o anticuerpo Ab-15 anti-HER2 monoclonal en ratón (Labvision, Cat. No. MS-599-P0).

Análisis de escisión HER2 in vitro

Se determinó la capacidad de proteasas candidatas de escindir ECD de HER2 *in vitro* usando proteína de fusión gDHER2-Fc purificada como sustrato. Los dominios catalíticos purificados de MT1-MMP (MMP-14), MT2- MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-19), y MT5-MMP (MMP-25) se adquirieron de R&D systems. Proteínas MMP purificadas se mezclaron con gDHER2-Fc, u otras proteínas HER-Fc tal como se describe, en amortiguador de análisis (100mM Tris (pH=7.4), 100 mM NaCl, 2.5 μM $ZnCl_2$, 10 mM $CaCl_2$, 0.001% Brij35) en una relación de 1:100, enzima:sustrato, y se incubaron a 37°C durante 20 minutos. Las reacciones se detuvieron usando un volumen igual de amortiguador de muestra 2x (Invitrogen), y se sometieron a ebullición durante 5 minutos antes de cargarlas en un gel de gradiente 4-20% Tris-glicina (Invitrogen). Las proteínas se visualizaron coloreando con reactivo de coloración GEL CODE BLUE™ (Pierce Cat. No. 24592), siguiendo las recomendaciones del fabricante. El sitio de segmentación de MMP se determinó mediante secuenciación de proteínas N-terminales mediante degradación automatizada de Edman usando un secuenciador de proteína automatizado (Kishiyama, *A., Anal. Chem.* 72(21):5431-6 (2000)).

Análisis de Inmunoprecipitación y Western Blotting

Células cos-7 se co-transfectaron con constructos pRK5.Flag-HER2 y pcDNA3.MMP-15 o vector pcDNA3.1 únicamente usando LIPOFECTAMINE™ 2000. Se dejó a las células recuperarse durante 48 horas. Las células transfectadas se enjuagaron con PBS y las células se desintegraron en amortiguador de lisis (1%TRITON X- 100™, 50mMTris (pH=7.4), 150mM NaCl, 1mM PMSF, 10μg/ml leupeptina, 10U/ml aprotinina, y 2mM Na_2VO_4). Los lisados se depuraron de material

insoluble mediante centrifugación, y los niveles de proteína total se determinaron utilizando un kit de análisis de proteína BCA (Pierce Cat. No. 23229). Doscientos microgramos de proteína celular total se agregaron al amortiguador de lisis a un volumen final de 1 ml, y el Flag-HER2 se inmunoprecipitó usando anti-Flag M2-agarosa (Sigma) o con anticuerpo anti-MMP-15 policlonal, acomplejado a Proteína A/agarosa G. Los complejos inmunitarios se lavaron dos veces con amortiguador de lisis y se resuspendieron en amortiguador de muestra SDS y se sometieron a ebullición. Las muestras se separaron en un gel de gradiente 4-12% Tris-glicina (Invitrogen) y se pasaron a membranas de nitrocelulosa. Se bloquearon las inmunotransferencias (***"blots"***) en 5%BSA/TBST y se sondearon con anticuerpos contra MMP-15 o HER2, seguidos por un anticuerpo secundario en conejo a anti-ratón conjugado a peroxidasa, tal como se describe. Las inmunotransferencias (***"blots"***) se desarrollaron por quimioluminiscencia incrementada (ECL, Amersham Pharmacia Biotech).

Tumores Fo5 y f2:1282 se resuspendieron en amortiguador de lisis con inhibidores de proteasa y se homogeneizaron usando un POLYTRON TISSUEMIZER™ (PT2100) sobre hielo. Se depuraron los lisados de tumor de material insoluble mediante centrifugación, y los niveles de proteína total se determinaron usando un kit de análisis de proteína BCA. Se inmunoprecipitó HER2 de 1 mg de proteína total de por lo menos tres lisados de tumor independientes usando anticuerpo monoclonal de ratón Ab-15 (Labvision, Cat. No. MS-599-P0) acomplejado a Proteína A/sefarosa G de un día para otro a 4°C. Los complejos se sedimentaron (***"were pelleted"***) por centrifugación, se lavaron dos veces con amortiguador de lisis y se resuspendieron en amortiguador de muestra SDS y se sometieron a ebullición. Las muestras se separaron en gel 4-12% Tris-Glicina y se pasaron a membranas de nitrocelulosa. Las inmunotransferencias (***"blots"***) se sondearon con anticuerpo monoclonal de ratón Ab-18 para detectar HER2 fosforilado (Labvision, Cat. No. MS-1072-P) o con Ab-15 (Labvsion Cat. No., MS-599-P0). Se evaluó la expresión de MMP-14, MMP-15, y MMP-25 mediante Western blotting de 50 ug de lisado de tumor o de 50 ug

de BT 474, o lisado de células MCF-7, o SKBR-3 usando Ab-1 policlonal de conejo (Labvision Cat. No. RB-1544-P) para detectar MMP-14, Ab-1 policlonal de conejo (Labvision Cat. No. RB-1546-P) para detectar MMP-15, o Ab-1 oliclonal de conejo (Oncogene Research Products Cat. No. PC499) para detectar MMP-25.

MAPK activada (Cell Signaling Technology Cat. No. 9101), PhosphoAkt Ser473 (Cell Signaling Technology Cat. No. 9271), Akt total (Cell Signaling Technology Cat. No. 9272), Erk total (Cell Signaling Technology Cat. No. 9102, o phosphoHER3 (Cell Signaling Technology Cat. No. 4791) se determinó por Western blotting de 50 μg de lisado celular.

Análisis de Proliferación

Las células se sembraron en placas de 96 pocillos (Nunc) con una densidad de 10^4 células por pocillo en cuadruplicado y se dejaron adherir de un día para otro. Las células se trataron como se indica, o se transfectaron como se indica. Luego de una incubación de 3 días, se agregaron 25 μl de reactivo Alamar blue (Trek Diagnostic Systems, Cat. No. 00-100) a cada pocillo, y se incubaron durante 3 horas más. Las placas se leyeron en un fluorómetro con longitud de onda de excitación de 530nm y longitud de onda de emisión de 590nm, de acuerdo con las recomendaciones del fabricante. Se determinó la proliferación relativa a células transfectadas con control o no estimuladas, como se describe previamente (Lewis *et al, Cancer Res.* 56(6): 1457-65 (1996)).

RESULTADOS

El ECD de HER2 se libera proteolíticamente de células de carcinoma de mama en cultivo y se detectó en el suero de pacientes con cáncer de mama metastásico, donde se asocia con un pronóstico clínico deficiente (Molina *et al. Cancer Res.* 61: 4744-4749 (2001)). No obstante, el papel biológico de la liberación HER2 no es claro. Los experimentos en esta solicitud se llevaron a cabo para identificar la ***sheddase HER2***.

La identificación de la sheddase HER2 puede ser igualmente importante, ya que previamente se ha demostrado que el trastuzumab inhibe la liberación en líneas celulares de carcinoma de mama (Molina *et al. Cancer Res.* 61:4744-4749 (2001)). Consistente con los resultados previamente informados, trastuzumab redujo la liberación en las líneas de células de cáncer de mama BT 474 y en las células SKBR-3 en más del 60% en una concentración de 10 µg/ml (Fig. 5, panel izquierdo) y disminuyó la proliferación celular en estas líneas de células en 50% (Fig. 5, panel derecho).

Un sistema de modelo animal, en donde tumores mamarios se derivaron de un ratón transgénico MMTVHER2 (Finkle *et al. Clin. Cancer Res.* 10:2499-2511 (2004)), se utilizó para identificar la sheddase HER2. Este sistema de modelo animal liberó en forma reproducible niveles diferentes de HER2 en el suero y exhibió una sensibilidad diferencial al trastuzumab (Fig. 6, panel derecho). Esta diferencia en el nivel de HER2 sérico se correlacionó igualmente bien con la presencia de un fragmento de proteína inmunoprecipitado de tres lisados células de tumor Fo5 independientes, con un peso molecular aparente de 95 kD, consistente con el tamaño de p95 HER2 (Figura 6, panel izquierdo inferior). Este fragmento está constitutivamente fosforilado en los lisados de células de tumor Fo5, indicando que este fragmento puede tener actividad biológica en estos tumores. Los niveles incrementados de una sheddase HER2 pueden contribuir a la resistencia al trastuzumab de esta línea, puesto que el epítopo de trastuzumab se perdería por segmentación proteolítica (Cho *et al. Nature* 421: 756-760 (2003)).

Puesto que la línea de tumor Fo5 libera niveles más altos de HER2 y tiene niveles elevados del fragmento p95HER2, se utilizó análisis de expresión diferencial como un enfoque para identificar transcritos que se regulan por aumento en tumores Fo5, en relación con tumores f2:1282. Se preparó ARN de 3 muestras de tumor individuales o bien de tumores Fo5 o bien de tumores f2:1282 con un tamaño promedio de tumor de 300-600mm^3. Se hibridó ARN de cada muestra de tumor independiente en

triplicado a un chip de arreglo simple de genoma de ratón (***"mouse genome single array chip"***) (MOE430P) AFFYMETRIX™ y a un arreglo de genes conocido (MOE430A). Se realizó una comparación Mann-Whitney en grupos de dos, identificando 638 transcritos regulados por aumento en muestras de tumor Fo5. Puesto que las líneas de células BT 474 y SKBR-3 también liberaron ECD de HER2 en medio acondicionado (Fig. 5, panel izquierdo), esta lista se comparó con transcritos que se expresan en ambas líneas de células utilizando la base de datos Genelogic Bioexpress. El enfoque adoptado para identificar la sheddase HER2 se ilustra en Fig. 7.

Con el fin de estrechar el número de genes candidatos, se usó el inhibidor de metaloproteasa general GM6001 para evaluar si la actividad MMP juega un papel en regular la liberación de HER2. La dosis de GM6001, pero no su estereoisómero, inhibió dependientemente la liberación de HER2 en células SKBR-3 como se determinó por un ELISA de ECD de HER2 (Fig. 8, panel izquierdo). Este resultado es consistente con informes publicados previamente de que el inhibidor de metaloproteasa general BB-94 inhibió la liberación de ECD de HER2 en líneas de células de carcinoma de mama (Codony-Servat *et al. Cancer Res.* 59:1196-1201 (1999)). Timp-1, Timp-2 y Timp-3 son péptidos de ocurrencia natural que regulan la actividad de metaloproteasa (Overall and López-Otin *Nature Reviews Cancer* 2:657-672 (2002)). La adición de Timp-1, Timp-2, o Timp-3 purificado al medio acondicionado de células SKBR-3 en una concentración de 1 μg/ml redujo los niveles ECD de HER2 (Fig. 8, panel derecho). No obstante, Timp-2 redujo la liberación ECD de HER2 con la máxima eficiencia (Fig. 8, panel derecho). Timp-2 es conocido por inhibir eficientemente la subfamilia MT-MMP de metaloproteasas (Overall and López-Otin *Nature Reviews Cancer* 2:657-672 (2002)). Estos datos sugirieron que la proteasa responsable de la liberación de HER2 en células SKBR-3 y BT 474 es una metaloproteasa, que reduce significativamente el número de candidatos identificados de la detección bioinformática (***"the bioinformatics screen"***).

Varios miembros de la familia MT-MMP se expresan en células SKBR-3 y BT 474 basadas en la base de datos GENELOGIC™. Las dos MT1-MMP y MT2-MMP se expresan igualmente en tumores Fo5 de los datos de microarreglo AFFYMETRIX™. Para determinar si estos transcritos se expresan, lisados de líneas de células de carcinoma de mama y tumor Fo5 y f2:1282 se inmunotransfirieron (***"were immunoblotted"***) con anticuerpos policlonales contra estas MT-MMP que reconocen proteínas humanas y de ratón (Fig. 9). Todas las líneas de células expresan MMP-14, MMP-15, y MMP-25, en diferentes niveles. Aunque MMP-14 se expresa en ambos lisados de células de tumores f2:1282 y Fo5, MMP-15 se expresa abundantemente en lisado de tumores Fo5. Este resultado fue consistente con la comparación Mann-Whitney, la cual indicó un nivel 2 veces más alto de expresión mARN de MMP-15 en tumores Fo5. Para verificar que MMP-15 se expresaba en las células epiteliales del tumor, y no en las células de estroma circundantes, tumores Fo5 y f2:1282 se seccionaron y colorearon o bien con anticuerpo anti-HER2 (Dako) o bien con un anticuerpo anti-MMP-15 (Ab-1). Los dos tumores f2:1282 y Fo5 expresan HER2 humano, pero MMP-15 se expresa más abundantemente en los tumores Fo5 que los tumores f2:1282.

Tradicionalmente se ha considerado que los sustratos para miembros de las MT-MMP son proteínas de matriz extracelular. Se estableció análisis bioquímico para probar candidatos identificados de los datos de microarreglo para escindir HER2 de longitud total *in vitro*. Este análisis usa una proteína de fusión recombinante purificada como sustrato para proteasas candidatas y consta de un ECD de HER2, marcado con un epítopo gD N-terminalmente, en el marco de lectura (***"in-frame"***) con la cadena pesada de IgG humana. Se utilizaron varias proteínas distintas en estos estudios. gDHER2(+)-IgG contiene aminoácidos 2-656 de ECD de HER2 (GenBank accesión AAA75493) y gDHER2(-)-IgG contiene aminoácidos 2-626 de ECD de HER2. La proteína recombinante gDHER2(+)-IgG contiene la secuencia yuxtamembrana que previamente ha demostrado contener un sitio putativo de sheddase HER2 (PA/EQR/ASP; SEQ ID NO.

23) mientras que la proteína gDHER2(-)-IgG carece de esta secuencia (Yuan *et al. Protein Expression and Purification* 29:217-222 (2003)). Una versión truncada gDHER2(+)-IgG se usó asimismo en esta análisis, gDHER2(DIV)-IgG, que sólo tiene el dominio IV (DIV) de HER2 en el marco de lectura (***"in-frame"***) con Fc humana. Dominios catalíticos purificados de MMP-14, MMP-15, MMP-16, MMP-19, y MMP-25 se incubaron con gDHER2(DIV)-IgG durante 20 minutos a 37°C. Todas las MT-MMP, excepto que MMP-14 escinde eficientemente el sustrato gDHER2(DIV)-IgG (Fig. 13, panel izquierdo). Los productos de proteína segmentada se retiraron por incisión y se secuenciaron N-terminalmente para identificar el sitio de segmentación. Todas las cuatro MT-MMP segmentaron HER2 cerca del sitio de secuencia publicado (Yuan et al Protein Expression and Purification 29: 217-222 (2003)) (Fig. 13, panel derecho).

Proteínas de fusión HER2-FC que tienen la secuencia PINCTHSCVDLDDKGCPAEQRASPASPLTSIV (SEQ ID NO. 21) son sustratos para estas cuatro MMP *in vitro* (Fig. 14). Estos datos sugieren que HER2 es un sustrato para MMP-15.

El reconocimiento del sustrato de MT-MMP puede estar influenciado por el dominio de hemopexina (Overall and López-Otin *Nature Reviews Cancer* 2:657-672 (2002)). Una forma soluble de MMP-15 con un epítopo His V5 C-terminal se expresó transitoriamente en células 293 y se purificó de medio acondicionado mediante cromatografía de afinidad. Como se ilustra en Fig. 15, esta forma soluble de MMP-15 puede segmentar igualmente gDHER2(+)-IgG en análisis de sheddase *in vitro*.

Para determinar si MMP-15 y HER2 pueden asociarse en la membrana, se llevaron a cabo análisis de co-inmunoprecipitación utilizando células Cos-7 transfectadas transitoriamente. pRK5.FlagHER2 se co-transfectó con cualquiera de los vectores, pcDNA3.MMP-15V5His, pcDNA3.sMMP-15V5His, o pcDNA3.MMP-15(E260A). FlagHER2 se inmunoprecipitó con una resina anti-FLAG. Fig. 10 muestra que las dos, la MMP-15 soluble y un mutante catalíticamente muerto (E260A), pueden

asociarse con Flag-HER2. Esto es consistente con la idea de que MMP-15 puede segmentar HER2 en membranas, puesto que la versión natural de la proteasa, pcDNA3.MMP-15V5His, eliminaría el FlagHER2ECD y un complejo HER2-MMP-15 no se co-inmunoprecipitaría.

Mutaciones puntuales somáticas y deleciones y variantes de empalme se han identificado en tumores mamarios de animales transgénicos MMTVHER2 (Siegel *et al. EMBO J.* 18:2149-2164 (1999)); y Finkle *et al. Clin. Cancer Res.* 10:2499-2511 (2004)). Estas mutaciones muy frecuentemente ocurren en el dominio extracelular de HER2 cerca del dominio transmembrana. La línea de tumor Fo5 tiene una deleción de cinco aminoácidos DLDDK (SEQ ID NO. 22) que es adyacente al sitio de segmentación de HER2, mientras que la línea de tumor f2:1282 tiene una sola mutación puntual C para R (Fig. 12). Estas mutaciones no influyen en la asociación de MMP-15 (Fig. 11). No obstante, es posible que la proximidad de la deleción DLDDK (SEQ ID NO. 22) al sitio de segmentación HER2 MMP pueda afectar la actividad enzimática de la proteasa.

La inhibición de ARN (ARNi) es un método que puede regular por disminución el transcrito diana y los niveles de proteína. Para examinar el papel biológico de MMP-15 en células SKBR-3 y BT 474, se utilizó un constructo RNAi anti-MMP-15. Cuando se introdujo en células SKBR-3 o BT 474, este plásmido de expresión shRNAi redujo niveles de proteína endógena de MMP-15 en los dos tipos de células (Fig. 17, panel superior izquierdo). La transfección de este shRNA redujo asimismo la cantidad de p95 HER2 en ambas líneas de células (Fig. 17, panel inferior izquierdo). La pérdida de la proteína MMP-15 en estas células redujo significativamente la liberación HER2ECD en ambas líneas (Fig. 17, panel derecho). Sin embargo, no se observó reducción significativa en la liberación HER2ECD cuando shRNAi contra MMP-25 se introdujo en cualquiera de las dos líneas de células (Fig. 17, panel derecho). Estos experimentos sugieren firmemente que la pérdida de MMP-15 afecta dramáticamente la liberación HER2ECD.

La reducción de los niveles de proteína MMP-15 por RNAi tiene igualmente un efecto sobre las tasas de crecimiento de las células SKBR-3 y BT 474 (Fig. 18, panel izquierdo). Este efecto se duplicó también por un shRNAi contra MMP-25. Esto sugiere que la reducción de la liberación HER2ECD regula también por disminución la cantidad de p95 HER2 en células BT 474 y SKBR-3, disminuyendo así las tasas de crecimiento celular. Cuando se introdujo un constructo p95 HER2 marcado con gD en estas líneas de células, se detectó un aumento en las tasas de crecimiento celular en ambos tipos de células, en relación con células transfectada de control (Fig. 18, panel izquierdo). Este constructo se fosforila constitutivamente y activa MAPK cuando se introduce en células Cos-7 en una manera independientemente del ligando (Fig. 16). No obstante, este constructo tiene aún que heterodimerizar con HER3 o EGFR con el fin de activar vías señalizadoras de Akt, consistente con un estudio publicado previamente (Xia *et al. Oncogene* 23:646-653 (2004)). La sobreexpresión de gDp95HER2 en células SKBR-3 no inhibió significativamente la inhibición del crecimiento mediada por trastuzumab en estas células (Fig. 18, panel derecho).

Un enfoque farmacológico se utilizó para determinar si la actividad MMP juega un papel en regular la liberación HER2 y los niveles p95 HER2 en tumores Fo5. Ratones FVB con tumores Fo5 con un tamaño promedio de 400-600 mm^3 se inyectaron con GM6001 intraperitonealmente o con vehículo de control diariamente en el curso de tres días. El día cuarto, se recogió suero y se analizó con respecto a HER2ECD mediante ELISA, y los tumores se recogieron y se pesaron. HER2 se inmunoprecipitó de lisados de células de tumor y se analizó con respecto a niveles p95 HER2 mediante Western blotting. Los animales tratados con GM6001 mostraron una reducción significativa en la cantidad de p95 HER2, comparados con los animales de control (Fig. 19, panel derecho). Los niveles séricos HER2EDC se redujeron en los animales tratados con GM6001 y vehículo (Fig. 19, panel izquierdo); sin embargo, se observó una mayor reducción en los animales tratados con GM6001.

SUMARIO

Los anteriores experimentos demuestran que la metaloproteasa de matriz MMP-15 segmenta HER2 *in vitro* en un sitio que es consistente con ECD liberado purificado de células SKBR3, e interactúa con HER2 de longitud total. La reducción de los niveles de esta MMP se correlaciona bien con una liberación de receptor HER2 reducida, y disminuye los niveles de proliferación básicos en lineas de células SKBR3 y BT474. Trastuzumab parece inhibir la liberación del receptor HER2 regulando indirectamente la actividad MMP-15, pero no compite con MMP-15 por unión de sustrato y segmentación. La reducción de la liberación HER2 con un antagonista MMP, tal como un antagonista MMP-15, representa un enfoque terapéutico para tratar cáncer, incluido el cáncer resistente al trastuzumab.

Lo que se reivindica es:

1. Un método de inhibir la liberación de HER2, que comprende tratar una célula que expresa HER2 con un antagonista de metaloproteasa de matriz (MMP) en una cantidad efectiva para inhibir la liberación de HER2.

2. El método de la reivindicación 1 en donde el antagonista MMP es un antagonista MMP anclado a membrana (MT-MMP).

3. El método de la reivindicación 2 en donde la MT-MMP se selecciona del grupo conformado por MMP-15 (MT2- MMP), MMP-16 (MT3-MMP), MMP-24 (MT5-MMP), MMP-17 (MT4-MMP), y MMP-25 (MT6- MMP).

4. El método de la reivindicación 3 en donde la MT-MMP es MMP-15.

5. El método de la reivindicación 1 en donde la célula exhibe sobreexpresión, amplificación, o activación de HER2.

6. El método de la reivindicación 5 en donde la célula exhibe la sobreexpresión o amplificación de HER2.

7. El método de la reivindicación 1, que comprende además tratar la célula con un inhibidor HER.

8. El método de la reivindicación 7 en donde el inhibidor HER es un anticuerpo HER2.

9. El método de la reivindicación 8 en donde el anticuerpo HER2 es trastuzumab o pertuzumab.

10. El método de la reivindicación 7 en donde el inhibidor HER se selecciona del grupo conformado por trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, y TAK165.

11. Un método para reducir el nivel sérico del dominio extracelular (ECD) de HER2 en un mamífero, que comprende administrar un antagonista de metaloprotesa de matriz (MMP) al mamífero en una cantidad efectiva para reducir el nivel sérico ECD de HER2 en el mamífero.

12. El método de la reivindicación 11 en donde el mamífero tiene un nivel de MMP elevado.

13. Un método para tratar cáncer en un mamífero, que comprende administrar un antagonista de metaloproteasa de matriz (MMP) al mamífero en una cantidad efectiva para tratar el cáncer.

14. El método de la reivindicación 13 en donde el cáncer exhibe expresión, amplificación, o activación de HER.

15. El método de la reivindicación 14 en donde el cáncer exhibe sobreexpresión o amplificación de HER2.

16. El método de la reivindicación 13 en donde el mamífero tiene un nivel sérico de HER2 liberado elevado o un nivel de p95 HER2 elevado.

17. Un método de tratar un cáncer resistente al inhibidor HER en un mamífero, que comprende administrar al mamífero un antagonista de metaloprotesa de matriz (MMP) en una cantidad efectiva para tratar el cáncer.

18. El método de la reivindicación 17 en donde el inhibidor HER es trastuzumab.

19. Un método para reducir el nivel de p95 HER2 en una célula, que comprende exponer la célula a un antagonista de metaloproteasa de matriz (MMP) en una cantidad efectiva para reduce el nivel de p95 HER2.

20. Un método de diagnóstico que comprende evaluar MMP-15 (MT2-MMP) en una muestra de un paciente de cáncer, en donde un nivel de MMP-15 elevado o actividad indica que el paciente tiene un nivel sérico p95 HER2 o HER2 liberado elevado, o tendrá una respuesta clínica deficiente.

21. El método de la reivindicación 20 en donde un nivel de MMP-15 elevado indica que el paciente tiene una respuesta clínica deficiente.

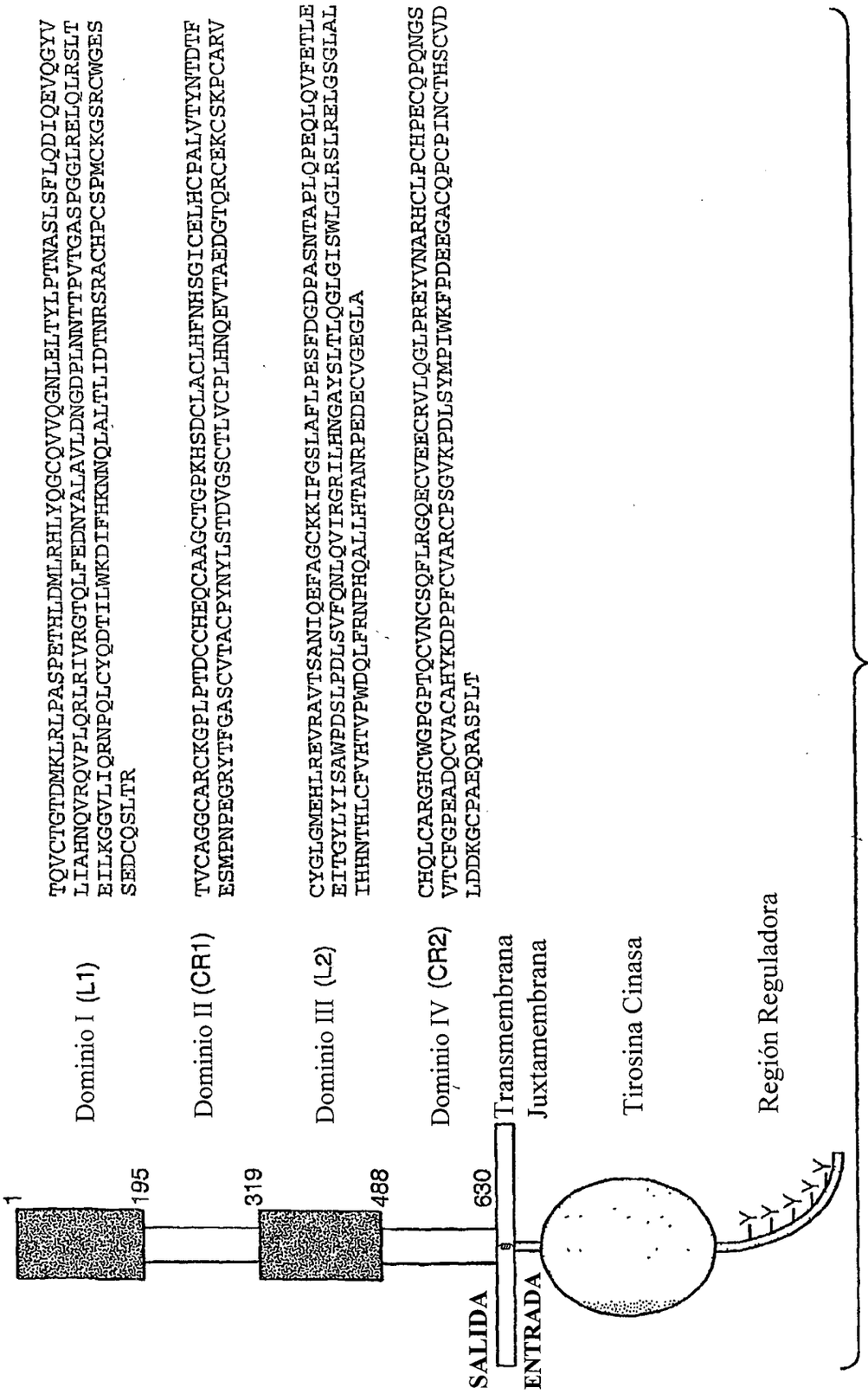

1
195
319
488
630
Dominio I (L1)
TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQGYV
LIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLT
EILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGES
SEDCQSLTR
Dominio II (CR1)
TVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTF
ESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARV
Dominio III (L2)
CYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLE
EITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLAL
IHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLA
Dominio IV (CR2)
CHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGS
VTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVD
LDDKGCPAEQRASPLT
Transmembrana
Juxtamembrana
Tirosina Cinasa
Región Reguladora
SALIDA
ENTRADA
Y
Y
Y
Y
Y

FIG. 1

Cadena Ligera

```
1                           15                              30                              45
D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q D V N T A V A W Y Q Q K P G K A P K

46                          60                              75                              90
L L I Y S A S F L Y S G V P S R F S G S R S G T D F T L T I S S L Q P E D F A T Y Y C Q Q

91                          105                             120                             135
H Y T T P P T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L

136                         150                             165                             180
L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T

181                         195                             210     214
L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
```

FIG. 2A

Cadena Pesada

```
1                            15                                      30                                       45
E V Q L V E S G G G L V Q P G G S L R L S C A A S G F N I K D T Y I H W V R Q A P G K G L

46                           60                                      75                                       90
E W V A R I Y P T N G Y T R Y A D S V K G R F T I S A D T S K N T A Y L Q M N S L R A E D

91                           105                                     120                                      135
T A V Y Y C S R W G G D G F Y A M D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S

136                          150                                     165                                      180
K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S

181                          195                                     210                                      225
G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K

226                          240                                     255                                      270
T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S

271                          285                                     300                                      315
H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D

316                          330                                     345                                      360
W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E

361                          375                                     390                                      405
M T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G

406                          420                                     435                                   449
S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
```

FIG. 2B

Secuencia de Aminoácidos para la Cadena Ligera de Pertuzumab

```
1         10        20        30        40        50        60
|         |         |         |         |         |         |
DIQMTQSPSSLSASVGDRVTITC**KASQDVSIGVA**WYQQKPGKAPKLLIYS**ASYRYT**GVPS

          70        80        90        100       110       120
          |         |         |         |         |         |
RFSGSGSGTDFTLTISSLQPEDFATYYC**QQYYIYPYT**FGQGTKVEIKRTVAAPSVFIFPP

          130       140       150       160       170       180
          |         |         |         |         |         |
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT

          190       200       210
          |         |         |
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
```

FIG. 3A

Secuencia de Aminoácidos para la Cadena Pesada de Pertuzumab

```
1         10        20        30        40        50        60
|         |         |         |         |         |         |
EVQLVESGGGLVQPGGSLRLSCAAS**GFTFTDYTMD**WVRQAPGKGLEWVA**DVNPNSGGSIY**

          70        80        90        100       110       120
          |         |         |         |         |         |
**NQRFK**GRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARN**LGPSFYFDY**WGQGTLVTVSSA

          130       140       150       160       170       180
          |         |         |         |         |         |
STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG

          190       200       210       220       230       240
          |         |         |         |         |         |
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGP

          250       260       270       280       290       300
          |         |         |         |         |         |*
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS

          310       320       330       340       350       360
          |         |         |         |         |         |
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM

          370       380       390       400       410       420
          |         |         |         |         |         |
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ

          430       440       448
          |         |         |
QGNVFSCSVMHEALHNHYTQKSLSLSPG
```

FIG. 3B

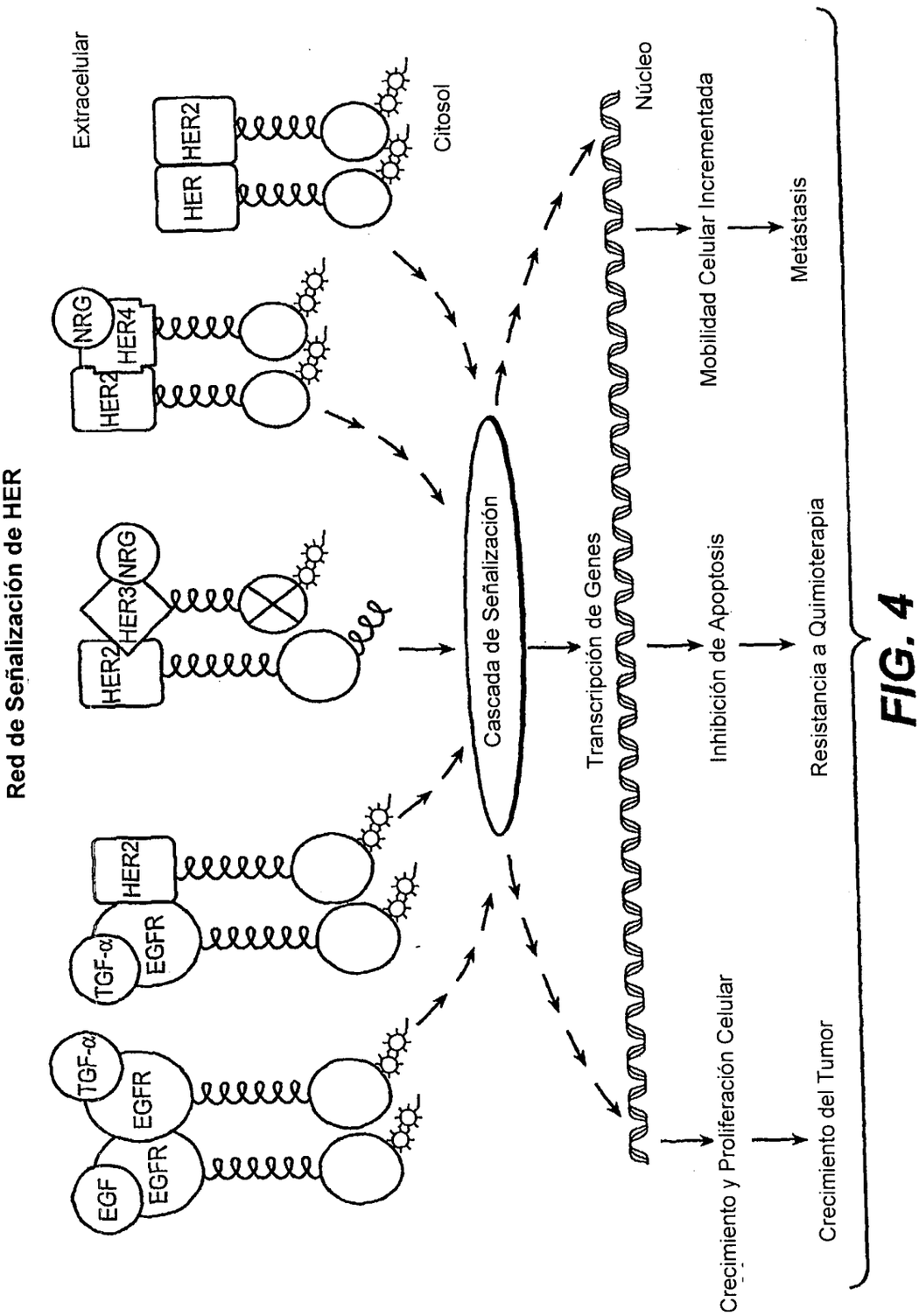

Red de Señalización de HER
Extracelular
Citosol
HER2
HER
NRG
HER4
HER2
HER3
NRG
HER2
HER2
EGFR
TGF-α
EGF
EGFR
EGFR
Cascada de Señalización
Transcripción de Genes
Núcleo
Mobilidad Celular Incrementada
Metástasis
Inhibición de Apoptosis
Resistancia a Quimioterapia
Crecimiento y Proliferación Celular
Crecimiento del Tumor

FIG. 4

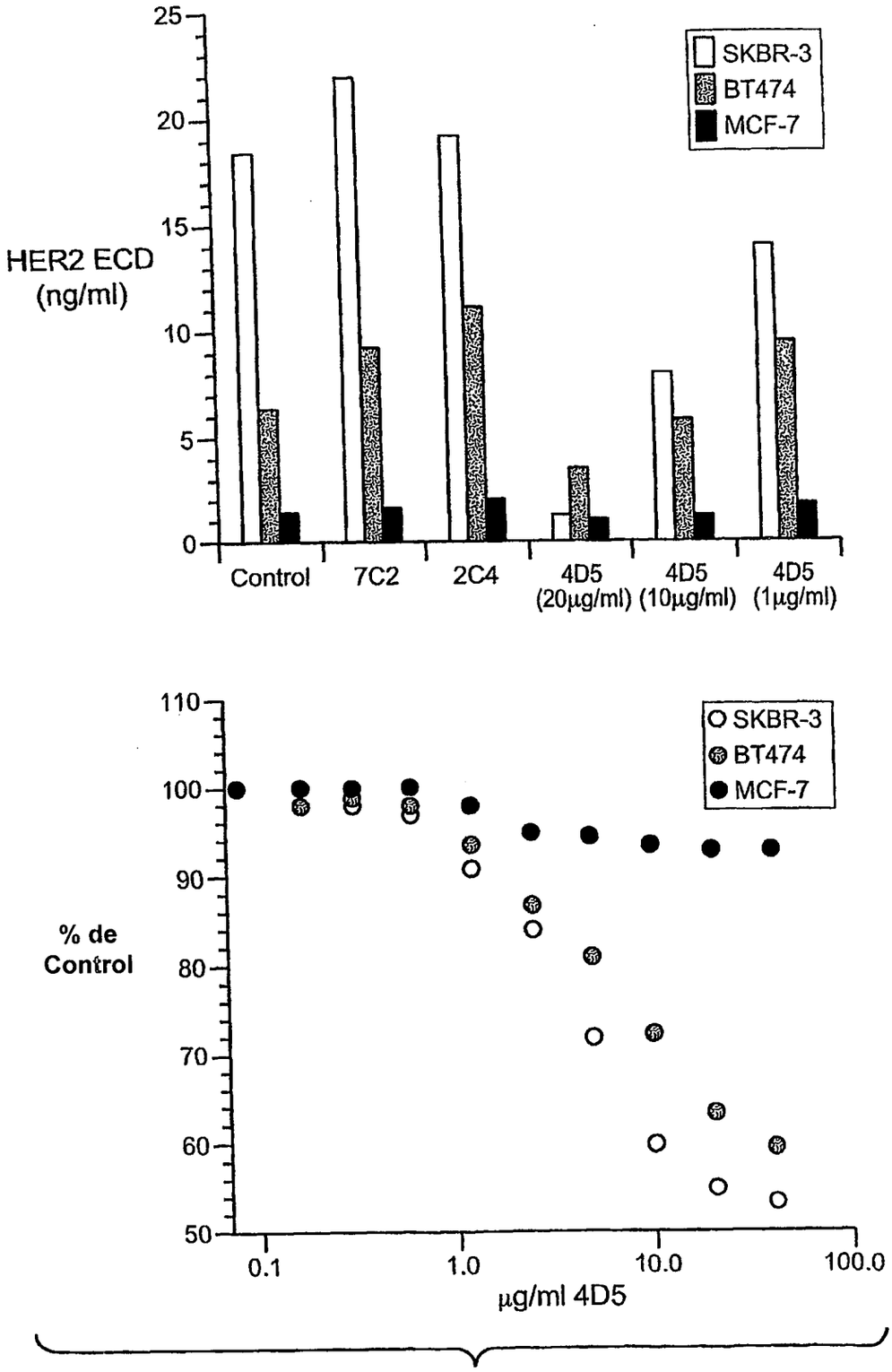
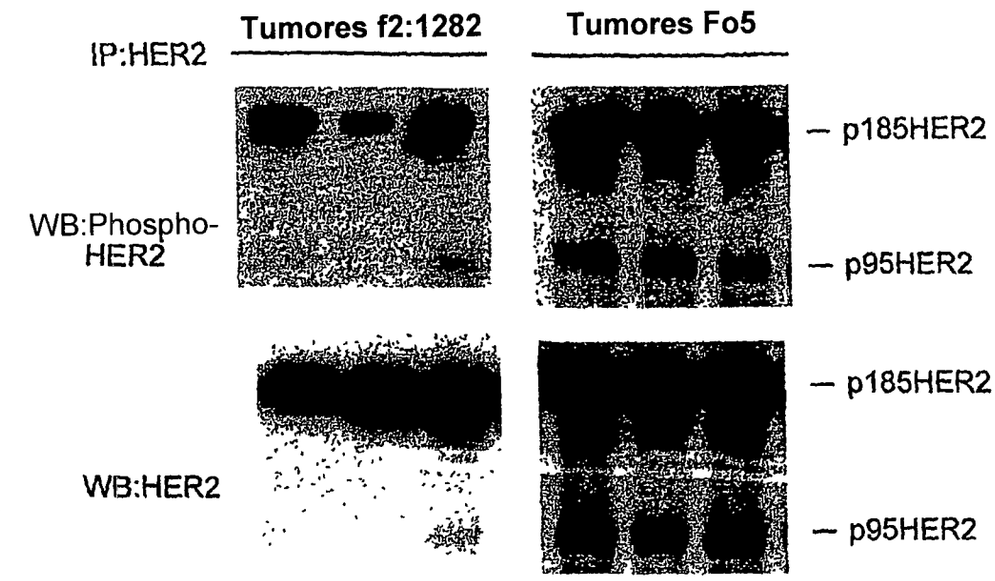

HER2 ECD (ng/ml)
25
20
15
10
5
0
SKBR-3
BT474
MCF-7
Control
7C2
2C4
4D5 (20µg/ml)
4D5 (10µg/ml)
4D5 (1µg/ml)
% de Control
110
100
90
80
70
60
50
0.1
1.0
10.0
100.0
µg/ml 4D5

FIG. 5


IP:HER2
Tumores f2:1282
Tumores Fo5
WB:Phospho-HER2
— p185HER2
— p95HER2
WB:HER2
— p185HER2
— p95HER2

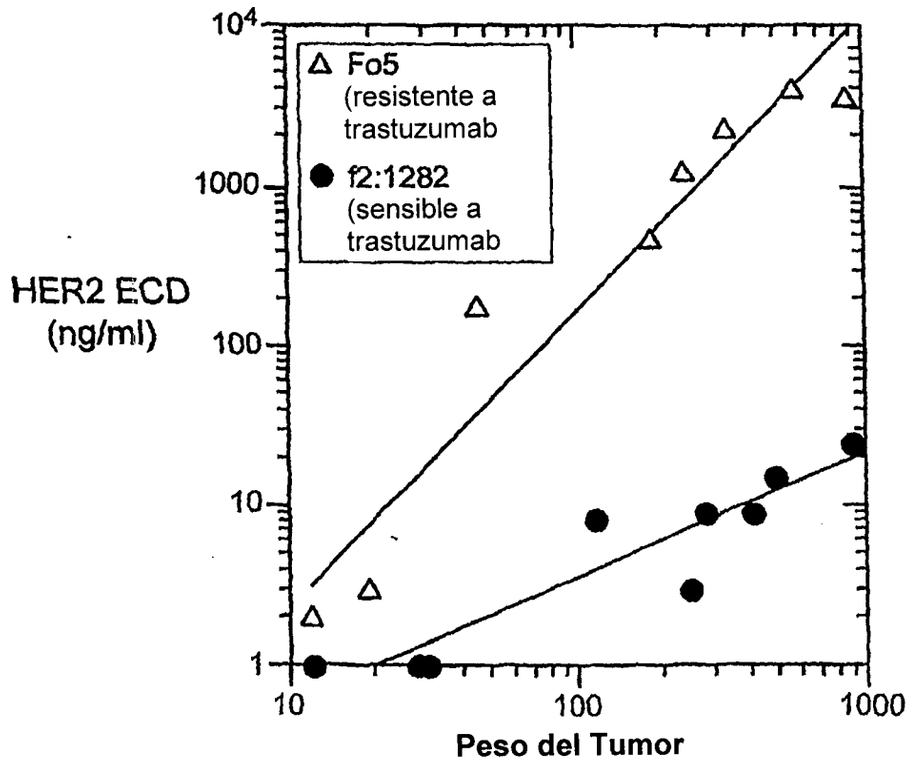
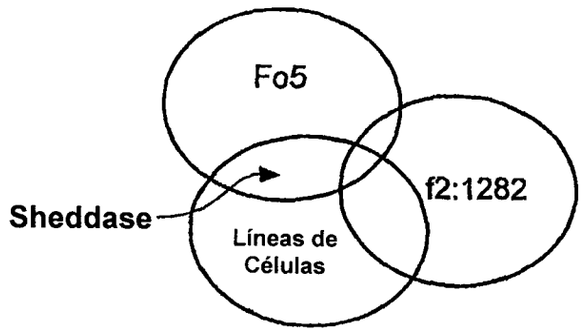

HER2 ECD (ng/ml)
10⁴
1000
100
10
1
Fo5 (resistente a trastuzumab
f2:1282 (sensible a trastuzumab
10
100
1000
Peso del Tumor

FIG. 6

Datos de Chip de Genes y Datos GENELOGIC™



Condiciones para la Detección

La proteasa tiene que expresarse en cada subconjunto
Estar regulada por aumento en tumores Fo5 vs. f2:1282
Expresada en líneas de células liberaron HER2ECD

FIG. 7

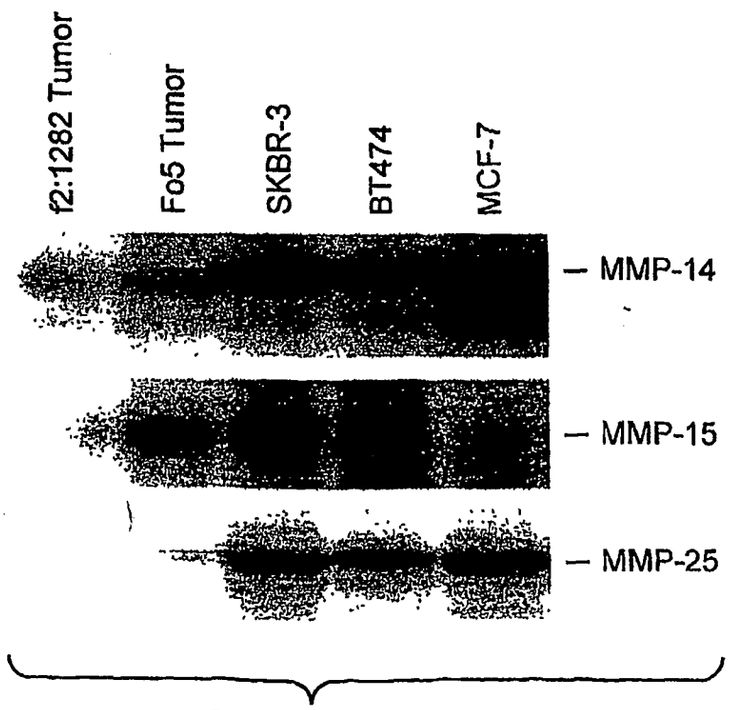
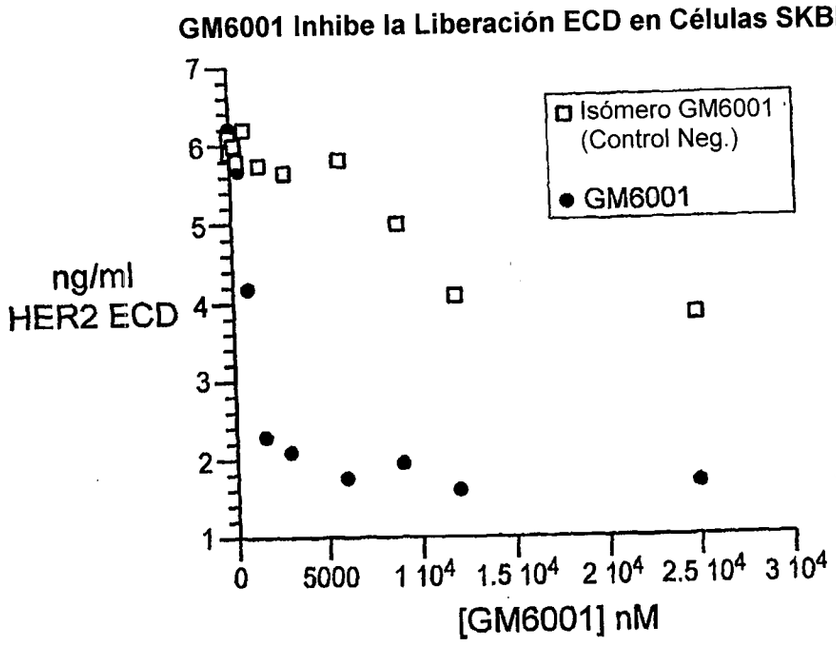
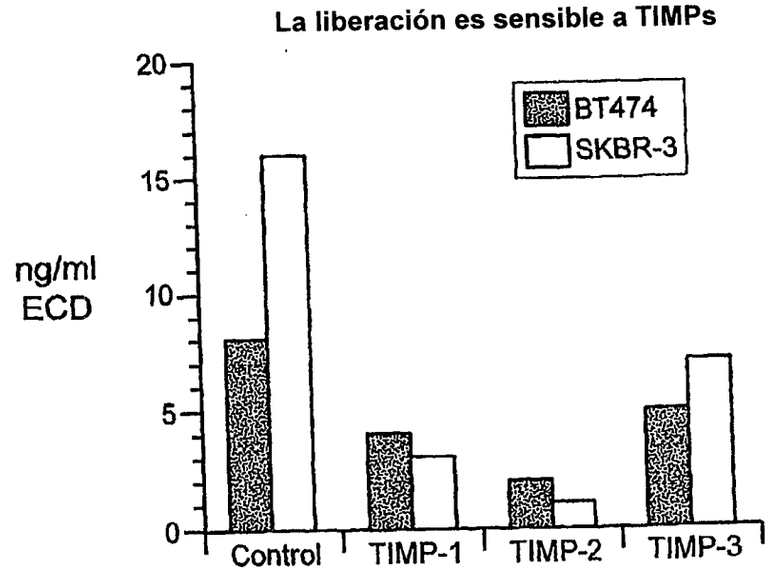


FIG. 9


GM6001 Inhibe la Liberación ECD en Células SKBR-3
Isómero GM6001 (Control Neg.)
GM6001
ng/ml HER2 ECD
[GM6001] nM


La liberación es sensible a TIMPs
BT474
SKBR-3
ng/ml ECD
Control
TIMP-1
TIMP-2
TIMP-3

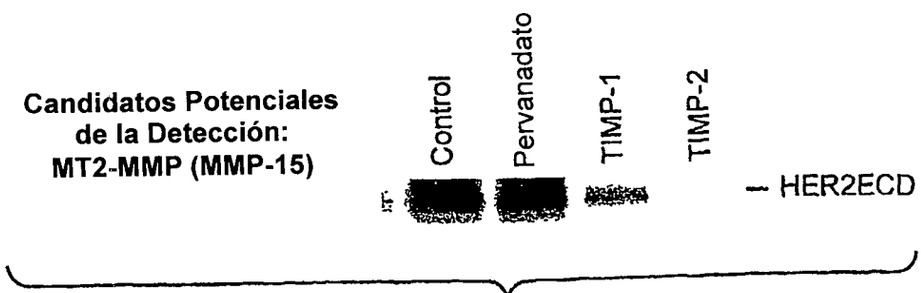

Candidatos Potenciales de la Detección: MT2-MMP (MMP-15)
Control
Pervanadato
TIMP-1
TIMP-2
HER2ECD

FIG. 8

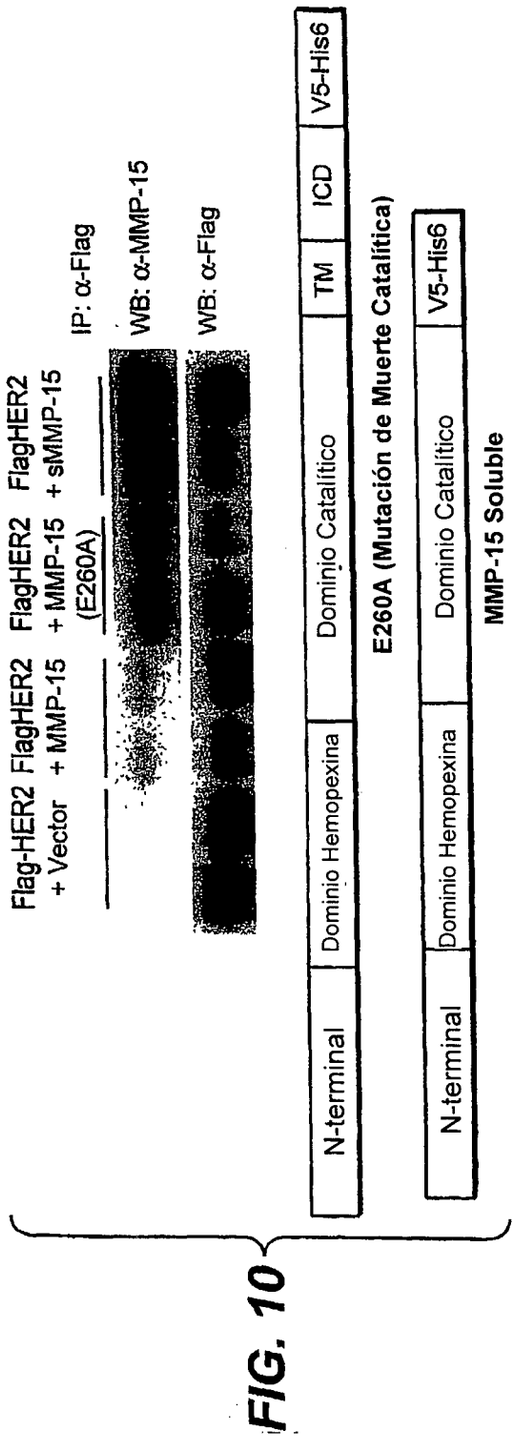

Flag-HER2 + Vector
FlagHER2 + MMP-15
FlagHER2 + MMP-15 (E260A)
FlagHER2 + sMMP-15
IP: α-Flag
WB: α-MMP-15
WB: α-Flag
N-terminal
Dominio Hemopexina
Dominio Catalítico
TM
ICD
V5-His6
E260A (Mutación de Muerte Catalítica)
N-terminal
Dominio Hemopexina
Dominio Catalítico
V5-His6
MMP-15 Soluble

FIG. 10

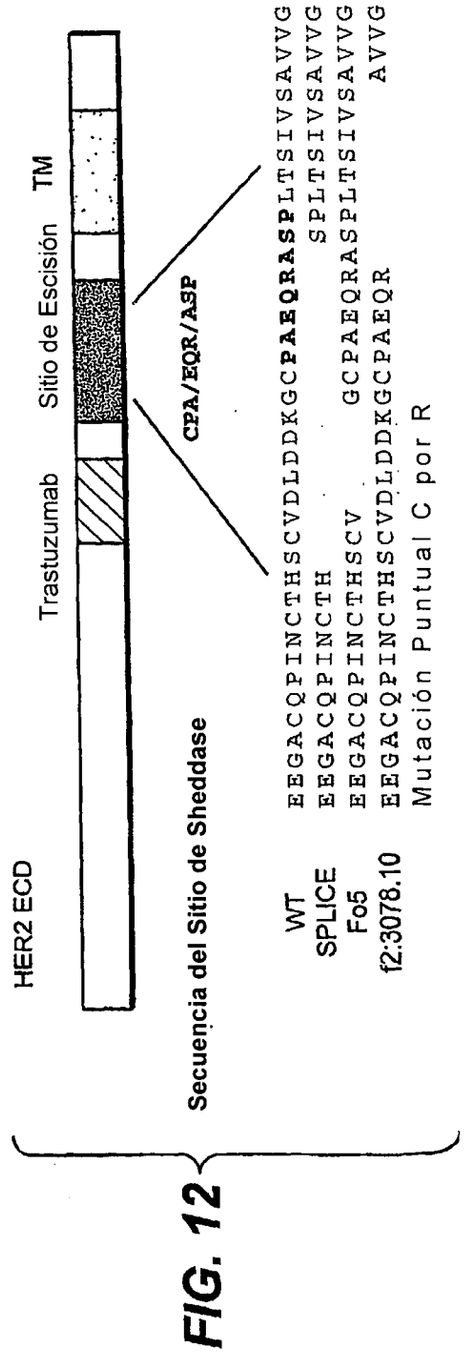

HER2 ECD
Trastuzumab
Sitio de Escisión
TM
CPA/EQR/ASP
Secuencia del Sitio de Sheddase
WT EEGACQPINCTHSCVDLDDKGCPAEQRASPLTSIVSAVVGIL
SPLICE EEGACQPINCTH SPLTSIVSAVVGIL
Fo5 EEGACQPINCTHSCV GCPAEQRASPLTSIVSAVVGIL
f2:3078.10 EEGACQPINCTHSCVDLDDKGCPAEQR AVVGIL
Mutación Puntual C por R

FIG. 12

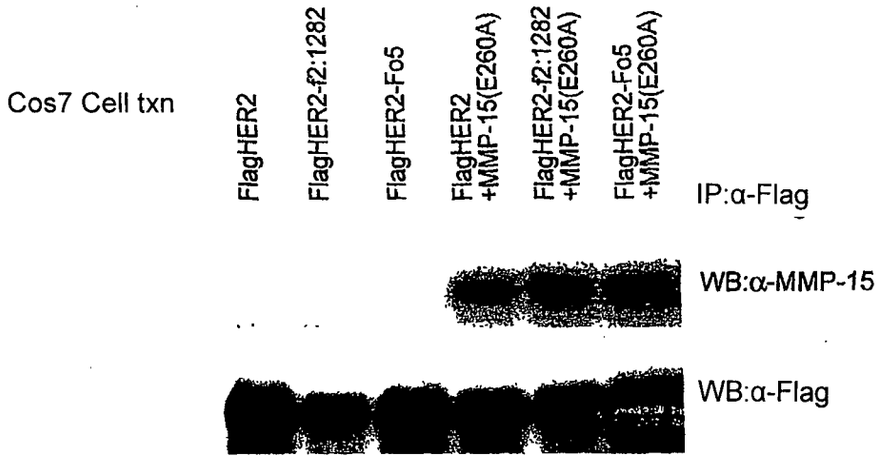
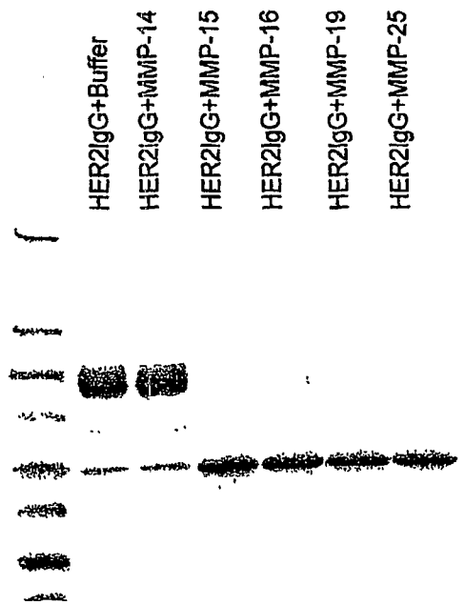


FIG. 11



SECUENCIA DE DEGRADACIONES (*DIGESTS*) DE PROTEASA

Sustrato escindido es doblete de:

IgG

1) MMP-15: N-LTSIVSTRDKTHTCPP...
MMP-16: N-LTSIVSTRDKTHTCPP...
MMP-19: N-LTSIVSTRDKTHTCPP...
MMP-25: N-LTSIVSTRDKTHTCPP...

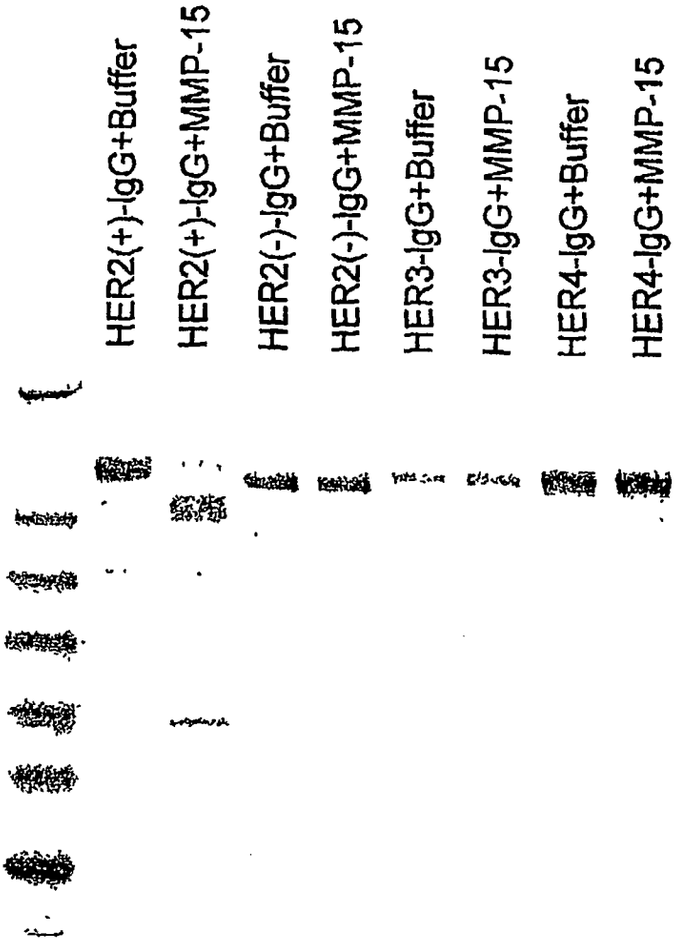
gD-tag

2) TODOS los demás:N-KYALADAS . . .

Sitio C-term. HER2ECD TM

CPINCTHSCVDLDDKGC**PA/EQR/ASP**LTSIVSAVVGILLVVV

FIG. 13



Variación de Secuencia en Familia HER2 cerca del Dominio TM

TM

HER2: ...DLDDKGC**PAEQRASP**LTSIVSAVVGIL..

EGFR: PTNGPKIPS

HER3: CLGQTLVLIGKTLT

HER4(Jma): CIYYPWTGHSTLPQHART

HER4(Jmb): CIGLMDRT...

FIG. 14

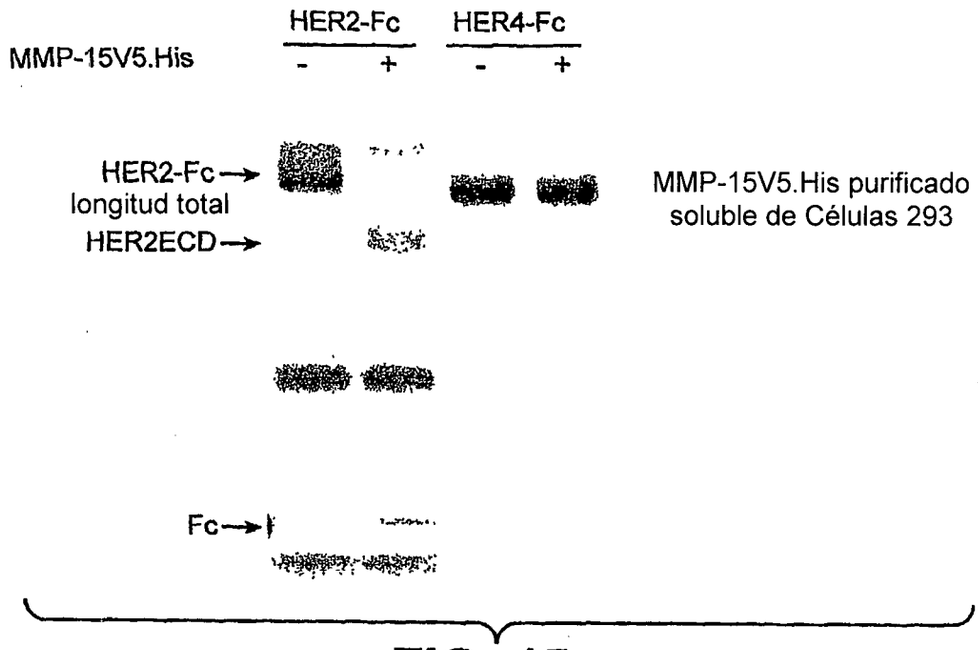

HER2-Fc
HER4-Fc
MMP-15V5.His
- + - +
HER2-Fc →
longitud total
HER2ECD →
MMP-15V5.His purificado
soluble de Células 293
Fc →

FIG. 15

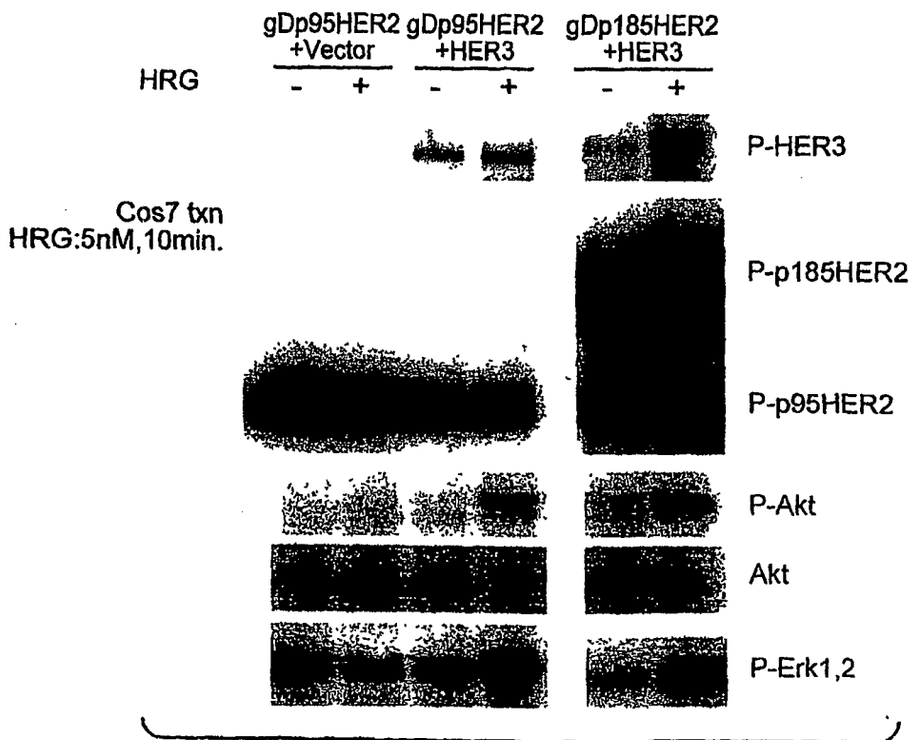

gDp95HER2 +Vector
gDp95HER2 +HER3
gDp185HER2 +HER3
HRG
- + - + - +
Cos7 txn
HRG:5nM,10min.
P-HER3
P-p185HER2
P-p95HER2
P-Akt
Akt
P-Erk1,2

FIG. 16

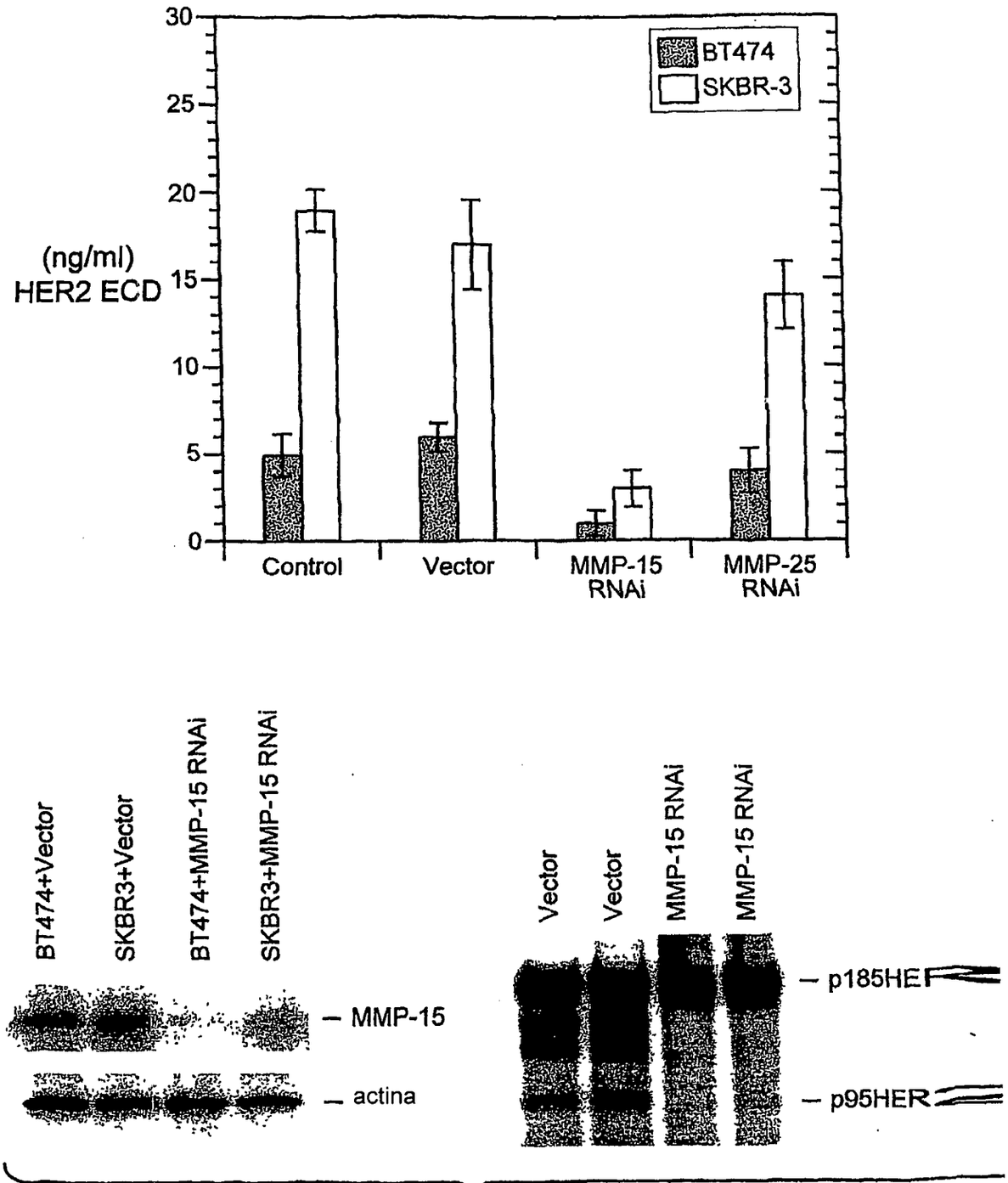
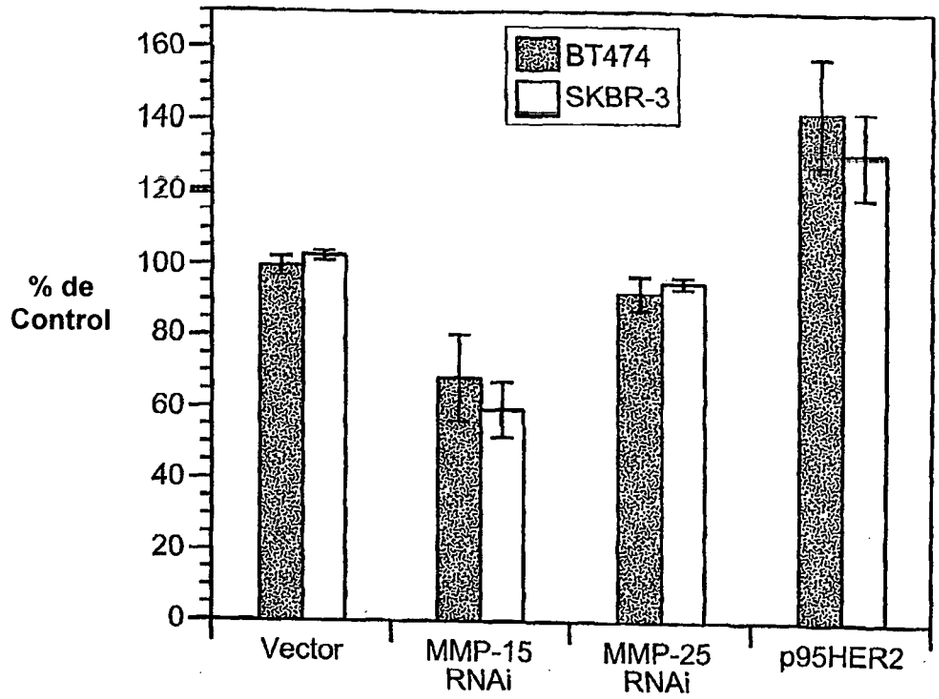

30
25
20
15
10
5
0
(ng/ml)
HER2 ECD
BT474
SKBR-3
Control
Vector
MMP-15 RNAi
MMP-25 RNAi
BT474+Vector
SKBR3+Vector
BT474+MMP-15 RNAi
SKBR3+MMP-15 RNAi
MMP-15
actina
Vector
Vector
MMP-15 RNAi
MMP-15 RNAi
p185HER
p95HER

FIG. 17

Reducción de Niveles de Proteína MMP-15 Inhibe Crecimiento Celular



Sobreexpresión de gDp95Her2 No Rescata la Inhibición del Crecimiento Mediada por Trastuzumab En Células SKBR-3 Infectadas

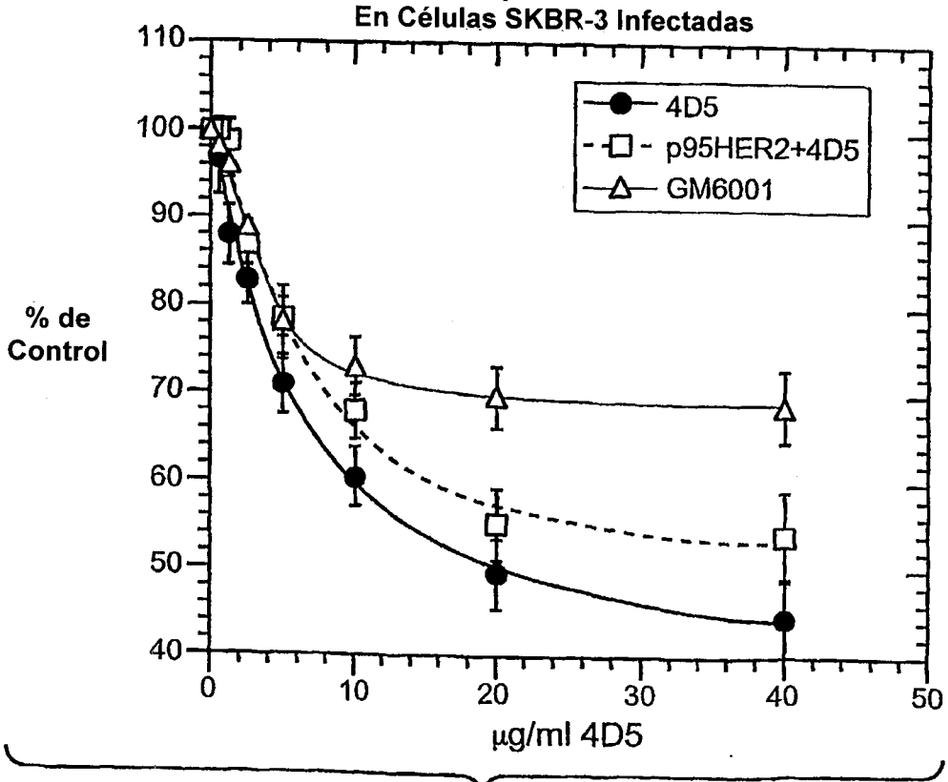
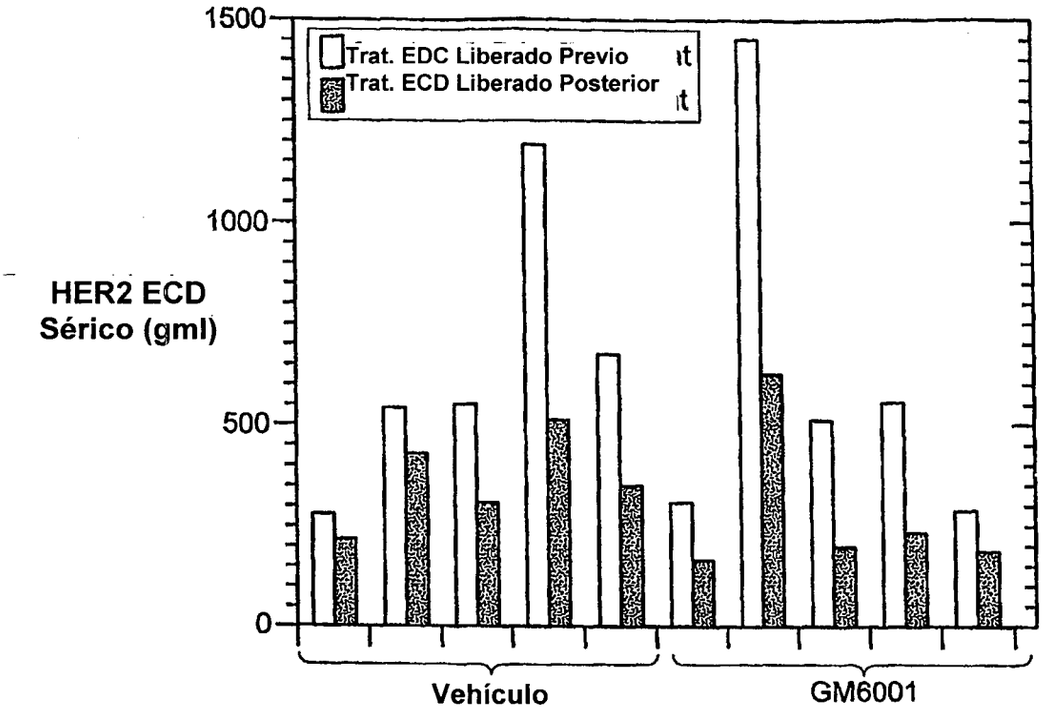


FIG. 18


1500
1000
500
0
HER2 ECD
Sérico (gml)
Trat. EDC Liberado Previo
Trat. ECD Liberado Posterior
Vehículo
GM6001

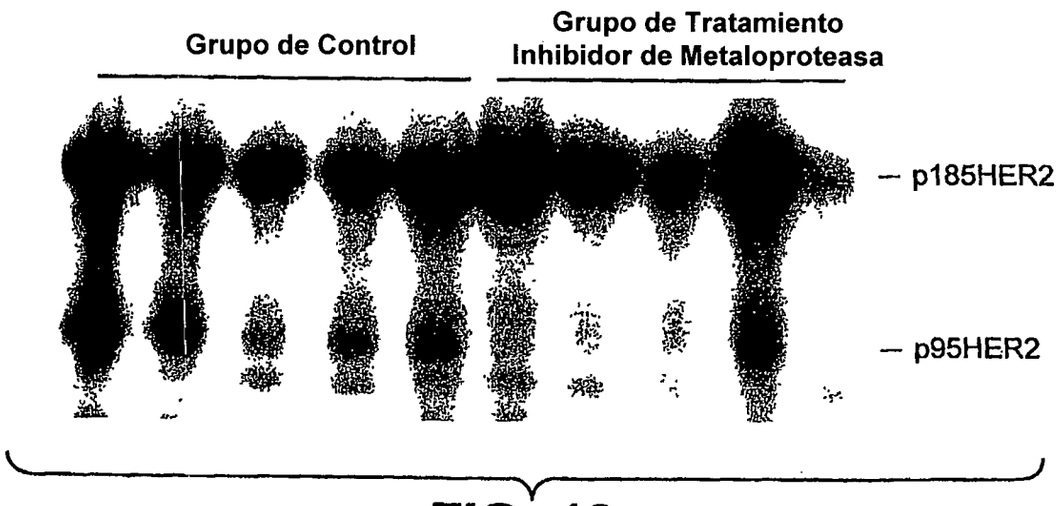

Grupo de Control
Grupo de Tratamiento
Inhibidor de Metaloproteasa
p185HER2
p95HER2

FIG. 19

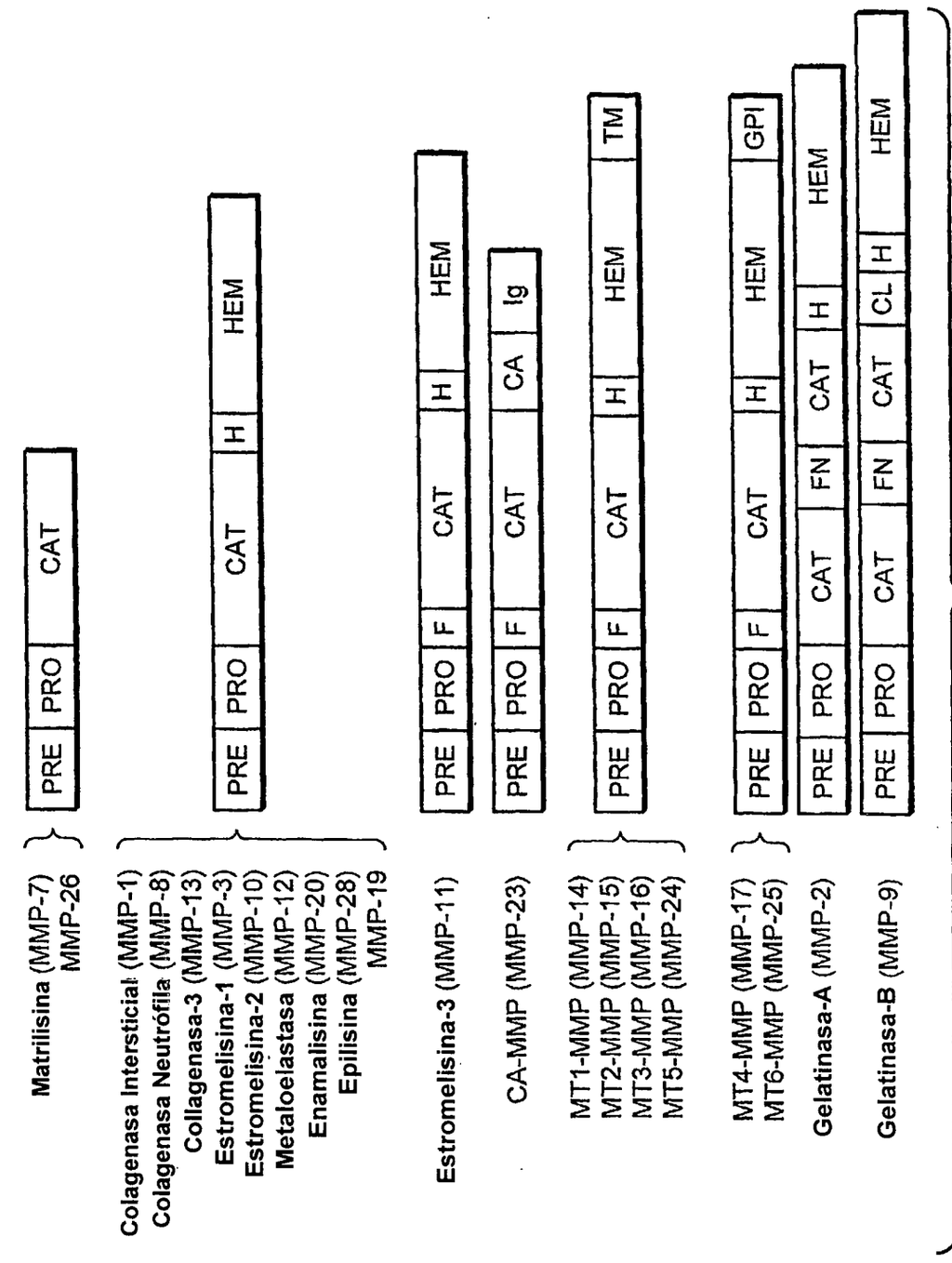

Matrilisina (MMP-7)
MMP-26
PRE
PRO
CAT
Colagenasa Intersticial (MMP-1)
Colagenasa Neutrófila (MMP-8)
Collagenasa-3 (MMP-13)
Estromelisina-1 (MMP-3)
Estromelisina-2 (MMP-10)
Metaloelastasa (MMP-12)
Enamalisina (MMP-20)
Epilisina (MMP-28)
MMP-19
H
HEM
Estromelisina-3 (MMP-11)
CA-MMP (MMP-23)
CA
Ig
MT1-MMP (MMP-14)
MT2-MMP (MMP-15)
MT3-MMP (MMP-16)
MT5-MMP (MMP-24)
TM
MT4-MMP (MMP-17)
MT6-MMP (MMP-25)
GPI
Gelatinasa-A (MMP-2)
FN
Gelatinasa-B (MMP-9)
CL

FIG. 20

Listado de Secuencias

<110> GENENTECH, INC.
CAREY, Kendall D.
SCHWALL, Ralph H.
SLIWKOWSKI, Mark X.

<120> INHIBICIÓN LIBERACIÓN DE HER2 CON ANTAGONISTAS DE METALOPROTEASA DE MATRIZ

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<151> 2005-02-09

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<213> Homo sapiens

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Ser	Pro	Glu	Thr	His	Leu	Asp	Met	Leu	Arg	His	Leu	Tyr	Gln	Gly	
				20				25						30	
Cys	Gln	Val	Val	Gln	Gly	Asn	Leu	Glu	Leu	Thr	Tyr	Leu	Pro	Thr	
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Asn	Ala	Ser	Leu	Ser	Phe	Leu	Gln	Asp	Ile	Gln	Glu	Val	Gln	Gly	
				50				55						60	
Tyr	Val	Leu	Ile	Ala	His	Asn	Gln	Val	Arg	Gln	Val	Pro	Leu	Gln	
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Arg	Leu	Arg	Ile	Val	Arg	Gly	Thr	Gln	Leu	Phe	Glu	Asp	Asn	Tyr	
				80				85						90	
Ala	Leu	Ala	Val	Leu	Asp	Asn	Gly	Asp	Pro	Leu	Asn	Asn	Thr	Thr	
				95				100						105	
Pro	Val	Thr	Gly	Ala	Ser	Pro	Gly	Gly	Leu	Arg	Glu	Leu	Gln	Leu	
				110				115						120	
Arg	Ser	Leu	Thr	Glu	Ile	Leu	Lys	Gly	Gly	Val	Leu	Ile	Gln	Arg	
				125				130						135	
Asn	Pro	Gln	Leu	Cys	Tyr	Gln	Asp	Thr	Ile	Leu	Trp	Lys	Asp	Ile	
				140				145						150	
Phe	His	Lys	Asn	Asn	Gln	Leu	Ala	Leu	Thr	Leu	Ile	Asp	Thr	Asn	
				155				160						165	
Arg	Ser	Arg	Ala	Cys	His	Pro	Cys	Ser	Pro	Met	Cys	Lys	Gly	Ser	
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Lys His Ser Asp Cys Leu Ala Cys Leu His Phe Asn His Ser Gly
35 40 45

Ile Cys Glu Leu His Cys Pro Ala Leu Val Thr Tyr Asn Thr Asp
50 55 60

Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg Tyr Thr Phe Gly
65 70 75

Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu Ser Thr Asp
80 85 90

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Cys Ala Arg Val

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Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp Pro Ala
35 40 45

Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe Glu
50 55 60

Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
65 70 75

Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile
80 85 90

Arg Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln
95 100 105

Gly Leu Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu
110 115 120

Gly Ser Gly Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe

10

125 130 135

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Glu Gly Leu Ala

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<212> PRT
<213> Homo sapiens

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20 25 30

Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val
35 40 45

Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
50 55 60

Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val
65 70 75

Ala Cys Ala His Tyr Lys Asp Pro Pro Phe Cys Val Ala Arg Cys
80 85 90

Pro Ser Gly Val Lys Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys
95 100 105

Phe Pro Asp Glu Glu Gly Ala Cys Gln Pro Cys Pro Ile Asn Cys
110 115 120

Thr His Ser Cys Val Asp Leu Asp Asp Lys Gly Cys Pro Ala Glu
125 130 135

Gln Arg Ala Ser Pro Leu Thr
140

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<212> PRT
<213> Secuencia artificial

<220>
<223> Se sintetiza la secuencia

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20 25 30

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267

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

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

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

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
110 115 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
125 130 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
140 145 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
155 160 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
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Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
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Arg Gly Glu Cys

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20 25 30

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35 40 45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
50 55 60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
65 70 75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp

204

80 85 90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
95 100 105

Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
110 115 120

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser
125 130 135

Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys
140 145 150

Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala
155 160 165

Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
170 175 180

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
185 190 195

Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
200 205 210

Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys
215 220 225

Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
245 250 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
260 265 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
275 280 285

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
290 295 300

Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
305 310 315

Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
320 325 330

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
335 340 345

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
350 355 360

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
365 370 375

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
380 385 390

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly

203

395 400 405

Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
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His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
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20 25 30

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

Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser
50 55 60

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

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

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

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
110 115 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
125 130 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
140 145 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
155 160 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
170 175 180

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Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
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Arg Gly Glu Cys

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Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
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Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
             35                  40                  45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
             50                  55                  60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
             65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
             80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
             95                 100                 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
            110                 115                 120

Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
            125                 130                 135

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp
            140                 145                 150

Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu
            155                 160                 165

Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly
            170                 175                 180

Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
            185                 190                 195

Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn
            200                 205                 210

Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr
            215                 220                 225

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
            230                 235                 240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
            245                 250                 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
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207

260 265 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
275 280 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
290 295 300

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp
305 310 315

Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
320 325 330

Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
335 340 345

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met
350 355 360

Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
365 370 375

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
380 385 390

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
395 400 405

Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
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aggtcagatg gtggcg 66

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Ile Val

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20 25

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Gly Ile Leu

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Pro

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Pro

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Pro

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1 5 10 15

Pro

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Ala Arg Thr

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30 DAY US IDS
• P2195R1 - 11 JAN 07

PATENT COOPERATION TREA /CK

RECEIVED
DEC 14 2006
GENENTECH, INC.
LEGAL DEPT.

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

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

(PCT Rule 44.1)

To:

GENENTECH, INC.
Attn. Kubinec, Jeffrey S.
MS 49
1 DNA Way
South San Francisco CA 94080
ETATS-UNIS D'AMERIQUE

Calendared/CLK
Resp to ISR/Consid CH2 - 12 FEB 07
Ch 2 Demand/Resp to WO - 12 MAR 07
Due Date

Applicant's or agent's file reference P2195R1	Date of mailing *(day/month/year)* 12/12/2006
International application No. PCT/US2006/004926	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
Applicant GENENTECH, INC.	International filing date *(day/month/year)* 09/02/2006

1. [x] 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 19:
   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, Fascimile No.: (41-22) 338.82.70

   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 90*bis*.1 and 90*bis*.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 **19 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) 340-3016	Authorized officer Odette Durand-Fleith

Form PCT/ISA/220 (October 2005)

(See notes on accompanying sheet)

NOTES TO FORM PCT/ISA/220

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 cancelling 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.

PATENT COOPERATION TREA

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
P2195R1		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2006/004926	09/02/2006	09/02/2005
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.

[X] 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:
      [X] 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. [X] 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,**
   [X] 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,**
   [X] 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. _____
      [ ] 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. [X] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2006/004926

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

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] on paper

   [X] in electronic form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [X] 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. C12N15/11 C12N9/64 A61K39/395 G01N33/574 A61P35/00

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)
C12N A61K G01N

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, WPI Data, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2004/096899 A1 (AOKI TAKANORI [JP] ET AL) 20 May 2004 (2004-05-20) whole document esp. [0002, 0004, 0006, 0027, 0042, 0074, 0108, 0111, 0208, 0213, 0217], claims 48,49,52	13,20,21 1-12, 14-19
X Y	WO 2004/096139 A2 (INCYTE CORP [US]; YAO WENQING [US]; ZHOU JINCONG [US]; XU MEIZHONG [US]) 11 November 2004 (2004-11-11) whole document esp. pages 4,7,8,9,146,152, claims 91-93	13-16 1,2, 5-12, 17-21

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[X] Further documents are listed in the continuation of Box C. [X] 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.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 November 2006	12/12/2006

Name and mailing address of the ISA	Authorized officer
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	Brück, Marianne

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2004/087042 A2 (YEDA RES & DEV [IL]; SAGI IRIT [IL]; DANON TAMAR [IL]) 14 October 2004 (2004-10-14) whole document esp. pages 1, 8, 22, 23, 32, claim 20	13 1-12, 14-21
X Y	WO 02/20020 A (PHARMACIA & UPJOHN SPA [IT]; UPJOHN CO [US]; SALLE ENRICO DI [IT]; PIS) 14 March 2002 (2002-03-14) whole document esp. pages 2-3, 8, claim 6	13-16 1-12, 17-21
X Y	WO 03/005971 A2 (PARATEK PHARMACEUTICALS INC [US]) 23 January 2003 (2003-01-23) whole document esp. pages 8, 11, 18, 134 claims 3, 5, 30-33	13-16 1-12, 17-21
Y	US 2002/164334 A1 (ARAKAWA TSUTOMU [US] ET AL) 7 November 2002 (2002-11-07) whole document esp. [0001, 0003], claim 1	1,2,5-21
Y	WO 03/026490 A2 (ELUSYS THERAPEUTICS INC [US]; JACKSON DAVID [US]; NARDONE LINDA [US]) 3 April 2003 (2003-04-03) whole document esp. pages 3,5-7,9,52, claims 1,3,8,16	5-10, 14-16
Y	WO 98/42865 A (UNIV MELBOURNE [AU]; PRINCE HENRYS INST MED RES [AU]; LOPATA ALEXANDER) 1 October 1998 (1998-10-01) whole document esp. pages 2-5	1-21
Y	MOLINA MA ET AL: "Trastuzumab (Herceptin), a Humanized Anti-HER2 Receptor Monoclonal Antibody, Inhibits Basal and Activated HER2 Ectodomain Cleavage in Breast Cancer Cells" CANCER RESEARCH, vol. 61, 2001, pages 4744-4749, XP002408598 whole document esp. abstract, pages 4748-4749	1-21
Y	WO 2004/046354 A (BEIERSDORF AG) 3 June 2004 (2004-06-03) whole document esp. pages 3,5,6,10-11	1-21
P,X	WO 2005/037826 A (INCYTE CORP [US]; LI YUN-LONG [US]; ZHOU JINCONG [US]; BURNS DAVID [US]) 28 April 2005 (2005-04-28) whole document esp. pages 2,3,12	1,2,5-19

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AGAMI, REUVEN: "RNAi and related mechanisms and their potential use for therapy" CURRENT OPINION IN CHEMICAL BIOLOGY, vol. 6, 2002, pages 829-834, XP002239988 the whole document	1-21

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004096899 A1	20-05-2004	AU 2312902 A	27-05-2002
		EP 1336851 A1	20-08-2003
		WO 0241000 A1	23-05-2002
WO 2004096139 A2	11-11-2004	AU 2004233863 A1	11-11-2004
		BR PI0409686 A	04-07-2006
		CA 2523426 A1	11-11-2004
		EP 1622569 A2	08-02-2006
		KR 20060008918 A	27-01-2006
		MX PA05011423 A	12-12-2005
		SE 0502338 A	23-12-2005
WO 2004087042 A2	14-10-2004	EP 1610678 A2	04-01-2006
WO 0220020 A	14-03-2002	AU 8986501 A	22-03-2002
		BR 0113625 A	22-07-2003
		CA 2419590 A1	14-03-2002
		CN 1729002 A	01-02-2006
		EP 1317270 A1	11-06-2003
		JP 2004508334 T	18-03-2004
		MX PA03001983 A	24-06-2003
		NZ 524104 A	24-12-2004
		US 2004024044 A1	05-02-2004
		ZA 200301309 A	18-02-2004
WO 03005971 A2	23-01-2003	AU 2002318238 A1	29-01-2003
		EP 1408987 A2	21-04-2004
		JP 2004537544 T	16-12-2004
US 2002164334 A1	07-11-2002	NONE	
WO 03026490 A2	03-04-2003	CA 2461631 A1	03-04-2003
		EP 1487487 A2	22-12-2004
		JP 2005510470 T	21-04-2005
WO 9842865 A	01-10-1998	AT 331039 T	15-07-2006
		CA 2284099 A1	01-10-1998
		CN 1257550 A	21-06-2000
		EP 0979304 A1	16-02-2000
		JP 2002501613 T	15-01-2002
		NZ 337868 A	31-05-2002
		US 6607894 B1	19-08-2003
		ZA 9802403 A	21-10-1998
WO 2004046354 A	03-06-2004	AU 2003298128 A1	15-06-2004
		DE 10254214 A1	09-06-2004
		EP 1576155 A1	21-09-2005
		US 2006058256 A1	16-03-2006
WO 2005037826 A	28-04-2005	AU 2004282189 A1	28-04-2005
		CA 2541509 A1	28-04-2005
		EP 1678167 A1	12-07-2006

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43*bis*.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.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2006/004926	09.02.2006	09.02.2005

International Patent Classification (IPC) or both national classification and IPC
INV. C12N15/11 C12N9/64 A61K39/395 G01N33/574 A61P35/00

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 43*bis*.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 notifed the International Bureau under Rule 66.1*bis*(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:	Date of completion of this opinion	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	see form PCT/ISA/210	Brück, Marianne Telephone No. +49 89 2399-8735

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 43*bis*.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 43*bis*.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. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-12,17-19,
	No:	Claims	13-16
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-21
Industrial applicability (IA)	Yes:	Claims	cf. SepSheet point V 5.
	No:	Claims	

2. Citations and explanations

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

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY (SEPARATE SHEET)

International application No.
PCT/US2006/004926

Section II

Priority

The present assessment of novelty and inventive step has been made on the assumption that the claims are entitled to their earliest declared priority date. D11, the cited P-document, discloses the claimed subject-matter and might, therefore, also be considered for the question of novelty or become state of the art in case of an invalid priority.

Section V

1. **Subject matter**

The independent claims relate to methods
- for inhibiting HER2 shedding with a matrix metalloprotease (MMP) antagonist (claim 1);
- for reducing HER2 extracellular domain (ECD) serum level in a mammal with a matrix metalloprotease (MMP) antagonist (claim 11);
- for treating cancer with a matrix metalloprotease (MMP) antagonist (claim 13);
- for treating a HER inhibitor resistant cancer with a matrix metalloprotease (MMP) antagonist (claim 17);
- for reducing p95 HER2 level in a cell with a matrix metalloprotease (MMP) antagonist (claim 19);
- of diagnosis by evaluating MMP-15 (MT2-MMP) as indication for an elevated p95 HER2 or shed HER2 serum level leading to a poor clinical prognosis (claim 20).

2. **Prior art**

The documents are numbered chronologically as they appear in the Search Report.

3. **Novelty**

Claims 13-16 and 20-21 are not novel because the treatment of cancer with matrix metalloprotease inhibitors has already been disclosed in the cited prior art (for details concerning the claims and relevant passages in the affected documents please cf. to the International Search Report).

4. Inventive Step

Claims 13-16 and 20-21 are not novel, therefore, also not inventive.

The remaining claims have been implicitly disclosed/ are obvious in the light of the numerous cited documents which have already disclosed the correlation of HER2 shedding - matrix metalloprotease - cancer (for details concerning the claims and relevant passages in the affected documents please cf. to the International Search Report).

5. Industrial applicability

For the assessment of the present claims 1-21 on the question whether it is industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims.
The EPO does not, for example, recognize as industrially applicable the subject matter of claims to the use of a compound in medical treatment, but may allow 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.
In case some methods are meant to be conducted only in vitro, "*in vitro*" should be introduced in the affected claims.

Section VIII

The application does not fulfill the requirements of Article 6, because dependant claim 18 is unclear due to the following:

- claim 18 is dependant from claim 17 relating to the treatment of HER inhibitor resistant cancer with a matrix metalloprotease (MMP) antagonist, but is specifying the HER inhibitor trastuzumab. This is inconsistent and contradictory and, renders, therefore, the claim unclear.

Bitte beachten Sie, dass angeführte Nichtpatentliteratur (wie z. B. wissenschaftliche oder technische Dokumente) je nach geltendem Recht dem Urheberrechtsschutz und/oder anderen Schutzarten für schriftliche Werke unterliegen könnte. Die Vervielfältigung urheberrechtlich geschützter Texte, ihre Verwendung in anderen elektronischen oder gedruckten Publikationen und ihre Weitergabe an Dritte ist ohne ausdrückliche Zustimmung des Rechtsinhabers nicht gestattet.

Veuillez noter que les ouvrages de la littérature non-brevets qui sont cités, par exemple les documents scientifiques ou techniques, etc., peuvent être protégés par des droits d'auteur et/ou toute autre protection des écrits prévue par les législations applicables. Les textes ainsi protégés ne peuvent être reproduits ni utilisés dans d'autres publications électroniques ou imprimées, ni rediffusés sans l'autorisation expresse du titulaire du droit d'auteur.

Please be aware that cited works of non-patent literature such as scientific or technical documents or the like may be subject to copyright protection and/or any other protection of written works as appropriate based on applicable laws. Copyrighted texts may not be copied or used in other electronic or printed publications or re-distributed without the express permission of the copyright holder.

XS CPRTENFRDE

22K

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**	
0-4-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.51.001.176 MT/FOP 20060101/0.20.4rc.2.7
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**	P2195R1
I	**Title of Invention**	INHIBITING HER2 SHEDDING WITH MATRIX METALLOPROTEASE ANTAGONISTS
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 MS 49 South San Francisco, California 94080 United States of America
II-6	State of nationality	US
II-7	State of residence	US
II-8	Telephone No.	650-225-1000
II-9	Facsimile No.	650-952-9881

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PCT REQUEST

Original (for **SUBMISSION**)

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)	CAREY, Kendall, D.
III-1-5	Address	545 Miller Avenue Apartment No. 5 South San Francisco, California 94080 United States of America
III-1-6	State of nationality	US
III-1-7	State of residence	US
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)	SCHWALL, Ralph, H.
III-2-5	Address	400 Griffin Avenue Pacifica, California 94044 United States of America
III-2-6	State of nationality	US
III-2-7	State of residence	US
III-3	**Applicant and/or inventor**	
III-3-1	This person is	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	SLIWKOWSKI, Mark, X.
III-3-5	Address	42 Oak Creek Lane San Carlos, California 94070 United States of America
III-3-6	State of nationality	US
III-3-7	State of residence	US

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PCT REQUEST

Original (for **SUBMISSION**)

IV-1	**Agent or common representative; or address for correspondence**	
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V	**DESIGNATIONS**	
V-1	**The filing of this request constitutes under Rule 4.9(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	09 February 2005 (09.02.2005)
VI-1-2	Number	60/651,348
VI-1-3	Country	US

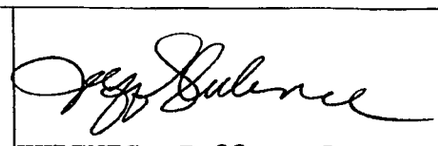
PCT REQUEST

Original (for **SUBMISSION**)

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)	
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)	5	✓
IX-2	Description	45	-
IX-3	Claims	1	-
IX-4	Abstract	1	✓
IX-5	Drawings	22	-
IX-7	TOTAL	74	
	Accompanying Items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-11	Copy of general power of attorney	✓	-
IX-16(A)	Sequence listing in electronic form		
IX-16(A)-(ii)	additional copies including, where applicable, the copy for the purposes of international search under Rule 13ter	-	1 Diskette
IX-17	PCT-SAFE physical media	-	✓
IX-18	other	Sequence listing in paper copy	
IX-18	other	Statement regarding sequence listing	
IX-19	**Figure of the drawings which should accompany the abstract**	NONE	
IX-20	**Language of filing of the international application**	English	

PCT REQUEST

Original (for **SUBMISSION**)

X-1	**Signature of applicant, agent or common representative**	
X-1-1	Name (LAST, First)	KUBINEC, Jeffrey S.
X-1-2	Name of signatory	
X-1-3	Capacity	

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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**	

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11-1	**Date of receipt of the record copy by the International Bureau**	

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INHIBITING HER2 SHEDDING WITH MATRIX METALLOPROTEASE ANTAGONISTS

This is a non-provisional application claiming priority under 35 USC §119 to provisional application no. 60/651,348 filed February 9, 2005, the entire disclosure of which is hereby incorporated by reference.

Field of the Invention

The present invention concerns using antagonists of matrix metalloproteases (MMPs), especially of MMP-15, for inhibiting HER2 shedding.

Background of the Invention

The HER family of receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes four distinct members including epidermal growth factor receptor (EGFR, ErbB1, or HER1), HER2 (ErbB2 or $p185^{neu}$), HER3 (ErbB3) and HER4 (ErbB4 or tyro2).

EGFR, encoded by the *erb*B1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, transforming growth factor alpha (TGF-α), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn *Pharmac. Ther.* 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-α and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, *e.g.*, Baselga and Mendelsohn., *supra*; Masui *et al. Cancer Research* 44:1002-1007 (1984); and Wu *et al. J. Clin. Invest.* 95:1897-1905 (1995).

The second member of the HER family, $p185^{neu}$, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the *neu* proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of *neu* is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon *et al.*, *Science*, 235:177-182 (1987); Slamon *et al.*, *Science*, 244:707-712 (1989); and US Pat No. 4,968,603). To date, no point mutation analogous to that in the *neu* proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. See, among others, King *et al.*, *Science*, 229:974 (1985); Yokota *et al.*, *Lancet*: 1:765-767 (1986); Fukushige *et al.*, *Mol Cell Biol.*, 6:955-958 (1986); Guerin *et al.*, *Oncogene Res.*, 3:21-31 (1988); Cohen *et al.*, *Oncogene*, 4:81-88 (1989); Yonemura *et al.*, *Cancer Res.*, 51:1034 (1991); Borst *et al.*, *Gynecol. Oncol.*, 38:364 (1990); Weiner *et al.*, *Cancer Res.*, 50:421-425 (1990); Kern *et al.*, *Cancer Res.*, 50:5184 (1990); Park *et al.*, *Cancer Res.*, 49:6605 (1989); Zhau *et al.*, *Mol. Carcinog.*, 3:254-257 (1990); Aasland *et al. Br. J. Cancer* 57:358-363 (1988); Williams *et al. Pathobiology* 59:46-52 (1991); and McCann *et al.*, *Cancer*, 65:88-92 (1990). HER2 may be overexpressed in prostate cancer (Gu

et al. Cancer Lett. 99:185-9 (1996); Ross *et al. Hum. Pathol.* 28:827-33 (1997); Ross *et al. Cancer* 79:2162-70 (1997); and Sadasivan *et al. J. Urol.* 150:126-31 (1993)).

Antibodies directed against the rat $p185^{neu}$ and human HER2 protein products have been described.

Drebin and colleagues have raised antibodies against the rat *neu* gene product, $p185^{neu}$ See, for example, Drebin *et al., Cell* 41:695-706 (1985); Myers *et al., Meth. Enzym.* 198:277-290 (1991); and WO94/22478. Drebin *et al. Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions of $p185^{neu}$ result in synergistic anti-tumor effects on *neu*-transformed NIH-3T3 cells implanted into nude mice. See also U.S. Patent 5,824,311 issued October 20, 1998.

Hudziak *et al., Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of HER2 antibodies which were characterized using the human breast tumor cell line SK-BR-3. Relative cell proliferation of the SK-BR-3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also U.S. Patent No. 5,677,171 issued October 14, 1997. The HER2 antibodies discussed in Hudziak *et al.* are further characterized in Fendly *et al. Cancer Research* 50:1550-1558 (1990); Kotts *et al. In Vitro* 26(3):59A (1990); Sarup *et al. Growth Regulation* 1:72-82 (1991); Shepard *et al. J. Clin. Immunol.* 11(3):117-127 (1991); Kumar *et al. Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis *et al. Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras *et al. Oncogene* 9:1829-1838 (1994); Vitetta *et al. Cancer Research* 54:5301-5309 (1994); Sliwkowski *et al. J. Biol. Chem.* 269(20):14661-14665 (1994); Scott *et al. J. Biol. Chem.* 266:14300-5 (1991); D'souza *et al. Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis *et al. Cancer Research* 56:1457-1465 (1996); and Schaefer *et al. Oncogene* 15:1385-1394 (1997).

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN™; U.S. Patent No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga *et al., J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration September 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein.

Other HER2 antibodies with various properties have been described in Tagliabue *et al. Int. J. Cancer* 47:933-937 (1991); McKenzie *et al. Oncogene* 4:543-548 (1989); Maier *et al. Cancer Res.* 51:5361-5369 (1991); Bacus *et al. Molecular Carcinogenesis* 3:350-362 (1990); Stancovski *et al. PNAS (USA)* 88:8691-8695 (1991); Bacus *et al. Cancer Research* 52:2580-2589 (1992); Xu *et al. Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk *et al. Cancer Research* 52:2771-2776 (1992);Hancock *et al. Cancer Res.* 51:4575-4580 (1991); Shawver *et al. Cancer Res.* 54:1367-1373 (1994); Arteaga *et al. Cancer Res.* 54:3758-3765 (1994); Harwerth *et al. J. Biol. Chem.* 267:15160-15167 (1992); U.S. Patent No. 5,783,186; and Klapper *et al. Oncogene* 14:2099-2109 (1997).

Homology screening has resulted in the identification of two other HER receptor family members; HER3 (US Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus *et al. PNAS (USA)* 86:9193-9197 (1989)) and HER4 (EP Pat Appln No 599,274; Plowman *et al., Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); and

Plowman *et al., Nature*, 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The HER receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of HER ligands (Earp *et al. Breast Cancer Research and Treatment* 35: 115-132 (1995)). EGFR is bound by six different ligands; epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin, heparin binding epidermal growth factor (HB-EGF), betacellulin and epiregulin (Groenen *et al. Growth Factors* 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta and gamma heregulins (Holmes *et al., Science*, 256:1205-1210 (1992); U.S. Patent No. 5,641,869; and Schaefer *et al. Oncogene* 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motor neuron derived factor (SMDF). For a review, see Groenen *et al. Growth Factors* 11:235-257 (1994); Lemke, G. *Molec. & Cell. Neurosci.* 7:247-262 (1996) and Lee *et al. Pharm. Rev.* 47:51-85 (1995). Recently three additional HER ligands were identified; neuregulin-2 (NRG-2) which is reported to bind either HER3 or HER4 (Chang *et al. Nature* 387 509-512 (1997); and Carraway *et al Nature* 387:512-516 (1997)); neuregulin-3 which binds HER4 (Zhang *et al. PNAS* (USA) 94(18):9562-7 (1997)); and neuregulin-4 which binds HER4 (Harari *et al. Oncogene* 18:2681-89 (1999)) HB-EGF, betacellulin and epiregulin also bind to HER4.

While EGF and TGFα do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase. See Earp *et al., supra.* Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski *et al., J. Biol. Chem.*, 269(20):14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi *et al., Journal of Neuroscience* 15: 1329-1340 (1995); Morrissey *et al., Proc. Natl. Acad. Sci. USA* 92: 1431-1435 (1995); and Lewis *et al., Cancer Res.*, 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, *Cell* 78:5-8 (1994)).

The HER signaling network is depicted in Fig. 4.

Patent publications related to HER antibodies include: US 5,677,171, US 5,720,937, US 5,720,954, US 5,725,856, US 5,770,195, US 5,772,997, US 6,165,464, US 6,387,371, US 6,399,063, US2002/0192211A1, US 6,015,567, US 6,333,169, US 4,968,603, US 5,821,337, US 6,054,297, US 6,407,213, US 6,719,971, US 6,800,738, US2004/0236078A1, US 5,648,237, US 6,267,958, US 6,685,940, US 6,821,515, WO98/17797, US 6,127,526, US 6,333,398, US 6,797,814, US 6,339,142, US 6,417,335, US 6,489,447, WO99/31140, US2003/0147884A1, US2003/0170234A1, US2005/0002928A1, US 6,573,043, US2003/0152987A1, WO99/48527, US2002/0141993A1, WO01/00245, US2003/0086924, US2004/0013667A1, WO00/69460, WO01/00238, WO01/15730, US 6,627,196B1, US6,632,979B1, WO01/00244, US2002/0090662A1, WO01/89566, US2002/0064785, US2003/0134344, WO 04/24866, US2004/0082047, US2003/0175845A1, WO03/087131, US2003/0228663, WO2004/008099A2, US2004/0106161, WO2004/048525, US2004/0258685A1, US 5,985,553, US 5,747,261, US 4,935,341, US 5,401,638, US 5,604,107, WO 87/07646, WO 89/10412, WO 91/05264, EP 412,116 B1, EP 494,135 B1, US 5,824,311, EP 444,181 B1, EP 1,006,194

A2, US 2002/0155527A1, WO 91/02062, US 5,571,894, US 5,939,531, EP 502,812 B1, WO 93/03741, EP 554,441 B1, EP 656,367 A1, US 5,288,477, US 5,514,554, US 5,587,458, WO 93/12220, WO 93/16185, US 5,877,305, WO 93/21319, WO 93/21232, US 5,856,089, WO 94/22478, US 5,910,486, US 6,028,059, WO 96/07321, US 5,804,396, US 5,846,749, EP 711,565, WO 96/16673, US 5,783,404, US 5,977,322, US 6,512,097, WO 97/00271, US 6,270,765, US 6,395,272, US 5,837,243, WO 96/40789, US 5,783,186, US 6,458,356, WO 97/20858, WO 97/38731, US 6,214,388, US 5,925,519, WO 98/02463, US 5,922,845, WO 98/18489, WO 98/33914, US 5,994,071, WO 98/45479, US 6,358,682 B1, US 2003/0059790, WO 99/55367, WO 01/20033, US 2002/0076695 A1, WO 00/78347, WO 01/09187, WO 01/21192, WO 01/32155, WO 01/53354, WO 01/56604, WO 01/76630, WO02/05791, WO 02/11677, US 6,582,919, US2002/0192652A1, US 2003/0211530A1, WO 02/44413, US 2002/0142328, US 6,602,670 B2, WO 02/45653, WO 02/055106, US 2003/0152572, US 2003/0165840, WO 02/087619, WO 03/006509, WO03/012072, WO 03/028638, US 2003/0068318, WO 03/041736, EP 1,357,132, US 2003/0202973, US 2004/0138160, US 5,705,157, US 6,123,939, EP 616,812 B1, US 2003/0103973, US 2003/0108545, US 6,403,630 B1, WO 00/61145, WO 00/61185, US 6,333,348 B1, WO 01/05425, WO 01/64246, US 2003/0022918, US 2002/0051785 A1, US 6,767,541, WO 01/76586, US 2003/0144252, WO 01/87336, US 2002/0031515 A1, WO 01/87334, WO 02/05791, WO 02/09754, US 2003/0157097, US 2002/0076408, WO 02/055106, WO 02/070008, WO 02/089842 and WO 03/86467.

The HER2 extracellular domain (ECD) is proteolytically shed from breast carcinoma cells in culture (Petch *et al., Mol. Cell. Biol.* 10:2973-2982 (1990); Scott *et al., Mol. Cell. Biol.* 13:2247-2257 (1993); and Lee and Maihle, *Oncogene* 16:3243-3252(1998)), and is found in the serum of some cancer patients (Leitzel *et al., J. Clin. Oncol.* 10:1436-1443 (1992)). HER2 ECD may be a serum marker of metastatic breast cancer (Leitzel *et al., J. Clin. Oncol.* 10:1436-1443 (1992)), and may allow escape of HER2 overexpressing tumors from immunological control (Baselga *et al., J. Clin. Oncol.* 14:737-744 (1997), Brodowicz *et al., Int. J. Cancer* 73:875-879 (1997)). Shed HER2 ECD serum levels represent an independent marker of poor clinical outcome in patients with HER2 overexpressing metastatic breast cancer (Ali *et al., Clin. Chem.* 48:1314-1320 (2002); Molina *et al., Clin. Cancer Res.* 8:347-353 (2002)).

A truncated extracellular domain of HER2 is also the product of a 2.3 kb alternative transcript generated by use of a polyadenylation signal within an intron (Scott *et al., Mol. Cell. Biol.* 13:2247-2257 (1993)). The alternative transcript was first identified in the gastric carcinoma cell line, MKN7 (Yamamoto *et al., Nature* 319:230-234 (1986); and Scott *et al., Mol. Cell. Biol.* 13:2247-2257 (1993)) and the truncated receptor was located within the perinuclear cytoplasm rather than secreted from these tumor cells (Scott *et al., Mol. Cell. Biol.* 13:2247-2257 (1993)).

Another alternatively spliced product of HER2, called "herstatin," has also been identified (Doherty *et al., Proc. Natl. Acad. Sci.* 96:10869-10874 (1999); Azios *et al., Oncogene* 20:5199-5209 (2001); Justman and Clinton, *J. Biol. Chem.* 277:20618-20624 (2002)). This protein consists of subdomains I and II from the extracellular domain followed by a unique C-terminal sequence encoded by intron 8.

Another mechanism that may account for poor clinical outcome in HER2 overexpressing tumors is suggested by the observation that, in some HER2 overexpressing tumor cells, the receptor is processed by an unknown metalloprotease (or metalloproteinase) to yield a truncated, membrane-associated receptor (sometimes

referred to as a "stub" and also known as p95), and a soluble extracellular domain (also known as ECD, ECD105, or p105).

As with other HER receptors, loss of the extracellular ligand binding domain renders the HER2 intracellular membrane-associated domain a constitutively active tyrosine kinase. It has therefore been postulated that the processing of the HER2 ECD creates a constitutively active receptor that can directly deliver growth and survival signals to the cancer cell. See, US Pat No. 6,541,214 (Clinton), and US Patent Appln No. 2004/0247602A1 (Friedman *et al.*)

Saez *et al. Clin Cancer Res.* 12(2): 424-431 (January, 2006) report that patients whose tumors express high levels of p95 have a significantly worse outcome than patients who do not. At present p95 level can only be determined by Western blot.

Summary of the Invention

In a first aspect, the invention concerns a method for inhibiting HER2 shedding comprising treating a HER2 expressing cell with a matrix metalloprotease (MMP) antagonist in an amount effective to inhibit HER2 shedding.

In addition, the invention provides a method for reducing HER2 extracellular domain (ECD) serum level in a mammal, comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to reduce the HER2 ECD serum level in the mammal.

In yet another aspect, a method for treating cancer in a mammal is provided, which comprises administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to treat the cancer.

Also, the invention concerns a method for treating a HER inhibitor-resistant cancer in a mammal comprising administering to the mammal a matrix metalloprotease (MMP) antagonist in an amount effective to treat the cancer.

In yet a further aspect, the invention relates to a method for reducing p95 HER2 level in a cell comprising exposing the cell to a matrix metalloprotease (MMP) antagonist in an amount effective to reduce the p95 HER2 level.

The invention also concerns a method of diagnosis (or prognosis) comprising evaluating MMP-15 (MT2-MMP) in a sample from a cancer patient, wherein elevated MMP-15 level or activity indicates the patient has an elevated p95 HER2 and/or shed HER2 serum level, and/or will have a poor clinical outcome. Preferably, MMP-15 level (protein or nucleic acid) is evaluated in the method and is used to identify patients with a poor prognosis, or who will have a poor clinical outcome. Optionally, the patient's cancer further displays HER expression, amplification, or activation, most preferably HER2 overexpression or amplification.

Brief Description of the Drawings

Figure 1 provides a schematic of the full length HER2 protein structure, and amino acid sequences for Domains I-IV (SEQ ID NOs. 1-4, respectively) of the extracellular domain thereof.

Figures 2A and 2B show the amino acid sequences of trastuzumab light chain (Fig. 2A; SEQ ID No. 5) and heavy chain (Fig. 2B; SEQ ID No. 6), respectively.

Figures 3A and 3B show the amino acid sequences of pertuzumab light chain (Fig. 3A; SEQ ID NO. 7) and heavy chain (Fig. 3B; SEQ ID NO. 8). CDRs are shown in bold. Calculated molecular mass of the light

chain and heavy chain are 23,526.22 Da and 49,216.56 Da (cysteines in reduced form). The carbohydrate moiety is attached to Asn 299 of the heavy chain.

Fig. 4 depicts the HER signaling network.

Fig. 5 illustrates trastuzumab inhibition of HER2 ECD shedding from HER2 overexpressing breast cancer cell lines (SKBR3, MT474) compared to nonoverexpressing breast cancer cell line (MCF-7).

Fig. 6 illustrates differences in p95 HER2 and shed HER2 ECD levels in MMTVHER2 transgenic tumors (f2:1282 tumors, trastuzumab-sensitive; and Fo5 tumors, trastuzumab-resistant).

Fig. 7 depicts the strategy used to identify HER2 sheddase.

Fig. 8 illustrates experiments which demonstrated sheddase had properties of a metalloprotease.

Fig. 9 shows expression of MMPs in MMTV-HER2 tumors and cell lines. MMP-15 is a candidate for explaining differences between f2:1282 tumors which are trastuzumab-sensitive, and Fo5 tumors which are trastuzumab-resistant.

Fig. 10 reflects interaction between flag-HER2 and MMP-15.

Fig. 11 shows interaction between flagHER2-f2:1282 and flagHER2-Fo5 and MMP-15. Differences between f2:1282 and Fo5 are not explainable by differential binding of mutants to MMP-15.

Fig. 12 illustrates somatic mutations found in MMTVHER2 transgenic mice. The sequences are: sheddase site (SEQ ID NO. 23), wild-type (WT) (SEQ ID NO. 24), splice (SEQ ID NO. 25), Fo5 (SEQ ID NO. 26), and f2:3078.10 (SEQ ID NO. 27).

Fig. 13 depicts results of the *in vitro* sheddase assay. gDHER29(DIV)-IgG is a substrate for the catalytic domains of MMP-15, MMP-16, MMP-19, and MMP-25. The sequences for protease digests are MMP-15 (SEQ ID NO. 28), MMP-16 (SEQ ID NO. 29), MMP-19 (SEQ ID NO. 30), MMP-25 (SEQ ID NO. 31), all other (SEQ ID NO. 32); and for the HER2 ECD C-terminal site (SEQ ID NO. 33).

Fig. 14 illustrates results of experiment demonstrating MMP-15 does not clip other HER receptors. The sequences demonstrating sequence variation near transmembrane domain are HER2 (SEQ ID NO. 34), EGFR (SEQ ID NO. 35), HER3 (SEQ ID NO. 36), HER4 (Jma) (SEQ ID NO. 37), and HER4 (Jmb) (SEQ ID NO. 38).

Fig. 15 shows MMP-15 "full length" clips HER2(+)-IgG.

Fig. 16 demonstrates an experiment which indicated p95HER2 is constitutively phosphorylated, but must heterdimerize with HER3 to activate Akt.

Fig. 17 shows MMP-15 RNA inhibitor (RNAi) reduces HER2 ECD shedding and p95 HER2 levels in SKBR3 and BT474 cells.

Fig. 18 illustrates how trastuzumab-mediated growth inhibition in SKBR3 cells is independent of inhibiting HER2 shedding.

Fig. 19 demonstrates that inhibition of metalloprotease activity in Fo5 xenograft tumors reduces HER2 shedding and inhibits p95HER2 levels.

Fig. 20 depicts members of the matrix metalloproteinase (MMP) family. MMP family members are grouped according to domain structure. The abbreviations used in this figure are: PRE, pre-domain; PRO, pro-domain; CAT, catalytic domain; H, hinge; HEM, hemopexin domain; F, furin-cleavage consensus domain; FN, fibronectin-like domain; GPI, glycophosphatidyl inositol anchor; TM, transmembrane domain; Ig, immunoglobulin-like domain; CA, cysteine array; CL, collagen-like domain.

Detailed Description of the Preferred Embodiments

I. Definitions

The terms "matrix metalloprotease" or "MMP" herein refer to a protein which is a member of a matrix metalloprotease (MMP) superfamily, dependent on Zn or Ca for activity. MMP herein includes the preproprotein, mature protein and variant forms thereof. See, also, Fig. 20 herein for examples of MMPs with various domains. MMPs are reviewed in Wagenaar-Miller *et al. Cancer and Metastasis Reviews* 23: 119-135 (2004).

A "membrane-tethered MMP" or "MT-MMP" herein is a MMP as defined above, where the MMP is capable of being attached to a cell membrane via either a transmembrane (TM) domain or a glycophosphatidyl inositol (GPI) anchor. Examples of MT-MMPs anchored by a transmembrane domain herein include MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT5-MMP (MMP-24). Examples of MT-MMPs anchored by a GPI anchor include MT4-MMP (MMP-17), and MT6-MMP (MMP-25). MMP-15 is the preferred MT-MMP herein.

"MT2-MMP" and "MMP-15"are synonyms herein and describe the preproprotein NP_002415 in the NCB1 database, the mature protein comprising amino acids 132-699 thereof, as well as variant forms thereof. Known substrates for MMP-15 include collagen, fibronectin, CD44, and complement. MMP-15 is upregulated in some cancers, and overexpression of this protease enhances tumor invasion and tumor cell growth.

A "MMP antagonist" is an agent that binds to and/or interferes to some extent with proteolytic activity of at least one MMP. Preferably, the MMP antagonist selectively binds to, or inhibits, the MMP, without significantly binding to, or inhibiting, other proteases, such as proteases in the ADAM (*a d*isintegrin *a*nd *m*etalloprotease) family. Examples of MMP antagonists herein include antibodies that bind to a MMP, small molecule inhibitors, pseudopeptides that mimic MMP substrates, nonpeptidic molecules that bind the catalytic zinc of MMPs, isolated natural tissue inhibitors of MMPs (TIMPs), nucleic acid inhibitors, such as RNA; or antisense inhibitors, etc.

A "MT-MMP antagonist" is an agent that binds to and/or interferes to some extent with proteolytic activity of at least one MT-MMP. Preferably, the MT-MMP antagonist selectively binds to, or inhibits, the MT-MMP, without significantly binding to, or inhibiting, other proteases (including other MMPs that are not membrane-tethered). Examples of MT-MMP antagonists include antibodies that bind to a MT-MMP, small molecule inhibitors, pseudopeptides that mimic MT-MMP substrates, nonpeptidic molecules that bind the catalytic zinc of a MT-MMP, isolated natural tissue inhibitors of MT-MMPs, MT-MMP nucleic acid inhibitors, such as RNA; or antisense inhibitors, etc.

A "MMP-15 antagonist" is an agent that binds to and/or interferes to some extent with proteolytic activity of MMP-15. Preferably, the MMP-15 antagonist selectively binds to, or inhibits, MMP-15 without significantly binding to, or inhibiting, other proteases (including MMPs other than MMP-15). Examples of MMP-15 antagonists include antibodies that bind to MMP-15, small molecule inhibitors, pseudopeptides that mimic MMP-15 substrates, nonpeptidic molecules that bind the catalytic zinc of MMP-15, isolated natural tissue inhibitors of MMP-15, MMP-15 nucleic acid inhibitors such as RNA; or antisense inhibitors etc.

By "elevated MMP level" is meant an amount of MMP in a biological sample, such as a tumor sample, that exceeds the normal amount of the MMP, for instance the amount in a normal, or non-tumor, sample of the

same tissue type. Such "normal amount" of MMP (*e.g.* of MMP-15) includes no or undetectable amount of MMP-15. Elevated MMP levels can be determined in various ways, including those which measure MMP protein or MMP nucleic acid.

A AHER receptor@ is a receptor protein tyrosine kinase which belongs to the HER receptor family and includes EGFR, HER2, HER3 and HER4 receptors. The HER receptor includes native sequence HER receptor, and variants thereof. Preferably the HER receptor is native sequence human HER receptor.

A "full length" HER receptor comprises an extracellular domain, which may bind an HER ligand and/or dimerize with another HER receptor molecule; a lipophilic transmembrane domain; an intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated.

The terms AErbB1," AHER1", Aepidermal growth factor receptor@ and AEGFR@ are used interchangeably herein and refer to EGFR as disclosed, for example, in Carpenter *et al. Ann. Rev. Biochem.* 56:881-914 (1987), including variant forms thereof (*e.g.* a deletion mutant EGFR as in Humphrey *et al. PNAS (USA)* 87:4207-4211 (1990)).

The expressions AErbB2" and AHER2" are used interchangeably herein and refer to human HER2 protein described, for example, in Semba *et al.*, *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto *et al. Nature* 319:230-234 (1986) (Genebank accession number X03363), as well as variant forms thereof, such as alternatively spliced forms (Siegel *et al. EMBO J.* 18(8):2149-2164 (1999)).

Herein, "HER2 extracellular domain" or "HER2 ECD" refers to a domain of HER2 that is outside of a cell, either anchored to a cell membrane, or in circulation, including fragments thereof. In one embodiment, the extracellular domain of HER2 may comprise four domains: ADomain I@ (amino acid residues from about 1-195; SEQ ID NO:1), ADomain II@ (amino acid residues from about 196-319; SEQ ID NO:2), ADomain III@ (amino acid residues from about 320-488: SEQ ID NO:3), and ADomain IV@ (amino acid residues from about 489-630; SEQ ID NO:4) (residue numbering without signal peptide). See Garrett *et al. Mol. Cell.*. 11: 495-505 (2003), Cho *et al. Nature* 421: 756-760 (2003), Franklin *et al. Cancer Cell* 5:317-328 (2004), and Plowman *et al. Proc. Natl. Acad. Sci.* 90:1746-1750 (1993), as well as Fig. 1 herein.

Herein, "HER2 shedding" refers to release of a soluble extracellular domain (ECD) fragment of HER2 from the cell surface of a cell which expresses HER2. Such shedding may be caused by proteolytic cleavage of cell surface HER2 resulting in release of an ECD fragment from the cell surface, or the soluble ECD or fragment thereof may be encoded by an alternate transcript.

By "shed HER2 serum level" is meant the amount of HER2 ECD present in the serum or circulation of a mammal. Such levels can be evaluated by various techniques including those described in: Ali *et al. Clin. Chem.* 48:1314-1320 (2002); Molina *et al. Clin. Cancer Res.* 8:347-353 (2002); U.S. Patent No. 4,933,294 issued June 12, 1990; WO91/05264 published April 18, 1991; U.S. Patent 5,401,638 issued March 28, 1995; or Sias *et al. J. Immunol. Methods* 132: 73-80 (1990).

Herein, "elevated shed HER2 serum level" refers to an amount of shed HER2 or HER2 ECD in the serum of a mammal (e.g. human) that exceeds the amount present in the serum a normal mammal (e.g. human). Elevated HER2 ECD serum levels may correlate with a poor prognosis and decreased responsiveness to endocrine therapy and chemotherapy in patients with advanced breast cancer.

The expression "p95 HER2" herein refers to NH2-terminal truncated HER2 protein. Generally, p95 is

a membrane-bound stub fragment which may arise from cleavage of full length HER2 by a protease or sheddase (Yuan *et al. Protein Expression and Purification* 29: 217-222 (2003)). p95 may have a M_r of about 95,000 and may be phosphorylated (Molina *et al. Cancer Research* 4744-4749 (2001)). p95 has been found in some breast cancer samples (Christianson *et al. Cancer Res.* 15:5123-5129 (1998)).

By "elevated p95 level" is meant a level of p95 in a cancer cell that exceeds the normal level, for instance the leval in a normal or non-cancerous cell of the same tissue type as the cancer cell. Such elevated p95 level may result in constitutive signaling, and nodal metastasis (Molina *et al. Clin. Cancer Research* 8:347-353 (2002); Christianson *et al. Cancer Res.* 15:5123-5129 (1998)).

By "evaluating" a marker, such as MMP-15, is intended a diagnostic and/or prognostic analysis thereof, including an analysis of the presence or absence of that marker, measurement of the amount thereof, and/or an analysis of activity thereof (*e.g.* increased activity).

A cancer patient with "a poor clinical outcome" is one with a poor prognosis, who is less likely to respond to cancer therapy, such as chemotherapy or therapy with a HER2 antibody, such as trastuzumab. The clinical outcome can be measured by standard means, such as survival, including disease free survival, etc.

AErbB3" and AHER3" refer to the receptor polypeptide as disclosed, for example, in US Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus *et al. PNAS (USA)* 86:9193-9197 (1989), including variant forms thereof.

The terms AErbB4" and AHER4" herein refer to the receptor polypeptide as disclosed, for example, in EP Pat Appln No 599,274; Plowman *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:1746-1750 (1993); and Plowman *et al., Nature*, 366:473-475 (1993), including variant forms thereof, such as the isoforms disclosed in WO99/19488, published April 22, 1999.

By AHER ligand@ is meant a polypeptide which binds to and/or activates a HER receptor. The HER ligand of particular interest herein is a native sequence human HER ligand such as epidermal growth factor (EGF) (Savage *et al., J. Biol. Chem.* 247:7612-7621 (1972)); transforming growth factor alpha (TGF-α) (Marquardt *et al., Science* 223:1079-1082 (1984)); amphiregulin also known as schwanoma or keratinocyte autocrine growth factor (Shoyab *et al. Science* 243:1074-1076 (1989); Kimura *et al. Nature* 348:257-260 (1990); and Cook *et al. Mol. Cell. Biol.* 11:2547-2557 (1991)); betacellulin (Shing *et al., Science* 259:1604-1607 (1993); and Sasada *et al. Biochem. Biophys. Res. Commun.* 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama *et al., Science* 251:936-939 (1991)); epiregulin (Toyoda *et al., J. Biol. Chem.* 270:7495-7500 (1995); and Komurasaki *et al. Oncogene* 15:2841-2848 (1997)); a heregulin (see below); neuregulin-2 (NRG-2) (Carraway *et al., Nature* 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang *et al., Proc. Natl. Acad. Sci.* 94:9562-9567 (1997)); neuregulin-4 (NRG-4) (Harari *et al. Oncogene* 18:2681-89 (1999)); and cripto (CR-1) (Kannan *et al. J. Biol. Chem.* 272(6):3330-3335 (1997)). HER ligands which bind EGFR include EGF, TGF-α, amphiregulin, betacellulin, HB-EGF and epiregulin. HER ligands which bind HER3 include heregulins. HER ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3, NRG-4, and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide encoded by the heregulin gene product as disclosed in U.S. Patent No. 5,641,869, or Marchionni *et al.*, *Nature*, 362:312-318 (1993). Examples of heregulins include heregulin-α, heregulin-β1, heregulin-β2 and heregulin-β3 (Holmes *et al.*, *Science*, 256:1205-1210 (1992); and U.S. Patent No. 5,641,869); *neu* differentiation factor (NDF) (Peles *et al. Cell* 69: 205-216

(1992)); acetylcholine receptor-inducing activity (ARIA) (Falls *et al. Cell* 72:801-815 (1993)); glial growth factors (GGFs) (Marchionni *et al.*, *Nature*, 362:312-318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho *et al. J. Biol. Chem.* 270:14523-14532 (1995)); γ-heregulin (Schaefer *et al. Oncogene* 15:1385-1394 (1997)).

A "HER dimer" herein is a noncovalently associated dimer comprising at least two HER receptors. Such complexes may form when a cell expressing two or more HER receptors is exposed to an HER ligand and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski *et al.*, *J. Biol. Chem.*, 269(20):14661-14665 (1994), for example. Examples of such HER dimers include EGFR-HER2, HER2-HER3 and HER3-HER4 heterodimers. Moreover, the HER dimer may comprise two or more HER2 receptors combined with a different HER receptor, such as HER3, HER4 or EGFR. Other proteins, such as a cytokine receptor subunit (*e.g.* gp130) may be associated with the dimer.

A cell, cancer, or biological sample which "displays HER expression, amplification, or activation" is one which, in a diagnostic test, expresses (including overexpresses) HER, has amplified HER gene, and/or otherwise demonstrates activation or phosphorylation of HER receptor(s). Such activation can be determined directly (*e.g.* by measuring HER phosphorylation) or indirectly (*e.g.* by gene expression profiling or by detecting HER heterodimers).

A cancer or tumor cell with AHER2 receptor overexpression or amplification@ is one which has significantly higher levels of a HER2 protein or gene compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. HER2 overexpression or amplification may be determined in a diagnostic or prognostic assay by evaluating increased levels of the HER2 protein present on the surface of a cell (*e.g.* via an immunohistochemistry assay; IHC). Alternatively, or additionally, one may measure levels of HER2 nucleic acid in the cell, *e.g.* via fluorescent *in situ* hybridization (FISH; see WO98/45479 published October, 1998), southern blotting, or polymerase chain reaction (PCR) techniques, such as quantitative real time PCR (qRT-PCR). One may also study HER2 overexpression or amplification by measuring shed HER2 in a biological fluid such as serum (see, *e.g.*, U.S. Patent No. 4,933,294 issued June 12, 1990; WO91/05264 published April 18, 1991; U.S. Patent 5,401,638 issued March 28, 1995; and Sias *et al. J. Immunol. Methods* 132: 73-80 (1990)). Aside from the above assays, various *in vivo* assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, *e.g.* a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, *e.g.* by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

Conversely, a cancer or tumor cell which Adoes not overexpress or amplify HER receptor@ is one which does not have higher than normal levels of HER receptor protein or gene compared to a noncancerous cell of the same tissue type. Antibodies that inhibit HER dimerization, such as pertuzumab, may be used to treat cancer which does not overexpress or amplify HER2 receptor.

A AHER inhibitor@ is an agent which interferes with HER activation or function. Examples of HER inhibitors include HER antibodies (*e.g.* EGFR, HER2, HER3, or HER4 antibodies); HER dimerization inhibitors; EGFR-targeted drugs; small molecule HER antagonists; HER tyrosine kinase inhibitors; HER2 and EGFR dual tyrosine kinase inhibitors such as lapatinib/GW572016; antisense molecules (see, for example, WO2004/87207); and/or agents that bind to, or interfere with function of, downstream signaling molecules,

such as MAPK or Akt. Preferably, the HER inhibitor is an antibody or small molecule which binds to a HER receptor. Specific examples of HER inhibitors include trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, and TAK165.

A "HER dimerization inhibitor" is an agent which inhibits formation of a HER dimer. Preferably, the HER dimerization inhibitor is an antibody, for example an antibody which binds to HER2 at the heterodimeric binding site thereof. The most preferred dimerization inhibitor herein is pertuzumab or monoclonal antibody 2C4 (MAb 2C4). Other examples of HER dimerization inhibitors include antibodies which bind to EGFR and inhibit dimerization thereof with one or more other HER receptors (for example EGFR monoclonal antibody 806, MAb 806, which binds to activated or "untethered" EGFR; see Johns *et al., J. Biol. Chem.* 279(29):30375-30384 (2004)); antibodies which bind to HER3 and inhibit dimerization thereof with one or more other HER receptors; antibodies which bind to HER4 and inhibit dimerization thereof with one or more other HER receptors; peptide dimerization inhibitors (US Patent No. 6,417,168); antisense dimerization inhibitors; etc.

A "HER antibody" is an antibody that binds to a HER receptor. Optionally, the HER antibody further interferes with HER activation or function. Preferably, the HER antibody binds to the HER2 receptor. HER2 antibodies of particular interest herein are trastuzumab and pertuzumab. Examples of EGFR antibodies include cetuximab, ABX0303, EMD7200 and IMC-11F5.

AHER activation@ refers to activation, or phosphorylation, of any one or more HER receptors. Generally, HER activation results in signal transduction (*e.g.* that caused by an intracellular kinase domain of a HER receptor phosphorylating tyrosine residues in the HER receptor or a substrate polypeptide). HER activation may be mediated by HER ligand binding to a HER dimer comprising the HER receptor of interest. HER ligand binding to a HER dimer may activate a kinase domain of one or more of the HER receptors in the dimer and thereby results in phosphorylation of tyrosine residues in one or more of the HER receptors and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s), such as Akt or MAPK intracellular kinases.

APhosphorylation@ refers to the addition of one or more phosphate group(s) to a protein, such as a HER receptor, or substrate thereof.

An antibody which "inhibits HER dimerization@ is an antibody which inhibits, or interferes with, formation of a HER dimer. Preferably, such an antibody binds to HER2 at the heterodimeric binding site thereof. The most preferred dimerization inhibiting antibody herein is pertuzumab or MAb 2C4. Other examples of antibodies which inhibit HER dimerization include antibodies which bind to EGFR and inhibit dimerization thereof with one or more other HER receptors (for example EGFR monoclonal antibody 806, MAb 806, which binds to activated or Auntethered@ EGFR; see Johns *et al., J. Biol. Chem.* 279(29):30375-30384 (2004)); antibodies which bind to HER3 and inhibit dimerization thereof with one or more other HER receptors; and antibodies which bind to HER4 and inhibit dimerization thereof with one or more other HER receptors.

A Aheterodimeric binding site@ on HER2, refers to a region in the extracellular domain of HER2 that contacts, or interfaces with, a region in the extracellular domain of EGFR, HER3 or HER4 upon formation of a dimer therewith. The region is found in Domain II of HER2. Franklin *et al. Cancer Cell* 5:317-328 (2004).

The HER2 antibody may be one which, like trastuzumab, Ainhibits HER2 ectodomain cleavage@ (Molina *et al. Cancer Res.* 61:4744-4749(2001)) or may be one which, like pertuzumab, does not significantly inhibit HER2 ectodomain cleavage.

A HER2 antibody that Abinds to a heterodimeric binding site@ of HER2, binds to residues in domain II (and optionally also binds to residues in other of the domains of the HER2 extracellular domain, such as domains I and III), and can sterically hinder, at least to some extent, formation of a HER2-EGFR, HER2-HER3, or HER2-HER4 heterodimer. Franklin *et al. Cancer Cell* 5:317-328 (2004) characterize the HER2-pertuzumab crystal structure, deposited with the RCSB Protein Data Bank (ID Code IS78), illustrating an exemplary antibody that binds to the heterodimeric binding site of HER2.

An antibody that Abinds to domain II@ of HER2 binds to residues in domain II and optionally residues in other domain(s) of HER2, such as domains I and III. Preferably the antibody that binds to domain II binds to the junction between domains I, II and III of HER2.

A Anative sequence@ polypeptide is one which has the same amino acid sequence as a polypeptide (*e.g.*, HER receptor or HER ligand) derived from nature. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.* bispecific antibodies) formed from at least two intact antibodies, and antibody fragments, so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope(s), except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. Such monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones or recombinant DNA clones. It should be understood that the selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity *in vivo*, to create a multispecific antibody, *etc.*, and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, the monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (*e.g.*, Kohler *et al., Nature*, 256:495 (1975); Harlow *et al., Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: Monoclonal Antibodies and T-Cell Hybridomas 563-681, (Elsevier, N.Y., 1981)), recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567), phage display technologies (see, *e.g.*, Clackson *et al., Nature*, 352:624-

628 (1991); Marks *et al., J. Mol. Biol.*, 222:581-597 (1991); Sidhu *et al., J. Mol. Biol.* 338(2):299-310 (2004); Lee *et al., J.Mol.Biol.*340(5):1073-1093 (2004); Fellouse, *Proc. Nat. Acad. Sci. USA* 101(34):12467-12472 (2004); and Lee *et al. J. Immunol. Methods* 284(1-2):119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, *e.g.,* WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); *Bruggemann et al., Year in Immuno.*, 7:33 (1993); U.S. Patent Nos. 5,545,806; 5,569,825; 5,591,669 (all of GenPharm); U.S. Patent No. 5,545,807; WO 1997/17852; U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks *et al., Bio/Technology*, 10: 779-783 (1992); Lonberg *et al., Nature*, 368: 856-859 (1994); Morrison, *Nature*, 368: 812-813 (1994); Fishwild *et al., Nature Biotechnology*, 14: 845-851 (1996); Neuberger, *Nature Biotechnology*, 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.*, 13: 65-93 (1995).

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)). Chimeric antibodies of interest herein include Aprimatized@ antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (*e.g.* Old World Monkey, Ape etc) and human constant region sequences.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragment(s).

An Aintact antibody@ herein is one which comprises two antigen binding regions, and an Fc region. Preferably, the intact antibody has one or more effector functions.

Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different Aclasses@. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into Asubclasses@ (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

Antibody Aeffector functions@ refer to those biological activities attributable to an Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor; BCR), etc.

AAntibody-dependent cell-mediated cytotoxicity@ and AADCC@ refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (*e.g.* Natural Killer (NK) cells, neutrophils,

and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. 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).

AHuman effector cells@ are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, *e.g.* from blood or PBMCs as described herein.

The terms "Fc receptor" or AFcR@ are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and Fcγ RIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al., Immunomethods* 4:25-34 (1994); and de Haas *et al., J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al., J. Immunol.* 117:587 (1976) and Kim *et al., J. Immunol.* 24:249 (1994)), and regulates homeostasis of immunoglobulins.

AComplement dependent cytotoxicity@ or ACDC@ refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (*e.g.* an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.* as described in Gazzano-Santoro *et al., J. Immunol. Methods* 202:163 (1996), may be performed.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is

aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term Ahypervariable region@ when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a Acomplementarity determining region@ or ACDR@ (*e.g.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a Ahypervariable loop@ (*e.g.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab= fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear

at least one free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994). HER2 antibody scFv fragments are described in WO93/16185; U.S. Patent No. 5,571,894; and U.S. Patent No. 5,587,458.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a variable heavy domain (V_H) connected to a variable light domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

"Humanized" forms of non-human (*e.g.*, rodent) 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 also will 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).

Humanized HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 or trastuzumab (HERCEPTIN7) as described in Table 3 of U.S. Patent 5,821,337 expressly incorporated herein by reference; humanized 520C9 (WO93/21319); and humanized 2C4 antibodies such as pertuzumab as described herein.

For the purposes herein, Atrastuzumab,@ AHERCEPTIN7,@ and AhuMAb4D5-8" refer to an antibody comprising the light and heavy chain amino acid sequences in SEQ ID NOS. 5 and 6, respectively.

Herein, Apertuzumab@ and AOMNITARGJ@ refer to an antibody comprising the light and heavy chain amino acid sequences in SEQ ID NOS. 7 and 8, respectively.

A Anaked antibody@ is an antibody that is not conjugated to a heterologous molecule, such as a

cytotoxic moiety or radiolabel.

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.

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

The term Amain species antibody@ herein refers to the antibody structure in a composition which is the quantitatively predominant antibody molecule in the composition.

An Aamino acid sequence variant@ antibody herein is an antibody with an amino acid sequence which differs from a main species antibody. Ordinarily, amino acid sequence variants will possess at least about 70% homology with the main species antibody, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with the main species antibody. The amino acid sequence variants possess substitutions, deletions, and/or additions at certain positions within or adjacent to the amino acid sequence of the main species antibody.

A Aglycosylation variant@ antibody herein is an antibody with one or more carbohydrate moeities attached thereto which differ from one or more carbohydate moieties attached to a main species antibody.

A Adeamidated@antibody is one in which one or more asparagine residues thereof has been derivitized, *e.g.* to an aspartic acid, a succinimide, or an iso-aspartic acid.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma (including medulloblastoma and retinoblastoma), sarcoma (including liposarcoma and synovial cell sarcoma), neuroendocrine tumors (including carcinoid tumors, gastrinoma, and islet cell cancer), mesothelioma, schwannoma (including acoustic neuroma), meningioma, adenocarcinoma, melanoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (*e.g.* epithelial squamous cell cancer), lung cancer including small-cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung and squamous carcinoma of the

lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer (including metastatic breast cancer), colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, testicular cancer, esophagael cancer, tumors of the biliary tract, as well as head and neck cancer.

A mammal with a "HER inhibitor-resistant cancer" is one who has progressed while receiving HER inhibitor-based therapy (*i.e.* the patient is "HER inhibitor refractory"), or the mammal has progressed within 12 months (for instance, within 6 months) after completing a HER inhibitor-based therapy regimen. The HER inhibitor-based therapy includes therapy with naked or conjugated HER inhibitor, where the HER inhibitor is administered as a single-agent, or in combination with other anti-tumor drug(s). The HER inhibitor may be trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, or TAK165, but preferably is trastuzumab.

A "mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

A Atumor sample@ herein is a sample derived from, or comprising tumor cells from, a patient=s tumor. Examples of tumor samples herein include, but are not limited to, tumor biopsies, circulating tumor cells, circulating plasma proteins, ascitic fluid, primary cell cultures or cell lines derived from tumors or exhibiting tumor-like properties, as well as preserved tumor samples, such as formalin-fixed, paraffin-embedded tumor samples or frozen tumor samples.

A Afixed@ tumor sample is one which has been histologically preserved using a fixative.

A Aformalin-fixed@ tumor sample is one which has been preserved using formaldehyde as the fixative.

An Aembedded@ tumor sample is one surrounded by a firm and generally hard medium such as paraffin, wax, celloidin, or a resin. Embedding makes possible the cutting of thin sections for microscopic examination or for generation of tissue microarrays (TMAs).

A Aparaffin-embedded@ tumor sample is one surrounded by a purified mixture of solid hydrocarbons derived from petroleum.

Herein, a Afrozen@ tumor sample refers to a tumor sample which is, or has been, frozen.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially a HER expressing cancer cell either *in vitro* or *in vivo.* Thus, the growth inhibitory agent may be one which significantly reduces the percentage of HER expressing 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 topo 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.

Examples of Agrowth inhibitory@ antibodies are those which bind to HER2 and inhibit the growth of cancer cells overexpressing HER2. Preferred growth inhibitory HER2 antibodies inhibit growth of SK-BR-3 breast tumor cells in cell culture by greater than 20%, and preferably greater than 50% (*e.g.* from about 50% to about 100%) at an antibody concentration of about 0.5 to 30 μg/ml, where the growth inhibition is determined six days after exposure of the SK-BR-3 cells to the antibody (see U.S. Patent No. 5,677,171 issued October 14, 1997). The SK-BR-3 cell growth inhibition assay is described in more detail in that patent and hereinbelow. The preferred growth inhibitory antibody is a humanized variant of murine monoclonal antibody 4D5, *e.g.*, trastuzumab.

An antibody which "induces apoptosis" is one which induces programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which overexpresses the HER2 receptor. Preferably the cell is a tumor cell, *e.g.* a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. *In vitro*, the cell may be a SK-BR-3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an annexin binding assay using BT474 cells (see below). Examples of HER2 antibodies that induce apoptosis are 7C2 and 7F3.

The "epitope 2C4" is the region in the extracellular domain of HER2 to which the antibody 2C4 binds. In order to screen for antibodies which bind to the 2C4 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Preferably the antibody blocks 2C4's binding to HER2 by about 50% or more. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 2C4 epitope of HER2. Epitope 2C4 comprises residues from Domain II in the extracellular domain of HER2. 2C4 and pertuzumab binds to the extracellular domain of HER2 at the junction of domains I, II and III. Franklin *et al. Cancer Cell* 5:317-328 (2004).

The "epitope 4D5" is the region in the extracellular domain of HER2 to which the antibody 4D5 (ATCC CRL 10463) and trastuzumab bind. This epitope is close to the transmembrane domain of HER2, and within Domain IV of HER2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of HER2 (*e.g.* any one or more residues in the region from about residue 529 to about residue 625, inclusive of the HER2 ECD, residue numbering including signal peptide).

The "epitope 7C2/7F3" is the region at the N terminus, within Domain I, of the extracellular domain of HER2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be

performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on HER2 (*e.g.* any one or more of residues in the region from about residue 22 to about residue 53 of the HER2 ECD, residue numbering including signal peptide).

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disease as well as those in which the disease is to be prevented. Hence, the patient to be treated herein may have been diagnosed as having the disease or may be predisposed or susceptible to the disease.

The term Aeffective amount@ refers to an amount of a drug effective to treat cancer in the patient. The effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. The effective amount may extend progression free survival, result in an objective response (including a partial response, PR, or complete response, CR), increase overall survival time, and/or improve one or more symptoms of cancer.

By Acomplete response@ or Acomplete remission@ is intended the disappearance of all signs of cancer in response to treatment. This does not always mean the cancer has been cured.

APartial response@ refers to a decrease in the size of one or more tumors or lesions, or in the extent of cancer in the body, in response to treatment.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.* At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN7 cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; TLK 286 (TELCYTAJ); acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL7); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN7), CPT-11 (irinotecan, CAMPTOSAR7), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; bisphosphonates, such as clodronate; antibiotics such as the enediyne antibiotics (*e. g.*, calicheamicin, especially calicheamicin

gammaI1 and calicheamicin omegaI1 (see, *e.g.*, Agnew, *Chem Intl. Ed. Engl.*, 33: 183-186 (1994)) and anthracyclines such as annamycin, AD 32, alcarubicin, daunorubicin, dexrazoxane, DX-52-1, epirubicin, GPX-100, idarubicin, KRN5500, menogaril, dynemicin, including dynemicin A, an esperamicin, neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN7 doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, liposomal doxorubicin, and deoxydoxorubicin), esorubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, and zorubicin; folic acid analogues such as denopterin, pteropterin, and trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, and floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, and testolactone; anti-adrenals such as aminoglutethimide, mitotane, and trilostane; folic acid replenisher such as folinic acid (leucovorin); aceglatone; anti-folate anti-neoplastic agents such as ALIMTA7, LY231514 pemetrexed, dihydrofolate reductase inhibitors such as methotrexate, anti-metabolites such as 5-fluorouracil (5-FU) and its prodrugs such as UFT, S-1 and capecitabine, and thymidylate synthase inhibitors and glycinamide ribonucleotide formyltransferase inhibitors such as raltitrexed (TOMUDEX^{RM}, TDX); inhibitors of dihydropyrimidine dehydrogenase such as eniluracil; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK7 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 (ELDISINE7, FILDESIN7); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids and taxanes, *e.g.*, TAXOL7 paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE^{TM} Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE7 docetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; antimetabolite chemotherapeutic agent such as gemcitabine (GEMZAR7), 5-fluorouracil (5-FU), capecitabine (XELODAJ), 6-mercaptopurine, methotrexate, 6-thioguanine, pemetrexed, raltitrexed, arabinosylcytosine ARA-C cytarabine (CYTOSAR-U7), dacarbazine (DTIC-DOME7), azocytosine, deoxycytosine, pyridmidene, fludarabine (FLUDARA7), cladrabine, and 2-deoxy-D-glucose; 6-thioguanine; mercaptopurine; platinum-based chemotherapeutic agent such as carboplatin, cisplatin, or oxaliplatinum; vinblastine (VELBAN7); etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN7); vinca alkaloid; vinorelbine (NAVELBINE7); novantrone; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; topoisomerase inhibitor RFS 2000; difluorometlhylornithine (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 or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX7 tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON7 toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE7 megestrol acetate, AROMASIN7 exemestane, formestanie, fadrozole, RIVISOR7 vorozole, FEMARA7 letrozole, and ARIMIDEX7 anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as gene therapy vaccines, for example, ALLOVECTIN7 vaccine, LEUVECTIN7 vaccine, and VAXID7 vaccine; PROLEUKIN7 rIL-2; LURTOTECAN7 topoisomerase 1 inhibitor; ABARELIX7 rmRH; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

An Aanti-angiogenic agent@ refers to a compound which blocks, or interferes with to some degree, the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or antibody that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. The preferred anti-angiogenic factor herein is an antibody that binds to vascular endothelial growth factor (VEGF), such as bevacizumab (AVASTIN7).

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

As used herein, the term AEGFR-targeted drug@ refers to a therapeutic agent that binds to EGFR and, optionally, inhibits EGFR activation. Examples of such agents include antibodies and small molecules that bind to EGFR. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455

(ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, US Patent No. 4,943, 533, Mendelsohn *et al.*) and variants thereof, such as chimerized 225 (C225 or Cetuximab; ERBUTIX7) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.); IMC-11F8, a fully human, EGFR-targeted antibody (Imclone); antibodies that bind type II mutant EGFR (US Patent No. 5,212,290); humanized and chimeric antibodies that bind EGFR as described in US Patent No. 5,891,996; and human antibodies that bind EGFR, such as ABX-EGF (see WO98/50433, Abgenix); EMD 55900 (Stragliotto *et al. Eur. J. Cancer* 32A:636-640 (1996)); EMD7200 (matuzumab) a humanized EGFR antibody directed against EGFR that competes with both EGF and TGF-alpha for EGFR binding; and mAb 806 or humanized mAb 806 (Johns *et al., J. Biol. Chem.* 279(29):30375-30384 (2004)). The anti-EGFR antibody may be conjugated with a cytotoxic agent, thus generating an immunoconjugate (see, *e.g.*, EP659,439A2, Merck Patent GmbH). Examples of small molecules that bind to EGFR include ZD1839 or Gefitinib (IRESSAJ; Astra Zeneca); CP-358774 or Erlotinib (TARCEVAJ; Genentech/OSI); and AG1478, AG1571 (SU 5271; Sugen); EMD-7200.

A Atyrosine kinase inhibitor@ is a molecule which inhibits tyrosine kinase activity of a tyrosine kinase such as a HER receptor. Examples of such inhibitors include the EGFR-targeted drugs noted in the preceding paragraph; small molecule HER2 tyrosine kinase inhibitor such as TAK165 available from Takeda; CP-724,714, an oral selective inhibitor of the ErbB2 receptor tyrosine kinase (Pfizer and OSI); dual-HER inhibitors such as EKB-569 (available from Wyeth) which preferentially binds EGFR but inhibits both HER2 and EGFR-overexpressing cells; GW572016 (available from Glaxo) an oral HER2 and EGFR tyrosine kinase inhibitor; PKI-166 (available from Novartis); pan-HER inhibitors such as canertinib (CI-1033; Pharmacia); Raf-1 inhibitors such as antisense agent ISIS-5132 available from ISIS Pharmaceuticals which inhibits Raf-1 signaling; non-HER targeted TK inhibitors such as Imatinib mesylate (GleevacJ) available from Glaxo; MAPK extracellular regulated kinase I inhibitor CI-1040 (available from Pharmacia); quinazolines, such as PD 153035,4-(3-chloroanilino) quinazoline; pyridopyrimidines; pyrimidopyrimidines; pyrrolopyrimidines, such as CGP 59326, CGP 60261 and CGP 62706; pyrazolopyrimidines, 4-(phenylamino)-7H-pyrrolo[2,3-d] pyrimidines; curcumin (diferuloyl methane, 4,5-bis (4-fluoroanilino)phthalimide); tyrphostines containing nitrothiophene moieties; PD-0183805 (Warner-Lamber); antisense molecules *(e.g.* those that bind to HER-encoding nucleic acid); quinoxalines (US Patent No. 5,804,396); tryphostins (US Patent No. 5,804,396); ZD6474 (Astra Zeneca); PTK-787 (Novartis/Schering AG); pan-HER inhibitors such as CI-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); Imatinib mesylate (Gleevac; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); CI-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Sugen); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-1C11 (Imclone); or as described in any of the following patent publications: US Patent No. 5,804,396; WO99/09016 (American Cyanimid); WO98/43960 (American Cyanamid); WO97/38983 (Warner Lambert); WO99/06378 (Warner Lambert); WO99/06396 (Warner Lambert); WO96/30347 (Pfizer, Inc); WO96/33978 (Zeneca); WO96/3397 (Zeneca); and WO96/33980 (Zeneca).

II. Inhibiting HER2 Shedding

The present application concerns a method for inhibiting HER2 shedding comprising treating, or exposing, a HER2 expressing cell with or to a matrix metalloprotease (MMP) antagonist in an amount effective to inhibit HER2 shedding. Preferably, the MMP antagonist is a membrane-tethered MMP (MT-MMP) antagonist, such as MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT5-MMP (MMP-

24), MT4-MMP (MMP-17), or MT6-MMP (MMP-25). Most preferably, the MT-MMP is MMP-15, and desirably, the antagonist binds selectively or preferentially to MMP-15, without significantly binding other proteases or MMPs other than MMP-15, and/or the antagonist interferes with MMP-15 proteolytic function without significantly interfering with function of other proteases or MMPs other than MMP-15.

In the preferred embodiment, the treated cell displays HER and/or MMP expression, amplification, or activation. For example, the cell may display HER2 and/or MMP-15 overexpression or amplification.

The activity of the MMP antagonist may be enhanced by combining it with another anti-tumor drug, HER inhibitor, or HER2 antibody (such as trastuzumab or pertuzumab). Examples of HER inhibitors that may be combined with the MMP antagonist include trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, and TAK165.

The invention also concerns a method for reducing HER2 extracellular domain (ECD) serum level in a mammal, comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to reduce the HER2 ECD serum level in the mammal. The mammal optionally has an elevated MMP level.

The invention also provides a method for treating cancer in a mammal comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to treat the cancer. The cancer may display HER and/or MMP expression, amplification, or activation. For example, the cancer may display HER2 or MMP-15 overexpression or amplification. In one embodiment, the treated mammal has an elevated shed HER2 serum level or elevated p95 HER2 level.

This invention also relates to a method for treating a HER inhibitor-resistant cancer in a mammal comprising administering to the mammal a matrix metalloprotease (MMP) antagonist in an amount effective to treat the cancer. For example, the mammal may be resistant to a HER2 antibody, such as trastuzumab.

Also provided is a method for reducing p95 HER2 level in a cell comprising exposing the cell to a matrix metalloprotease (MMP) antagonist in an amount effective to reduce the p95 HER2 level.

Various MMP antagonists may be used, but preferably the MMP antagonist is a small molecule inhibitor or an antibody. Methods for making antibodies are described hereinbelow.

III. Production of Antibodies

A description follows as to exemplary techniques for the production of antibodies used in accordance with the present invention. The antigen to be used for production of antibodies may be, *e.g.*, a soluble form of the antigen or a portion thereof, containing the desired epitope. Alternatively, cells expressing the antigen at their cell surface (*e.g.* NIH-3T3 cells transformed to overexpress HER2; or a carcinoma cell line such as SK-BR-3 cells, see Stancovski *et al. PNAS (USA)* 88:8691-8695 (1991)) can be used to generate antibodies. Other forms of antigen useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide

(through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies

Various methods for making monoclonal antibodies herein are available in the art. For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); and Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al., Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies 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 murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy chain and light chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; and Morrison, *et al.*, *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has 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.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-327 (1988); Verhoeyen *et al.*, *Science*, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the

corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

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

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 a preferred 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.

(iv) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al., Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al., Nature*, 362:255-258 (1993); Bruggermann *et al., Year in Immuno.*, 7:33 (1993); and U.S. Patent Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty *et al., Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and

displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, *e.g.*, Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, *Nature*, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), or Griffith *et al.*, *EMBO J.* 12:725-734 (1993). See, also, U.S. Patent Nos. 5,565,332 and 5,573,905.

As discussed above, human antibodies may also be generated by *in vitro* activated B cells (see U.S. Patents 5,567,610 and 5,229,275).

Human HER2 antibodies are described in U.S. Patent No. 5,772,997 issued June 30, 1998 and WO 97/00271 published January 3, 1997.

(v) Antibody fragments

Various techniques have been developed for the production of antibody fragments comprising one or more antigen binding regions. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.* , *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan *et al.*, *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Patent No. 5,571,894; and U.S. Patent No. 5,587,458. The antibody fragment may also be a Alinear antibody@, *e.g.*, as described in U.S. Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the HER2 protein. Other such antibodies may combine a HER2 binding site with binding site(s) for EGFR, HER3 and/or HER4. Alternatively, a HER2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the HER2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express HER2. These antibodies possess a HER2-binding arm and an arm which binds the cytotoxic agent (*e.g.* saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies).

WO 96/16673 describes a bispecific HER2/FcγRIII antibody and U.S. Patent No. 5,837,234 discloses a bispecific HER2/FcγRI antibody IDM1 (Osidem). A bispecific HER2/Fcα antibody is shown in WO98/02463. U.S. Patent No. 5,821,337 teaches a bispecific HER2/CD3 antibody. MDX-210 is a bispecific HER2-FcγRIII Ab.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al., Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al., EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al., Methods in Enzymology*, 121:210 (1986).

According to another approach described in U.S. Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli,* which can be chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

(vii) Other amino acid sequence modifications

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 changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites.

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 *Science*, 244:1081-1085 (1989). 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 antibody variants 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 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.

Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred 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 substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion 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 human HER2. 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.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that lacks fucose attached to an Fc region of the antibody are described in US Pat Appl No US 2003/0157108 A1, Presta, L. See also US 2004/0093621 A1 (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetylglucosamine (GlcNAc) in the carbohydrate attached to an Fc region of the antibody are referenced in WO03/011878, Jean-Mairet *et al.* and US Patent No. 6,602,684, Umana *et al.* Antibodies with at least one galactose residue in the oligosaccharide attached to an Fc region of the antibody are reported in WO97/30087, Patel *et al.* See, also, WO98/58964 (Raju, S.) and WO99/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof.

It may be desirable to modify the antibody of the invention with respect to effector function, *e.g.* so as to enhance antigen-dependent cell-mediated cyotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al. Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:219-230 (1989).

WO00/42072 (Presta, L.) describes antibodies with improved ADCC function in the presence of human effector cells, where the antibodies comprise amino acid substitutions in the Fc region thereof.

Preferably, the antibody with improved ADCC comprises substitutions at positions 298, 333, and/or 334 of the Fc region. Preferably the altered Fc region is a human IgG1 Fc region comprising or consisting of substitutions at one, two or three of these positions.

Antibodies with altered C1q binding and/or complement dependent cytotoxicity (CDC) are described in WO99/51642, US Patent No. 6,194,551B1, US Patent No. 6,242,195B1, US Patent No. 6,528,624B1 and US Patent No. 6,538,124 (Idusogie *et al.*). The antibodies comprise an amino acid substitution at one or more of amino acid positions 270, 322, 326, 327, 329, 313, 333 and/or 334 of the Fc region thereof (using Eu numbering of Fc region residues as in Kabat).

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in US Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

Antibodies with improved binding to the neonatal Fc receptor (FcRn), and increased half-lives, are described in WO00/42072 (Presta, L.) and US2005/0014934A1 (Hinton *et al.*). These antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. For example, the Fc region may have substitutions at one or more of positions 238, 250, 256, 265, 272, 286, 303, 305, 307, 311, 312, 314, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424, 428 or 434 thereof (using Eu numbering of Fc region residues as in Kabat). The preferred Fc region-comprising antibody variant with improved FcRn binding comprises amino acid substitutions at one, two or three of positions 307, 380 and 434 of the Fc region thereof.

Engineered antibodies with three or more (preferably four) functional antigen binding sites are also contemplated (US Appln No. US2002/0004587 A1, Miller *et al.*).

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.

(viii) Immunoconjugates

The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.* a small molecule toxin or an enzymatically active toxin of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, a maytansine (U.S. Patent No. 5,208,020), a trichothene, and CC1065 are also contemplated herein.

In one preferred embodiment of the invention, the antibody is conjugated to one or more maytansine molecules (*e.g.* about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody (Chari *et al. Cancer Research* 52: 127-131 (1992)) to generate a maytansinoid-antibody immunoconjugate.

Another immunoconjugate of interest comprises an antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ^I_1 (Hinman *et al. Cancer Research* 53: 3336-3342 (1993) and Lode *et al. Cancer Research* 58: 2925-2928 (1998)). See, also, US Patent Nos. 5,714,586; 5,712,374; 5,264,586; and 5,773,001 expressly incorporated herein by reference.

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).

A variety of radioactive isotopes are available for the production of radioconjugated HER2 antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) 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 glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 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 Acleavable linker@ facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari *et al. Cancer Research* 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, *e.g.* by recombinant techniques or peptide synthesis.

Other immunoconjugates are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, 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, Oslo, A., Ed., (1980).

The antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA*, 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published October 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al. J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon *et al. J. National Cancer Inst.* 81(19)1484 (1989).

IV. Pharmaceutical Formulations

Therapeutic formulations of the MMP antagonists used in accordance with the present invention are prepared for storage by mixing a MMP antagonist having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as TWEENJ, PLURONICSJ or polyethylene glycol (PEG). Lyophilized antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

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. Various drugs which can be combined with the MMP antagonist are described in the method of treatment section below. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, 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).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. 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 DEPOTJ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

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

V. Treatment

Examples of various cancers that can be treated with the MMP antagonist are listed in the definition section above. Administration of the MMP antagonist will result in an improvement in the signs or symptoms of cancer.

The MMP antagonist is administered to a human patient in accord with known methods, such as intravenous administration, *e.g.*, as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

For the prevention or treatment of disease, the dose of MMP antagonist will depend on the type of cancer to be treated, as defined above, the severity and course of the cancer, 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.

While the MMP antagonist may be the sole anti-tumor drug administered, the patient is optionally treated with a combination of the MMP antagonist, and one or more other anti-tumor agent(s). The combined administration includes coadministration or concurrent administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Thus, the other anti-tumor agent may be administered prior to, or following, administration of the MMP antagonist. In this embodiment, the timing between at least one administration of the MMP antagonist and at least one administration of the other anti-tumor agent is preferably approximately 1 month or less, and most preferably approximately 2 weeks or less. Alternatively, the MMP antagonist and other anti-tumor agent are administered concurrently to the patient, in a single formulation or separate formulations. Treatment with the combination of the MMP antagonist and the other anti-tumor agent may result in a synergistic, or greater than additive, therapeutic benefit to the patient.

Examples of second anti-tumor agents that may be combined with the MMP antagonist include: one or more chemotherapeutic agent(s); a HER inhibitor (*e.g.* trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, TAK165, etc); Raf and/or ras inhibitor (see, for example, WO 2003/86467); a growth inhibitory HER2 antibody such as trastuzumab; a HER dimerization inhibitor such as Pertuzumab; a HER2 antibody which induces apoptosis of a HER2-overexpressing cell, such as 7C2, 7F3 or humanized variants thereof; an antibody directed against a tumor

associated antigen, such as EGFR, HER3, HER4; anti-hormonal compound, *e.g.*, an anti-estrogen compound such as tamoxifen, or an aromatase inhibitor; a cardioprotectant (to prevent or reduce any myocardial dysfunction associated with the therapy); a cytokine; an EGFR-targeted drug (such as TARCEVA7, IRESSA7 or Cetuximab); an anti-angiogenic agent (especially bevacizumab sold by Genentech under the trademark AVASTINJ); a tyrosine kinase inhibitor; a COX inhibitor (for instance a COX-1 or COX-2 inhibitor); non-steroidal anti-inflammatory drug, Celecoxib (CELEBREX7); farnesyl transferase inhibitor (for example, Tipifarnib/ZARNESTRA7 R115777 available from Johnson and Johnson or Lonafarnib SCH66336 available from Schering-Plough); antibody that binds oncofetal protein CA 125 such as Oregovomab (MoAb B43.13); HER2 vaccine (such as HER2 AutoVac vaccine from Pharmexia, or APC8024 protein vaccine from Dendreon, or HER2 peptide vaccine from GSK/Corixa); doxorubicin HCl liposome injection (DOXIL®); topoisomerase I inhibitor such as topotecan; taxane; HER2 and EGFR dual tyrosine kinase inhibitor such as lapatinib/GW572016; TLK286 (TELCYTA®); EMD-7200; a temperature-reducing medicament such as acetaminophen, diphenhydramine, or meperidine; hematopoietic growth factor, etc.

Suitable dosages for any of the above coadministered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and MMP antagonist.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

Aside from administration of protein MMP antagonists to the patient, the present application contemplates administration of MMP antagonists by gene therapy. See, for example, WO96/07321 published March 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells; *in vivo* and *ex vivo*. For *in vivo* delivery the nucleic acid is injected directly into the patient, usually at the site where the antibody is required. For *ex vivo* treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, *e.g.* U.S. Patent Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for *ex vivo* delivery of the gene is a retrovirus.

The currently preferred *in vivo* nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.* capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated

endocytosis is described, for example, by Wu *et al.*, *J. Biol. Chem.* 262:4429-4432 (1987); and Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson *et al.*, *Science* 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

VI. Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA (ATCC):

Antibody Designation	**ATCC No.**	**Deposit Date**
7C2	ATCC HB-12215	October 17, 1996
7F3	ATCC HB-12216	October 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	April 8, 1999

Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1

The following example investigated the role of matrix metalloproteases (MMPs) in HER2 shedding.

MATERIALS AND METHODS

Cell Culture and Transfections

All cell lines used in this study were obtained from the American Type Tissue Culture Collection (ATCC, Manassas, VA). BT 474, MCF-7, and SKBR-3 cells were maintained in high-glucose DMEM:Ham's F12 (50:50) supplemented with 10% heat-inactivated FBS and 2mM L-Glutamine. Cos-7 cells were maintained in High-Glucose DMEM supplemented with 10% heat-inactivated FBS and 2mM L-Glutamine. Cell lines were maintained at 37^{0}C in a humidified incubator supplied with 5% CO_2. BT 474 and SKBR-3 cells were transiently transfected with constructs as described by electroporation using kit V according to the manufacturer's instructions (AMAXA™). Cos-7 cells were transfected as described using LIPOFECTAMINE™ 2000 (Invitrogen) according to the manufacturer's recommendations.

Fo5 and f2:1282 Mouse Xenograft Tumor lines

The Fo5 and f2:1282 lines have been previously described (Finkle *et al., Clin. Cancer Res.* 10: 2499-2511 (2004)). These lines were derived from primary tumors from a MMTV HER2 transgenic mouse and passaged in the mammary fat pad of FVB mice.

GM6001 injections

Fo5 tumors were transplanted into FVB mice and allowed to grow to an average size of 400-600 mm^3 prior to initiating the study. GM6001 (3-[N-hydroxycarbomyl]-[2R]-isobutylpropionyl-L-tryptophan methylamide) was obtained from Calbiochem and reconstituted in a slurry of 4% carboxymethylcellulose/0.9% PBS and administered daily by intraperitoneal injection at 100mg/kg body weight for 3 days. Twenty-four

hours after the third day of injection, animals were sacrificed and serum collected by cardiac puncture. Tumors size was measured at day 0 and at day 4. Tumors were removed and flash frozen for further analysis.

RNA preparation and AFFYMETRIX™ arrays

Total RNA was isolated from flash frozen tumor samples using a RNAEASY™ kit (Qiagen, Chatsworth, CA). Residual genomic DNA was removed by DNAse I treatment (Roche Molecular Biochemicals). Microarray experiments were performed and analyzed as previously described (Jin, H *et al., Circulation* 103:736-742 (2001)). Samples were hybridized to the AFFYMETRIX™ mouse genome single array (MOE430P) and known genes array (MOE430A) (AFFYMETRIX™, Inc., Santa Clara, CA). Experiments were done in three replicates for three Fo5 and three f2:1282 tumor samples. A Mann-Whitney pairwise comparison was performed and those genes with a 2-fold increase in expression having a concordance of greater than 80% were considered significant. The GeneLogic Bioexpress Database (GeneLogic, Gaithersburg, MD), a collection of gene expression data from AFFYMETRIX™ microarray analysis of cell lines and tissues, was used to examine the expression of proteases identified from the Fo5-f2:1282 tumor differential screen for expression in SKBR-3 and BT 474 cell lines.

HER2 ECD ELISA

Serum from mice harboring Fo5 or f2:1282 xenograft tumors was collected by cardiac puncture and HER2 ECD levels detected using a previously described HER2 ELISA (Finkle *et al. Clin. Cancer Res.* 10: 2499-2511 (2004)). Serum was diluted 1:50 using assay buffer (PBS/0.5% BSA, 0.05% TWEEN® 20/10 ppm PROCLIN® 300/0.2% BGG/0.25% CHAPS/0.15 M NaCl/5mM EDTA (pH 7.4) followed by additional 1:2 serial dilutions. Shed HER2 was captured using a goat anti-HER2 polyclonal antibody (Genentech) onto NUNC® Maxisorp plates and detected using a biotin-conjugated rabbit anti-HER2 polyclonal antibody (Genentech) followed by a AMDEX™-strepavidin-horseradish peroxidase antibody (Amersham Pharmacia Biotech) following a procedure described previously (Sias et *al., J. Immunol. Meth.* 109:219-27 (1990)). For cell culture experiments, cells were treated as indicated and conditioned media was collected and diluted in assay buffer serially 1:2. The concentration of shed HER2 in serum or in conditioned media was determined from a four-parameter fit of a standard curve using purified recombinant HER2 ECD protein (Genentech) as a standard.

DNA constructs and Protein Purification

Human MMP-15 was cloned into pRK5 by PCR using an origen clone as a template. A C-terminally V5His tagged full length version was generated by PCR using primers
5'-GCCGAAGCTTGCCACCATGGGCAGCGACCCGAGCGCG-3' (SEQ ID NO. 9) and
5'-GCTGTCTAGAGTTCACCGTCCGTGCCAGTGCCACCTCCTCC-3' (SEQ ID NO. 10)
and cloned in pcDNA3.1V5His.

A soluble version of MMP-15, which lacks the transmembrane domain, was generated by PCR using primers
5'-GCCGAAGCTTGCCACCATGGGCAGCGACCCGAGCGCG-3' (SEQ ID NO. 11) and
5'-GCTGTCTAGAGACCCACTCCTGCAGCGAGCG-3' (SEQ ID NO. 12).

A catalytically dead mutant of MMP-15 was generated by substituting an alanine residue for glutamine at amino acid 260 of the mature protein by site-directed mutagenesis.

pRK5.gDHER2-Fc (pRK5.gDHER2-IgG) was generated by PCR using primers 5'-CGAGTCGACCTCGAGGCCAGCCCTCTGACGTCC-3' (SEQ ID NO. 13) and 5'-GGCACGCGTCGTCAGAGGGCTGGCTCTCTG-3' (SEQ ID NO. 14) and cloned into the XhoI-MluI digested pRK5.gDHER2-Fc (Fitzpatrick *et. al., FEBBS Lett.* 431(1):102-6 (1998)). This introduced the putative cleavage site of HER2 identified from purified HER2 ECD isolated from SKBR-3 conditioned media (Yuan et. *al., Protein Expression and Purification* 29:217-222 (2003)).

A truncated version of gDHER2-Fc was also generated that only contains domain IV of HER2 using PCR primers 5'-GGCCTCGAGGGCCTGGCCTGCCACCAG-3' (SEQ ID NO. 15) and 5'-GGCACGCGTCGTCAGAGGGCTGGCTCTCTG-3' (SEQ ID NO. 16).

pRK5.HER3-Fc and pRK5.HER4-Fc have been previously described (Fitzpatrick *et al., FEBBS Lett.* 431(1): 102-6 (1998)). A FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; (SEQ ID NO. 17)) was introduced into the N-terminus of pRK5. HER-Fc fusion proteins were transfected into 293 cells and purified from serum free conditioned media as previously described (Fitzpatrick *et. al., FEBBS Lett.*, 431(1):102-6 (1998)). Soluble MMP-15V5His and sMMP-15V5His(E260A) protein was generated from transiently transfected 293 cell conditioned medium and purified using Ni-NTA agarose according to manufacturer's recommendations (Qiagen).

shRNA knockdown of MMP-15

Several different shRNAs were evaluated for the ability to reduce MMP-15 protein levels when introduced into cells. The most effective shRNA directed against human MMP-15 was found to recognize bp 5'-CCACCATCTGACCTTTAGCTT-3' (SEQ ID NO. 18) corresponding to nt 1396-1414 (GENBANK™ accession number NM_002428). The RNAi to MMP-15 was introduced and into the BamH1-EcoRI sites pSIREN vector (BD Biosciences) as oligos:

5'-GATCCGCCACCATCTGACCTTTAGCTTCAAGAGAGCTAAAGGTCAGATGGTGGTTTTTTGCTAGCG-3' (SEQ ID NO. 19) and

5'-AATTCGCTAGCAAAAAACCACCATCTGACCTTTAGCTCTCTTGAAGCTAAAGGTCAGATGGTGGCG-3' (SEQ ID NO. 20) which contain an internal hairpin to form a shRNA. A shRNA to MMP-25 was purchased from OPENBIOSYSTEMS™. The pSIREN shRNA construct(s) or vector alone were transiently transfected into SKBR-3 and BT 474 cells using AMAXA™ kit V following manufacturer's recommendations. After 48 hours, conditioned media was collected and assayed for shed HER2 by HER2 ELISA and the cells were lysed directly on the plate using 2xSample buffer. MMP-15, full length HER2, and p95 HER2 proteins were detected by Western blotting using a rabbit polyclonal anti-MMP-15 antibody (Labvision cat. no. RB-1546-P) or mouse monoclonal anti-HER2 antibody Ab-15 (Labvision, cat. no. MS-599-P0).

***In Vitro* HER2 cleavage assay**

The ability of candidate proteases to cleave HER2 ECD was determined *in vitro* using purified gDHER2-Fc fusion protein as a substrate. The purified catalytic domains of MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-19), and MT5-MMP (MMP-25) were purchased from R&D systems. MMP purified proteins were mixed with gDHER2-Fc, or other HER-Fc proteins as described, in assay buffer (100mM Tris (pH=7.4), 100 mM NaCl, 2.5 μM $ZnCl_2$, 10 mM $CaCl_2$, 0.001% Brij35) in a ratio of 1:100, enzyme:substrate, and incubated at 37^{0}C for 20 minutes. Reactions were stopped using an equal volume of 2x Sample buffer (Invitrogen), and boiled for 5 min. prior to loading onto a 4-20% Tris-glycine gradient gel (Invitrogen). Proteins were visualized by staining with GEL CODE BLUE™ stain reagent (Pierce cat. no. 24592) following manufacturer's recommendations. The MMP cleavage site was determined by N-terminal protein sequencing by automated Edman degradation using an automated protein sequencer (Kishiyama, A., *Anal. Chem.* 72(21):5431-6 (2000)).

Immunoprecipitation assays and Western Blotting

Cos-7 cells were co-transfected with pRK5.Flag-HER2 and pcDNA3.MMP-15 constructs or pcDNA3.1 vector alone using LIPOFECTAMINE™ 2000. Cells were allowed to recover for 48 hrs. Transfected cells were rinsed with PBS and cells were ruptured in lysis buffer (1%TRITON X-100™, 50mMTris (pH=7.4), 150mM NaCl, 1mM PMSF, 10μg/ml leupeptin, 10U/ml aprotinin, and 2mM Na_2VO_4). Lysates were cleared of insoluble material by centrifugation and total protein levels were determined using a BCA protein assay kit (Pierce cat. no. 23229). Two-hundred micrograms of total cellular protein was added to lysis buffer to a final volume of 1 ml and Flag-HER2 was immunoprecipitated using anti-Flag M2-agarose (Sigma) or with polyclonal anti-MMP-15 antibody complexed to ProteinA/G agarose. Immune complexes were washed twice with lysis buffer and resuspended in SDS sample buffer and boiled. Samples were separated on a 4-12% Tris-glycine gradient gel (Invitrogen) and transferred to nitrocellulose membranes. Blots were blocked in 5%BSA/TBST and probed with antibodies to either MMP-15 or HER2 followed by a peroxidase conjugated anti-mouse or rabbit secondary antibody as described. Blots were developed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Fo5 and f2:1282 tumors were resuspended in lysis buffer with protease inhibitors and homogenized using a POLYTRON TISSUEMIZER™ (PT2100) on ice. Tumor lysates were cleared of insoluble material by centrifugation and total protein levels were determined using a BCA protein assay kit. HER2 was immunoprecipitated from 1 mg total protein from at least three independent tumor lysates using mouse monoclonal antibody Ab-15 (Labvision, cat. no. MS-599-P0) complexed to Protein A/G sepharaose overnight at 4^{0}C. Complexes were pelleted by centrifugation, washed twice with lysis buffer and resuspended in SDS sample buffer and boiled. Samples were separated on a 4-12% Tris-Glycine gel and transferred to nitrocellulose membranes. Blots were probed with mouse monoclonal antibody Ab-18 to detect phosphorylated HER2 (Labvision, cat. no. MS-1072-P) or with Ab-15 (Labvsion cat. no., MS-599-P0). Expression of MMP-14, MMP-15, and MMP-25 was evaluated by Western blotting from 50 ug of tumor lysate or from 50 ug from BT 474, or MCF-7, or SKBR-3 cellular lysate using rabbit polyclonal Ab-1 (Labvision cat. no. RB-1544-P) to detect MMP-14, rabbit polyclonal Ab-1 (Labvision cat. no. RB-1546-P) to detect MMP-15,or rabbit polyclonal Ab-1 (Oncogene Research Products cat. no. PC499) to detect MMP-25.

Activated MAPK (Cell Signaling Technology cat. no. 9101), PhosphoAkt Ser473 (Cell Signaling Technology cat. no. 9271), total Akt (Cell Signaling Technology cat. no. 9272), total Erk (Cell Signaling Technology cat. no. 9102, or phosphoHER3 (Cell Signaling Technology cat. no. 4791) was determined by Western blotting of 50 μg of cellular lysate.

Proliferation assays

Cells were seeded into 96-well dishes (Nunc) at a density of 10^4 cells per well in quadruplicate and allowed to adhere overnight. Cells were treated as indicated, or transfected as indicated. Following a 3 day incubation, 25μl of Alamar blue reagent (Trek Diagnostic Systems, cat. no. 00-100) was added to each well, and incubated for an additional 3 hours. Plates were read in a flourometer with excitation wavelength of 530nm and emission wavelength of 590nm according to manufacturer's recommendations. Proliferation relative to non-stimulated or control transfected cells was determined as previously described (Lewis *et al., Cancer Res.* 56(6):1457-65 (1996)).

RESULTS

The ECD of HER2 is proteolytically shed from breast carcinoma cells in culture and has been detected in the serum of patients with metastatic breast cancer, where it is associated with a poor clinical prognosis (Molina *et al. Cancer Res.* 61: 4744-4749 (2001)). However, the biological role of HER2 shedding is unclear. The experiments herein were carried out in order to identify the HER2 sheddase.

Identification of the HER2 sheddase may also be important as trastuzumab has previously been demonstrated to inhibit shedding in breast carcinoma cell lines (Molina *et al. Cancer Res.* 61:4744-4749 (2001)). Consistent with previously reported results, trastuzumab reduced shedding in the breast cancer cell lines BT 474 and SKBR-3 cells by more than 60% at a concentration of 10 μg/ml (Fig. 5, left panel) and reduced cellular proliferation in these cell lines by 50% (Fig. 5, right panel).

An animal model system where mammary tumors derived from a MMTVHER2 transgenic mouse (Finkle *et al. Clin. Cancer Res.* 10:2499-2511 (2004)) was used to identify the HER2 sheddase. This animal model system reproducibly shed different levels of HER2 into serum and exhibited differential sensitivity to trastuzumab (Fig. 6, right panel). This difference in the level of serum HER2 also correlated well with the presence of a protein fragment immunoprecipitated from three independent Fo5 tumor cell lysates having an apparent molecular weight of 95 kD, consistent with the size of p95 HER2 (Figure 6, lower left panel). This fragment is constitutively phosphorylated in the Fo5 tumor cell lysates, indicating that this fragment may have biological activity in these tumors. The increased levels of a HER2 sheddase may contribute to the trastuzumab resistance of this line, since the epitope of trastuzumab would be lost by proteolytic cleavage (Cho *et al. Nature* 421: 756-760 (2003)).

Since the Fo5 tumor line sheds higher levels of HER2 and has high levels of p95HER2 fragment, differential expression analysis was used as an approach to identify transcripts that are upregulated in Fo5 tumors relative to f2:1282 tumors. RNA was prepared from 3 individual tumor samples from either Fo5 tumors or f2:1282 tumors with an average tumor size of 300-600mm^3. RNA from each independent tumor sample was hybridized in triplicate to AFFYMETRIX™ mouse genome single array chip (MOE430P) and known gene array (MOE430A). A Mann-Whitney pairwise comparison was performed identifying 638 upregulated

transcripts in Fo5 tumor samples. Since BT 474 and SKBR-3 cell lines also shed HER2 ECD into conditioned medium (Fig. 5, left panel), this list was compared to transcripts that are expressed in both cell lines using the Genelogic Bioexpress database. The approach taken to identify the HER2 sheddase is illustrated in Fig. 7.

In order to narrow down the number of candidate genes, the general metalloprotease inhibitor GM6001 was used to evaluate whether MMP activity plays a role in regulating HER2 shedding. GM6001, but not its stereoisomer, dose dependently inhibited HER2 shedding in SKBR-3 cells as determined by a HER2 ECD ELISA (Fig. 8, left panel). This result is consistent with previously published reports that the general metalloprotease inhibitor BB-94 inhibited HER2 ECD shedding in breast carcinoma cell lines (Codony-Servat *et al. Cancer Res.* 59:1196-1201 (1999)). Timp-1, Timp-2 and Timp-3 are naturally occurring peptides that regulate metalloprotease activity (Overall and Lopez-Otin *Nature Reviews Cancer* 2:657-672 (2002)). Adding purified Timp-1, Timp-2, or Timp-3 to the conditioned media of SKBR-3 cells at a concentration of 1 μg/ml reduced HER2 ECD levels (Fig. 8, right panel). However, Timp-2 reduced HER2 ECD shedding the most efficiently (Fig. 8, right panel). Timp-2 is known to efficiently inhibit the MT-MMP subfamily of metalloproteases (Overall and Lopez-Otin *Nature Reviews Cancer* 2:657-672 (2002)). These data suggested that the protease responsible for HER2 shedding in SKBR-3 and BT 474 cells is a metalloprotease, significantly reducing the number of candidates identified from the bioinformatics screen.

Several members of the MT-MMP family are expressed in SKBR-3 and BT 474 cells based on the GENELOGIC™ database. Both MT1-MMP and MT2-MMP are also expressed in Fo5 tumors from the AFFYMETRIX™ microarray data. To determine if these transcripts are expressed, Fo5 and f2:1282 tumor and breast carcinoma cell line lysates were immunoblotted with polyclonal antibodies to these MT-MMPs that recognize both human and mouse proteins (Fig. 9). The cell lines all express MMP-14, MMP-15, and MMP-25, at different levels. While MMP-14 is expressed in both f2:1282 and Fo5 tumor cell lysates, MMP-15 is abundantly expressed in Fo5 tumors lysates. This result was consistent with the Mann-Whitney comparison which indicated a 2-fold higher level of mRNA expression of MMP-15 in Fo5 tumors. To verify that MMP-15 was expressed in the epithelial cells of the tumor, and not the surrounding stromal cells, Fo5 and f2:1282 tumors were sectioned and stained with either an anti-HER2 antibody (Dako) or an anti-MMP-15 antibody (Ab-1). Both f2:1282 and Fo5 tumors express human HER2, but MMP-15 is more abundantly expressed in the Fo5 tumors than the f2:1282 tumors.

Substrates for members of the MT-MMPs have traditionally been thought to be extracellular matrix proteins. A biochemical assay was set up to test candidates identified from the microarray data to cleave full length HER2 *in vitro*. This assay uses a purified recombinant fusion protein as a substrate for candidate proteases and consists of a N-terminally gD epitope-tagged HER2 ECD in-frame with the Fc heavy chain of human IgG. Several different proteins were used in these studies. gDHER2(+)-IgG contains amino acids 2-656 of HER2 ECD (GenBank accession AAA75493) and gDHER2(-)-IgG contains amino acids 2-626 of HER2 ECD. The gDHER2(+)-IgG recombinant protein contains juxtamembrane sequence that have previously been shown to contain a putative HER2 sheddase site (PA/EQR/ASP; SEQ ID NO. 23) while the gDHER2(-)-IgG protein lacks this sequence (Yuan *et al. Protein Expression and Purification* 29:217-222 (2003)). A truncated version gDHER2(+)-IgG was also used in this assay, gDHER2(DIV)-IgG, which has only domain IV (DIV) of HER2 in-frame with human Fc. Purified catalytic domains of MMP-14, MMP-15, MMP-16, MMP-19, and MMP-25 were incubated with gDHER2(DIV)-IgG for 20 minutes at 37^{0}C. All MT-MMPs except MMP-14

efficiently cleave gDHER2(DIV)-IgG substrate (Fig. 13, left panel). The cleaved protein products were excised and N-terminally sequenced to identify the cleavage site. All four MT-MMPs cleaved HER2 near the published sequence site (Yuan *et al. Protein Expression and Purification* 29: 217-222 (2003)) (Fig. 13, right panel).

HER2-Fc fusion proteins that have the sequence PINCTHSCVDLDDKGCPAEQRASPASPLTSIV (SEQ ID NO. 21) are substrates for these four MMPs *in vitro* (Fig. 14). This data suggest that HER2 is a substrate for MMP-15.

MT-MMP substrate recognition may be influenced by the hemopexin domain (Overall and Lopez-Otin *Nature Reviews Cancer* 2:657-672 (2002)). A soluble form of MMP-15 with a C-terminal V5 His epitope was transiently expressed in 293 cells and purified from 293 conditioned media by affinity chromatography. As shown in Fig. 15, this soluble form of MMP-15 can also cleave gDHER2(+)-IgG in the *in vitro* sheddase assay.

To determine if MMP-15 and HER2 can associate in the membrane, co-immunoprecipitation assays were performed using transiently transfected Cos-7 cells. pRK5.FlagHER2 was co-transfected with either vector, pcDNA3.MMP-15V5His, pcDNA3.sMMP-15V5His, or pcDNA3.MMP-15(E260A). FlagHER2 was immunoprecipitated with an anti-FLAG resin. Fig. 10 shows that both soluble MMP-15 and a catalytically dead (E260A) mutant can associate with Flag-HER2. This is consistent with the notion that MMP-15 can cleave HER2 in membranes since the wild-type version of the protease, pcDNA3.MMP-15V5His, would remove the FlagHER2 ECD and a HER2-MMP-15 complex would not be co-immunoprecipitated.

Somatic point mutations and deletions and splice variants have been identified mammary tumors from MMTVHER2 transgenic animals (Siegel *et al. EMBO J.* 18:2149-2164 (1999)); and Finkle *et al. Clin. Cancer Res.* 10:2499-2511 (2004)). These mutations most frequently occur in the HER2 extracellular domain near the transmembrane domain. The Fo5 tumor line has a five amino acid deletion DLDDK (SEQ ID NO. 22) that is adjacent to the HER2 cleavage site while the f2:1282 tumor line has a single point mutation C for R (Fig. 12). These mutations do not influence MMP-15 association (Fig. 11). However, it is possible that the proximity of the DLDDK (SEQ ID NO. 22) deletion to the HER2 MMP cleavage site may influence enzymatic activity of the protease.

RNA inhibition (RNAi) is a method that can downregulate target transcript and protein levels. To examine the biological role of MMP-15 in SKBR-3 and BT 474 cells, an anti-MMP-15 RNAi construct was used. When introduced into SKBR-3 or BT 474 cells, this shRNAi expression plasmid reduced endogenous protein levels of MMP-15 in both cell types (Fig. 17, left upper panel). Transfection of this shRNA also reduced the amount of p95 HER2 in both cell lines (Fig. 17, left lower panel). Loss of MMP-15 protein in these cells significantly reduced HER2 ECD shedding in both lines (Fig. 17, right panel). However, no significant reduction in HER2 ECD shedding was observed when a shRNAi to MMP-25 was introduced into either cell line (Fig. 17, right panel). These experiments strongly suggest that loss of MMP-15 dramatically effects HER2 ECD shedding.

Reducing MMP-15 protein levels by RNAi also has an effect on the growth rates of SKBR-3 and BT 474 cells (Fig. 18, left panel). This effect is also duplicated by a shRNAi to MMP-25. This suggests that reducing HER2 ECD shedding also downregulates the amount of p95 HER2 in BT 474 and SKBR-3 cells, thereby reducing cell growth rates. When a gD-tagged p95 HER2 construct was introduced into these cell lines, an increase in cell growth rates was detected in both cell types relative to control transfected cells (Fig. 18, left panel). This construct is constitutively phosphorylated and activates MAPK when introduced into Cos-7 cells in

a ligand-independent manner (Fig. 16). However, this construct must still heterodimerize with HER3 or EGFR in order to activate Akt signaling pathways consistent with a previously published study (Xia *et al. Oncogene* 23:646-653 (2004)). Overexpression of gDp95HER2 in SKBR-3 cells did not significantly inhibit trastuzumab-mediated growth inhibition in these cells (Fig. 18, right panel).

A pharmacological approach was used to determine if MMP activity plays a role in regulating HER2 shedding and p95 HER2 levels in Fo5 tumors. FVB mice with Fo5 tumors with average size of 400-600 mm^3 were injected with GM6001 intraperitoneally or control vehicle daily over the course of three days. On day four, serum was collected and assayed for HER2 ECD by ELISA, and tumors collected and weighed. HER2 was immunoprecipitated from tumor cell lysates and assayed for p95 HER2 levels by Western blotting. GM6001 treated animals showed a significant reduction in the amount of p95 HER2 as compared to control animals (Fig. 19, right panel). Serum HER2 ECD levels were reduced in both vehicle and GM6001 treated animals (Fig. 19, left panel), however, a greater reduction was observed in GM6001 treated animals.

SUMMARY

The experiments above demonstrate that the matrix metalloprotease MMP-15 cleaves HER2 *in vitro* at a site that is consistent with purified shed ECD from SKBR3 cells, and interacts with full length HER2. Reducing levels of this MMP correlates well with reduced HER2 receptor shedding, and decreases basal proliferation levels in SKBR3 and BT474 cell lines. Trastuzumab appears to inhibit HER2 receptor shedding by indirectly regulating MMP-15 activity, but does not compete with MMP-15 for substrate binding and cleavage. Reducing HER2 shedding with a MMP antagonist, such as an MMP-15 antagonist, represents a therapeutic approach for treating cancer, including trastuzumab-resistant cancer.

What is claimed is:

1. A method for inhibiting HER2 shedding comprising treating a HER2 expressing cell with a matrix metalloprotease (MMP) antagonist in an amount effective to inhibit HER2 shedding.
2. The method of claim 1 wherein the MMP antagonist is a membrane-tethered MMP (MT-MMP) antagonist.
3. The method of claim 2 wherein the MT-MMP is selected from the group consisting of MMP-15 (MT2-MMP), MMP-16 (MT3-MMP), MMP-24 (MT5-MMP), MMP-17 (MT4-MMP), and MMP-25 (MT6-MMP).
4. The method of claim 3 wherein the MT-MMP is MMP-15.
5. The method of claim 1 wherein the cell displays HER2 overexpression, amplification, or activation.
6. The method of claim 5 wherein the cell displays HER2 overexpression or amplification.
7. The method of claim 1 further comprising treating the cell with a HER inhibitor.
8. The method of claim 7 wherein the HER inhibitor is a HER2 antibody.
9. The method of claim 8 wherein the HER2 antibody is trastuzumab or pertuzumab.
10. The method of claim 7 wherein the HER inhibitor is selected from the group consisting of trastuzumab, pertuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib, CP724714, CI1033, GW572016, IMC-11F8, and TAK165.
11. A method for reducing HER2 extracellular domain (ECD) serum level in a mammal, comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to reduce the HER2 ECD serum level in the mammal.
12. The method of claim 11 wherein the mammal has an elevated MMP level.
13. A method for treating cancer in a mammal comprising administering a matrix metalloprotease (MMP) antagonist to the mammal in an amount effective to treat the cancer.
14. The method of claim 13 wherein the cancer displays HER expression, amplification, or activation.
15. The method of claim 14 wherein the cancer displays HER2 overexpression or amplification.
16. The method of claim 13 wherein the mammal has an elevated shed HER2 serum level or elevated p95 HER2 level.
17. A method for treating a HER inhibitor-resistant cancer in a mammal comprising administering to the mammal a matrix metalloprotease (MMP) antagonist in an amount effective to treat the cancer.
18. The method of claim 17 wherein the HER inhibitor is trastuzumab.
19. A method for reducing p95 HER2 level in a cell comprising exposing the cell to a matrix metalloprotease (MMP) antagonist in an amount effective to reduce the p95 HER2 level.
20. A method of diagnosis comprising evaluating MMP-15 (MT2-MMP) in a sample from a cancer patient, wherein elevated MMP-15 level or activity indicates the patient has an elevated p95 HER2 or shed HER2 serum level, or will have a poor clinical outcome.
21. The method of claim 20 wherein an elevated MMP-15 level indicates the patient will have a poor clinical outcome.

INHIBITING HER2 SHEDDING WITH MATRIX METALLOPROTEASE ANTAGONISTS

ABSTRACT OF THE DISCLOSURE

The present application describes using antagonists of matrix metalloproteases (MMPs), especially of MMP-15, for inhibiting HER2 shedding.

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Mark Sliwkowski, San Carlos, CA

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6. Total number of applications and patents involved: 1

7. Total fee (37 CFR 3.41): $ 40.00

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ASSIGNMENT
(joint provisional appln.)

WHEREAS, **Kendall Carey**, a citizen of the **USA**, residing at **545 Miller Ave., #5, South San Francisco, CA 94080**, and **Ralph Schwall**, a citizen of the **USA**, residing at **400 Griffin Ave. Pacifica, CA 94044** and **Mark Sliwkowski**, a citizen of the **USA**, residing at **42 Oak Lane, San Carlos, CA 94070,** (hereinafter "ASSIGNORS"), have invented a new and useful invention in

INHIBITING HER2 SHEDDING WITH MATRIX METALLOPROTEASE ANTAGONISTS

for which a provisional application Serial No. **60/651,348** (Docket No. PR2195) has been filed by them on **February 9, 2005**; 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.

IN WITNESS WHEREOF we undersign as follows;

South San Francisco

5/28/05

Date

Name: Kendall Carey

South San Francisco

Date

Name: Ralph Schwall

South San Francisco

Date

Name: Mark Sliwkowski

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.

IN WITNESS WHEREOF we undersign as follows;

South San Francisco

Date

Name: Kendall Carey

South San Francisco

4-12-05

Date

Name: Ralph Schwall

South San Francisco

Date

Name: Mark Sliwkowski

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.

IN WITNESS WHEREOF we undersign as follows;

South San Francisco

Date

Name: Kendall Carey

South San Francisco

Date

Name: Ralph Schwall

South San Francisco

March 18, 2005

Date

Name: Mark Sliwkowski

AS:104752

APOSTILLE

(Convention de La Haye du 5 octobre 1961)

1. Country: *United States of America*

This public document

2. has been signed by W. Montgomery

3. acting in the capacity of Certifying Officer

4. bears the seal/stamp of U.S. Patent and Trademark Office

Certified

5. at Washington, D.C.

6. the thirty-first of July, 2007

7. by *Assistant Authentication Officer, United States Department of State*

8. No. 07031470-1

9. Seal/Stamp:

10. Signature:

Patrick O. Hatchett

D.C. Colombia

A 7077453

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

July 17, 2007

THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF A DOCUMENT RECORDED ON JUNE 13, 2005.

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

W. Montgomery

W. MONTGOMERY
Certifying Officer

UNITED STATES PATENT AND TRADEMARK OFFICE
DEPARTMENT OF COMMERCE

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

RECORDATION FORM COVER SHEET
PATENTS ONLY

Atty Dkt No: PR2195

Commissioner for Patents: Please record the attached original documents or copy thereof.

1. Name of conveying party(ies):

Kendall Carey, South San Francisco, CA
Ralph Schwall, Pacifica, CA
Mark Sliwkowski, San Carlos, CA

Additional name(s) of conveying party(ies) attached? _ Yes X No

2. Name and address of receiving party(ies):

Name: GENENTECH, INC.

Internal Address:

Street Address: 1 DNA Way

City: South San Francisco State: California

ZIP: 94080-4990

Additional name(s) & address(es) attached? _ Yes X No

3. Nature of conveyance:

[X] Assignment Merger

Security Agreement

Change of Name

Other:

Execution Date: Kendall Carey, 5-28-05, Ralph Schwall, 4-12-05 & Mark Sliwkowski 3-18-05

4. Application number(s) or patent number(s):

If this document is being filed together with a new application, the execution date of the application is:

A. Patent Application No.(s) 60/651,348

B. Patent No.(s)

Additional numbers attached? _ Yes X No

5. Name and address of party to whom correspondence concerning document should be mailed:

Wendy Lee
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080

6. Total number of applications and patents involved: 1

7. Total fee (37 CFR 3.41): $ 40.00

_ Enclosed

X Authorized to be charged to deposit account

8. Deposit account number:
07-0630

DO NOT USE THIS SPACE

9. Statement and signature.

To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document.

Wendy Lee
Name of Person Signing
Registration No. 40,378

WM Lee
Signature

June 13, 2005
Date

Total number of pages comprising cover sheet, attachments and document:

Revised (10/11/95)

CH $40.00 070630 60651348

#182619

Attorney Docket No. PR2195

ASSIGNMENT
(joint provisional appln.)

WHEREAS, **Kendall Carey**, a citizen of the **USA**, residing at **545 Miller Ave., #5, South San Francisco, CA 94080**, and **Ralph Schwall**, a citizen of the **USA**, residing at **400 Griffin Ave. Pacifica, CA 94044** and **Mark Sliwkowski**, a citizen of the **USA**, residing at **42 Oak Lane, San Carlos, CA 94070,** (hereinafter "ASSIGNORS"), have invented a new and useful invention in

INHIBITING HER2 SHEDDING WITH MATRIX METALLOPROTEASE ANTAGONISTS

for which a provisional application Serial No. **60/651,348** (Docket No. PR2195) has been filed by them on **February 9, 2005**; 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.

IN WITNESS WHEREOF we undersign as follows;

South San Francisco
5/28/05
Date

Name: Kendall Carey

South San Francisco

Date

Name: Ralph Schwall

South San Francisco

Date

Name: Mark Sliwkowski

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.

IN WITNESS WHEREOF we undersign as follows;

South San Francisco

Date

Name: Kendall Carey

South San Francisco
4-12-05
Date

Name: Ralph Schwall

South San Francisco

Date

Name: Mark Sliwkowski

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.

IN WITNESS WHEREOF we undersign as follows;

South San Francisco

Date

Name: Kendall Carey

South San Francisco

Date

Name: Ralph Schwall

South San Francisco

March 18, 2005

Date

Name: Mark Sliwkowski

294

TRADUCCION AL ESPAÑOL del Documento de Cesión a **GENENTECH, INC.** firmado por los cedentes y el cesionario.

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 W. Montgomery
3. actuando en capacidad de Funcionario que Certifica
4. lleva el sello/timbre de la Oficina de marcas y patentes

Certificado

5. en Washington D.C.
6. el treinta y uno de Julio de 2007
7. por el Funcionario de Autenticación Asistente, Departamento de Estado de Estados Unidos
8. No. 07031470-1
9. Sello/timbre

10. Firma

(Firma)

Patrick O. Hatchett

[sello]

LOS ESTADOS UNIDOS DE AMÉRICA
A TODO AQUEL QUE PUEDA LLEGARLE LA PRESENTE:

DEPARTAMENTO DE COMERCIO DE ESTADOS UNIDOS
Oficina de Marcas y Patentes de Estados Unidos

Julio 17 de 2007

ESTE ES PARA CERTIFICAR QUE EL ANEXO ES UNA COPIA VERDADERA DE LOS ARCHIVOS DE ESTA OFICINA DE UN DOCUMENTO ARCHIVADO EL 13 DE JUNIO DE 2005.

Por autoridad del
Subsecretario de Comercio para la Propiedad Intelectual
y Director de la Oficina de Marcas y Patente de Estados Unidos

W. MONTGOMERY
Funcionario que Certifica

Hay un sello que dice:
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Departamento de Comercio

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SOLAMENTE PATENTES

Departamento de Comercio
Oficina de Marcas y Patentes
Expediente de abogado No. PR2027

Comisionado Asistente para patentes: Por favor archive los documentos originales anexos o copias de los mismos

1. Nombre de la(s) parte(s) cedente(s):

   Kendall Carey, South San Francisco, CA
   Ralph Schwall, Pacifica, CA
   Mark Sliwkowski, San Carlos, CA

   ¡Se anexa(n) nombre(s) adicional(es) de parte(s) cedente(s)? _ Si X No

2. Nombre y dirección de la(s) parte(s) receptora(s)

   Nombre: GENENTECH, INC.

   Dirección interna:

   Dirección: 1 DNA Way

   Ciudad: South San Francisco Estado: California

   ZIP: 94080-4990

   ¿Se anexan Nombre(s) y dirección(es) adicional(es)? _ Si X No

3. Naturaleza del traspaso:

   X Cesión Fusión

   Acuerdo de Seguridad

   Cambio de Nombre

   Otro:

   Fecha de Ejecución: Kendall Carey, 5-28-05, Ralph Schwall, 4-12-05 & Mark Sliwkowski 3-18-05

4. Numero(s) de solicitud o número(s) de patente:

   Si este documento se presenta junto con una nueva solicitud, la fecha de ejecución de la solicitud es:

   A. Solicitud de patente No(s). B. Patente No(s).

   60/651,348

   ¿Se anexa números adicionales? __ Si X No

5. Nombre y dirección de la parte a quien se le debe enviar la correspondencia:

   Wendy Lee
   GENENTECH, INC.
   1DNA Way
   South San Francisco, CA 94080

6. Numero total de solicitudes y patentes involucradas: 1

7. Tarifa total (37 C.F.R 3.41): $40.00

   __ Adjunta
   X Autorizado para cargarla a cuenta de depósito

8. Número de cuenta de Depósito: 07-0630

NO USE ESTE ESPACIO

9. Declaración y firma.
   hasta donde se y creo, la información anterior es verdadera y correcta y cualquier copia anexa es una copia verdadera del documento original.

Wendy Lee	(Firma)	Junio 13, 2005
Nombre de la persona que firma Registro No. 36,487	Firma	Fecha

Número total de páginas que comprenden portada, anexos y documento:

700186926

PATENTE
ROLLO: 016325 CUADRO: 0799

2004

CESIÓN
(cesión conjunta provisional)

CONSIDERANDO QUE, Kendall Carey, un ciudadano de los E.U.A., residente en 545 Miller Ave., #5, South San Francisco, CA 94080, y Ralph Schwall, un ciudadano de los E.U.A., residente en 400 Griffin Ave., Pacifica, CA 94044 y Mark Sliwkowski, un ciudadano de los E.U.A., residente en 42 Oak Lane, San Carlos, CA 94070, (en adelante referidos como "CEDENTE"), han inventado una invención nueva y útil

DESPRENDIMIENTO INHIBIDOR DE HER2 CON ANTAGONISTAS DE MATRIZ DE METALOPROTEASA

para la cual se presentó la solicitud provisional con Serie No. 60/651,348 (Expediente No. PR2195) por ellos el 9 de Febrero, 2005; y

CONSIDERANDO QUE, GENENTECH, INC., una corporación organizada y existente bajo y por virtud de las leyes del Estado de Delaware, que tiene oficinas en 1 DNA Way, South San Francisco, California 94080-4990, está deseosa de adquirir un interés en y para dicha invención, y en y para las patentes que se obtendrán de la misma;

AHORA, POR LO TANTO, téngase por sabido por todo aquel que le concierna:

Que por una consideración buena y valiosa el recibo de la cual se reconoce por la presente, dicho CEDENTE ha y por la presente vende, cede, transfiere y traspasa a dicha GENENTECH, INC. sus sucesores y cesionarios, el derecho, título e interés completo y exclusivo incluyendo todos los derechos bajo la Convención de Paris para la Protección de la Propiedad Industrial, en y para dicha invención, y en y para cualquiera y todas las patentes que se concedan y emitan para la misma o cualquier continuación, división, renovación, o sustitución de la misma, y en cuanto a patentes cualquier reemisión o reexaminación de las mismas, no solo para, por y en los Estados Unidos de América, sus territorios y posesiones, sin para, por y en todos los otros países; y se ha y por la presente se autoriza y solicita que las agencias gubernamentales respectivas emitan dichas patentes a GENENTECH, INC. de acuerdo con esta cesión.

Dicho CEDENTE garantiza y acuerda cooperar con GENENTECH, INC., para posibilitar a dicha GENETECH, INC., a disfrutar hasta su máxima extensión el derecho, título e interés aquí cedido en Estados Unidos y los países extranjeros. Tal cooperación por dichos CEDENTE incluye la pronta producción de hechos y documentos pertinentes, dando testimonio, ejecutando peticiones, juramentos, especificaciones, declaraciones u otros documentos, y otra ayuda todas hasta el punto considerado necesario o deseable por dichas GENETECH, INC. (a) para perfeccionar el derecho, título e interés aquí cedido; (b) para procesar cualquiera de dichas solicitudes; (c) para presentar y procesar solicitudes de reemisión de cualquiera de dichas patentes; (d) para procesos de interferencia u otros procesos de prioridad que involucran dicha invención; y (e) para procedimientos legales que involucran dicha invención y cualquier solicitud de la misma y cualquier patente concedida sobre la misma, incluyendo sin limitación procedimientos de oposición, procedimientos de cancelación, disputas de prioridad, procesos de uso público, acciones de violación y acciones de la corte; con la condición que los gastos incurridos por dicho CEDENTE al proporcionar dicha cooperación deban ser pagados por GENETECH, INC.

Los términos y garantías de esta cesión deben actuar en beneficio de dicha GENETECH, INC. sus sucesores, cesionarios y otros representantes legales, y deberá ser obligante sobre dichos CEDENTE, sus respectivos herederos, representantes legales y cesionarios.

Dicho CEDENTE por la presente garantiza y manifiesta que no ha entrado y no entrará en ninguna cesión, contrato o entendimiento en conflicto con la presente.

PATENTE
ROLLO: 016325 CUADRO: 0800

300

EN TESTIMONIO DE LO CUAL suscribimos como sigue:

South San Francisco
5/28/05
Fecha

(firma)
Nombre: Kendall Carey

South San Francisco
Fecha

Nombre: Ralph Schwall

South San Francisco
Fecha

Nombre: Mark Sliwkowski

PATENTE
ROLLO: 016325 CUADRO: 0801

EN TESTIMONIO DE LO CUAL suscribimos como sigue:

South San Francisco

Fecha

Nombre: Kendall Carey

South San Francisco
4-12-05
Fecha

(firma)
Nombre: Ralph Schwall

South San Francisco

Fecha

Nombre: Mark Sliwkowski

EN TESTIMONIO DE LO CUAL suscribimos como sigue:

South San Francisco

Fecha

Nombre: Kendall Carey

South San Francisco

Fecha

Nombre: Ralph Schwall

South San Francisco

Marzo 18, 2005
Fecha

(firma)
Nombre: Mark Sliwkowski

State of California

SECRETARY OF STATE

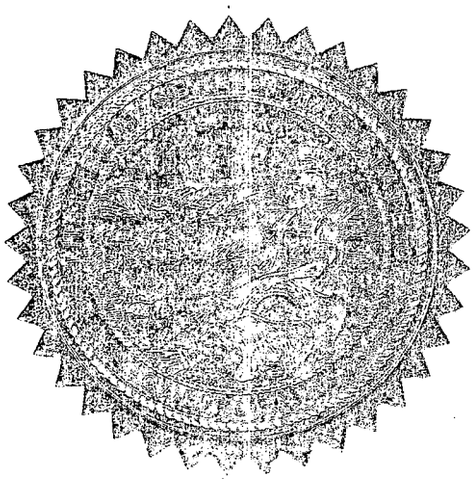
APOSTILLE

(Convention de La Haye du 5 octobre 1961)

1. *Country: United States of America*
   This public document
2. *has been signed by AIDA MICLAT GUIAM*
3. *acting in the capacity of Notary Public, State of California*
4. *bears the seal/stamp of AIDA MICLAT GUIAM, Notary Public, 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:*



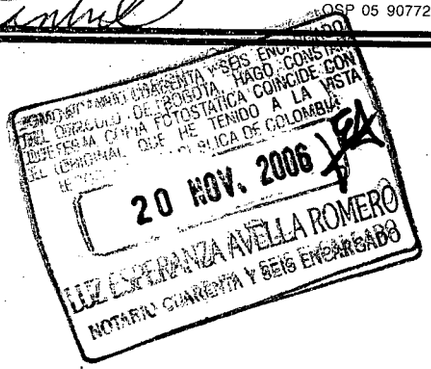
10. *Signature*

Secretary of State

BY

NP-24 A (REV. 4-05)

OSP 05 90772



304

CALIFORNIA ALL-PURPOSE ACKNOWLEDGMENT

State of California

County of San Mateo } ss.

On December 6, 2005 (Date) before me, Aida Miclat Guiam (Name and Title of Officer (e.g., "Jane Doe, Notary Public")),

personally appeared Timothy R. Schwartz (Name(s) of Signer(s)),

☐ personally known to me

☒ proved to me on the basis of satisfactory evidence

to be the person~~(s)~~ whose name~~(s)~~ is/~~are~~ subscribed to the within instrument and acknowledged to me that he/~~she/they~~ executed the same in his/~~her/their~~ authorized capacity~~(ies)~~, and that by his/~~her/their~~ signature~~(s)~~ on the instrument the person~~(s)~~, or the entity upon behalf of which the person~~(s)~~ acted, executed the instrument.

WITNESS my hand and official seal.

Aida Miclat Guiam
Signature of Notary Public

AIDA MICLAT GUIAM
Commission # 1455662
Notary Public - California
San Mateo County
My Comm. Expires Dec 9, 2007

OPTIONAL

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☐ Corporate Officer — Title(s): _____
☐ Partner — ☐ Limited ☐ General
☐ Attorney-in-Fact
☐ Trustee
☐ Guardian or Conservator
☐ Other: _____

Signer Is Representing: _____

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BOGOTA D.C. REPUBLICA DE COLOMBIA
20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

Brigard & Castro
BOGOTA - COLOMBIA

PODER

Yo (nosotros)

domiciliado(s) en

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 enseñas, 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 y/o domicilio; elaborar, tramitar y registrar contratos de licencia y licencias de cualesquiera clases; intervenir como mandante 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.

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.

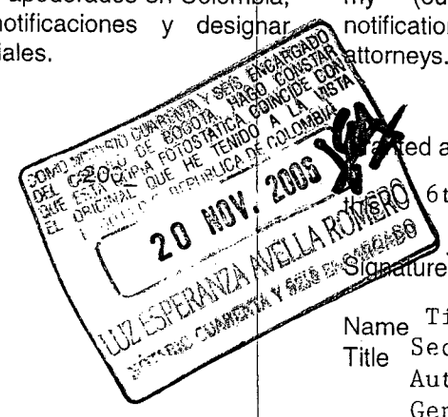
Este poder comprende la facultad de ratificar o confirmar en mi (nuestro) nombre lo actuado así como la facultad de desistir, transigir, comprometer, y la de sustituirlo en todo o en parte y revocar sustituciones. Asimismo nombro(amos) a: **JUAN PABLO CADENA SARMIENTO** y/o **BEATRIZ JARAMILLO** y/o **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.

Otorgado y firmado en

hoy de de

Firma

Nombre
Cargo



POWER OF ATTORNEY

I (We) **Genentech, Inc.**
1 DNA Way
domiciled in **South San Francisco, California 94080-4990**
USA

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 explotation 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.

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 of 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) interest(s) with all the other necessary legal powers.

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.

Executed and signed in **South San Francisco, California USA**

this 6th day of December 2005

Signature

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.



30X

TRADUCCION AL ESPAÑOL del Documento de Poder otorgado por **Genentech, Inc.** domiciliada en **1 DNA Way, South San Francisco, California 94080-4990, Estados Unidos de América**.

ESTADO DE CALIFORNIA
SECRETARIA DE ESTADO

APOSTILLA
(Convención de La Haya del 5 de Octubre de 1961)

1 País: Estados Unidos de América
Esta documento público
2. ha sido firmado por AIDA MICLAT GUIAM
3. actuando en capacidad de Notario Público, Estado de California
4. lleva el sello/timbre de AIDA MICLAT GUIAM, Notario Público
Estado de California

CERTIFICADO

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

NOTARIO CUARENTA Y SEIS ENCARGADO
DEL CIRCULO DE BOGOTA HAGO CONSTAR
QUE ESTA COPIA FOTOSTATICA COINCIDE CON
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BOGOTA D.C. REPUBLICA DE COLOMBIA
20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

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RECONOCIMIENTO TODO-PROPOSITO DE CALIFORNIA

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 evidencia satisfactoria ser la persona cuyo nombre esta suscrito en el instrumento y quien reconoció ante mi que ejecutó el mismo en su capacidad autorizada, y que por su firma en el instrumento la persona o la entidad a nombre de la cual la persona actuó, ejecutó el instrumento.

Hay un sello que dice:
AIDA MICLAT GUIAM
COMISIÓN # 14556612
Notario Público-California
Condado de San Mateo
Mi comisión expira Dic. 9 de 2007

Atestigua mi firma y sello oficial,

AIDA MICLAT GUIAM

----------**OPCIONAL**----------

Aunque la información en esta sección no es requerida por la ley, puede probar ser valiosa para las personas que se basan en el documento y puede prevenir la remoción y readhesión fraudulenta de esta forma a otro documento

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Nombre del firmante: _____

- ☐ Individuo
- ☐ Funcionario Corporativo – Titulo(s): _____
- ☐ Socio - ☐ Limitado ☐ General
- ☐ Abogado de hecho
- ☐ Fideicomisario
- ☐ Guardián o conservador
- ☐ Otro: _____

El firmante representa: _____

Huella Índice derecho del firmante
Parte alta del dedo aquí



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AUTORIZACION

Genentech, Inc. por la presente autoriza a cualquiera de Janet E. Hasak, Mark T. Kresnak, Jeffrey S. Kubinec y Thomity R. Schwartz, mientras permanezcan como empleados de Genentech, Inc., a ejecutar, a su nombre, cualquier solicitud asociada con presentación, procesamiento, consecución, y extensión y mantenimiento de concesión de solicitudes de patente y marca registrada en los Estados Unidos y países extranjeros, contratos relacionados con depósitos biológicos con la Colección Americana de Tipo de Cultivo, y cesiones a publicistas de derechos de autor que cubran los trabajos de autoría de los empleados de Genentech.

27 de Agosto de 2002
Fechado

(Firma)
Sean A. Johnston
Vicepresidente, Propiedad Intelectual
Genentech, Inc.



17.000
1/6

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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. 0254030

9. Seal/Stamp:

10. Signature:

Harriet Smith Windsor

Secretary of State

DECANO NOTARIA CUARENTA Y SEIS ENCARGADO
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NOTARIO CUARENTA Y SEIS ENCARGADO

Delaware

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: 3900384

DATE: 05-24-05

COMO NOTARIO CUARENTA Y SEIS DEL CIRCULO DE BOGOTA D.C. REPUBLICA DE COLOMBIA CERTIFICO QUE ESTA COPIA FOTOSTATICA CONCUERDA CON EL ORIGINAL QUE HE TENIDO A LA VISTA

20 NOV. 2005

LUZ ESPERANZA AVELLA ROMERO

NOTARIO CUARENTA Y SEIS ENCARGADO

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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**").

STATE OF DELAWARE
SECRETARY OF STATE
DIVISION OF CORPORATIONS
FILED 09:00 AM 07/22/1999
991302331 - 2112549



ARTICLE 4

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 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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20 NOV. 2006
LUZ ESPERANZA MEJIA CARDENAS
NOTARIO CUARENTA Y SEIS (E) DEL CIRCULO

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.

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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, as 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

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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

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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 recission 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, recission, 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



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.

DECIMO NOTARIO CUARENTA Y SEIS DEL CIRCULO DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA CONCUERDA CON EL ORIGINAL QUE HE TENIDO A LA VISTA
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NOTARIO CUARENTA Y SEIS ENCARGADO

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 the

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NOTARIA CUARENTA Y SEIS ENCARGADO

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 of 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.



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.

COMO NOTARIO CUARENTA Y SEIS (46) DEL CIRCULO DE BOGOTA D.C. HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA CONCUERDA CON LA VISTA EL ORIGINAL QUE HE TENIDO A LA REPRODUCIDA DE
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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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TRADUCCION AL ESPAÑOL del Certificado de Incorporación de Genentech, Inc.

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. 0254030
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado



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



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TRADUCCION AL ESPAÑOL del Certificado de Incorporación de Genentech, Inc.

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. 0254030
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 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

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 del 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) *Series 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 acto 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 se 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



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 serien caso de [illegible] liquidación, disolución o cierre voluntaria o involuntaria de la Corporación

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los derechos relativos de prioridad, de haber, de pago de acciones de esa serie; y

(viii) cualquier otro derecho relativo, preferencia y limitación de tal serie.

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 le 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, repelerse, 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 Acciones Preferencial.* Durante cualquier periodo cuando los accionistas de cualquier

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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, o esta 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

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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.



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ARTICULO 8

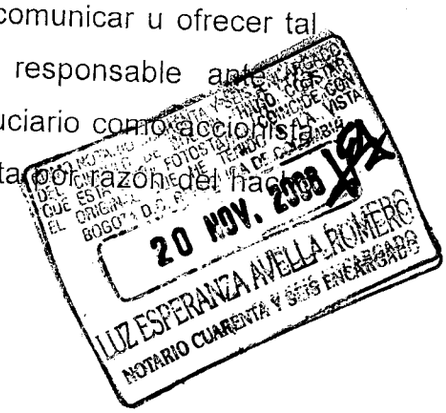
SECCION 8.01. *Relación con Roche.* Anticipándose a que la Corporación cese de ser un subsidiaria completamente de propiedad de Roche Holdings, Inc., pero que Roche (como se define en la sección 8.05) permanezca siendo un accionista de la Corporación, y anticipándose a que la Corporación (como se define 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 artículos 8 se establecen para regular, definir y guiar la conducta de ciertas cuestiones de la Corporación ya que pueden involucrar a Roche y sus funcionaros 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 (directa o indirectamente) cincuenta por ciento o más de las acciones con voto, 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 y definida de acuerdo con la cláusula (i) de esta sección 8.05) que son ***"afiliados"*** (como se

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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 cualquiera de tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo) estará gobernado por las condiciones de este Cerificado 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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LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

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.

NOTARIO CUARENTA Y SEIS (46) DEL CIRCULO DE BOGOTA D.C. HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA COINCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA BOGOTA D.C. REPUBLICA DE COLOMBIA
20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

338

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



97.200

2/

334

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

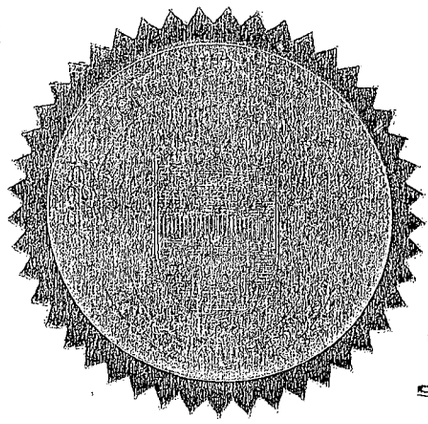
5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

8. No. 0254031

9. Seal/Stamp:



10. Signature:

Harriet Smith Windsor

Secretary of State



Delaware

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.

2112549

050426506

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

AUTHENTICATION: 3900385

DATE: 05-24-05



STATE OF DELAWARE
SECRETARY OF STATE
DIVISION OF CORPORATIONS
FILED 05:00 PM 05/15/2000
001245261 – 2212549

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

75841



342

TRADUCCION AL ESPAÑOL del Certificado de Incorporación de Genentech, Inc.

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



345

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
AUTENTICACION: 2[illegible]0385
FECHA: 05-24-05

20 NOV. 2005
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

744

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 enmiende 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.



346

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

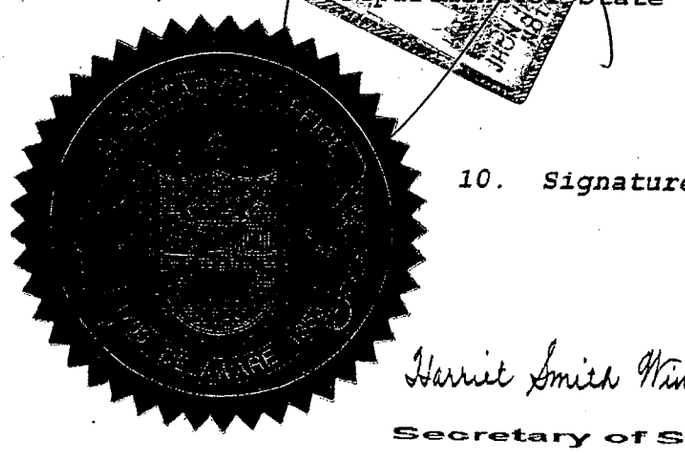
5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

8. No. 0254033

9. Seal/Stamp:



10. Signature:

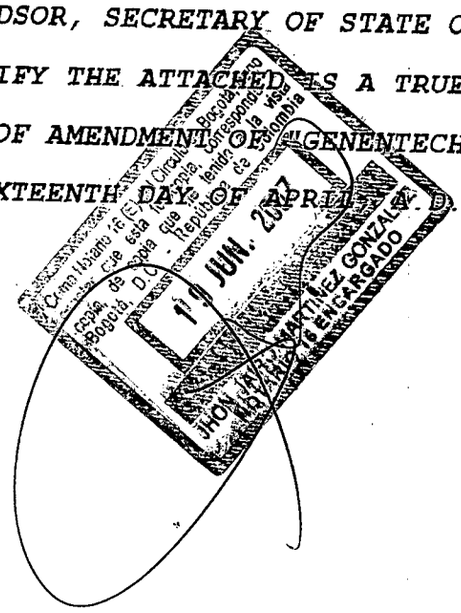
Harriet Smith Windsor

Secretary of State

Delaware

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

State of Delaware
Secretary of State
Division of Corporations
Delivered 07:44 PM 04/16/2004
FILED 07:39 PM 04/16/2004
SRV 040281669 - 2112549 FILE

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: _____
Name: Stephen G. Juelsgaard
Title: Executive Vice President, General Counsel and Secretary

#153087

344

TRÁDUCCION AL ESPAÑOL del Certificado de Enmienda de Genentech, Inc.

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

Como Notario 16 (E) del Círculo de Bogotá, hago constar que la presente copia corresponde a la vista de copia que me fue presentada. Bogotá, D.C., 10 JUN. 2005

Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado

310

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 DECIMOSEXTO DIA DE ABRIL, A. D. 2004, A LAS 7:39 EN PUNTO DE LA TARDE.



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 enmiende 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.

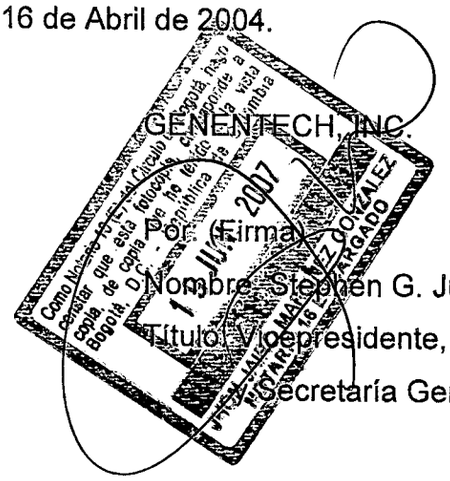
SEGUNDO: Que por lo tanto, de acuerdo con la resolución de su Junta Directiva, la reunión ordinaria de accionistas de dicha corporación se convocó debidamente

312

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.

TA 205
4-6y

317

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

COMO NOTARIO CUARENTA Y SEIS ENCARGADO DEL CIRCULO DE BOGOTA, PAGO CONSTAR QUE ESTA COPIA FOTOSTATICA CONCUERDA CON EL ORIGINAL QUE HE TENIDO A LA VISTA
BOGOTA D.C. REPUBLICA DE COLOMBIA
20 NOV. 2005
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

Delaware

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 Smith Windsor

Harriet Smith Windsor, Secretary of State

AUTHENTICATION: 3900386

DATE: 05-24-05

COMO NOTARIO CUARENTA Y SEIS ENCARGADO DEL CIRCULO DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA CONCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA BOGOTA D.C. REPUBLICA DE COLOMBIA

20 NOV. 2006

LUZ ESPERANZA AVELLA ROMERO

NOTARIO CUARENTA Y SEIS ENCARGADO

STATE OF DELAWARE
SECRETARY OF STATE
DIVISION OF CORPORATIONS
FILED 02:05 PM 05/10/2001
010226224 – 2112549

S ////STATE/OF/DELAWARE///// V
T ///SECRETARY/OF/STATE///// O
A DIVISION/OF/CORPORATIONS// I
M FILED/02/00/PM/05/10/2001/ D
P ///010226224/+/2112549////
BY Sandy Miller

CERTIFICATE OF AMENDMENT OF AMENDED AND RESTATED CERTIFICATE OF INCORPORATION

335

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

86747

COMO NOTARIO CUARENTA Y SEIS (46) DEL CIRCULO DE BOGOTA, PARA LOS FINES QUE ESTA COPIA FOTOSTATICA CORRESPONDE AL ORIGINAL QUE HE TENIDO A LA VISTA EN BOGOTA D.C. REPUBLICA D
20 NOV. 2009
LUZ ESPERANZA AVELLANEDA O
NOTARIO CUARENTA Y SEIS ENCARGADO

TRADUCCION AL ESPAÑOL del Certificado de Enmienda de Genentech, Inc.

354

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

357

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: 05-240-05

JUEZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

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 enmiende 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 $0.02 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 convoco 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.

LUZ ESPERANZA AVELLA ROLDÁN
NOTARIO CUARENTA Y SEIS ENCARGADO
23 NOV. 2005

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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.



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
No. 07-080736- -00000-0000
Fecha: 2007-08-08 16:14:23 Dep: 2020 NUECREACIONI
Tra: 11 PATENTEIYII Eve: 378 FASENACIONALI
Act 411 PRESENTACION Folios: 359

Industria y Comercio
SUPERINTENDENCIA

REQUISITOS MINIMOS PARA ADMISION A TRAMITE PCT

PATENTE DE INVENCION [X] MODELO DE UTILIDAD [ ]

- Indicación de que se solicita la concesión de una patente [X]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [X]
- Descripción de la invención [X]
- Dibujos de ser estos pertinentes [X]
- Comprobante de pago de las tasas establecidas [X]

COMPLETA [ ] INCOMPLETA [ ]

DISEÑO INDUSTRIAL [ ]

- Indicación de que se solicita el registro de Diseño Industrial [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [ ]
- Representación gráfica y fotográfica del Diseño Industrial o muestra del material que incorpora el diseño [ ]
- Comprobante de pago de las tasas establecidas [ ]

COMPLETA [ ] INCOMPLETA [ ]

ESQUEMA DE TRAZADO [ ]

- Indicación de que se solicita el registro de un Esquema de Trazado [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [ ]
- Representación gráfica del esquema trazado [ ]
- Comprobante de pago de las tasas establecidas [ ]

COMPLETA [ ] INCOMPLETA [ ]

Firma Responsable

Fecha 08 AGO. 2007

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E-mail: info@sic.gov.co
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Bogotá, D.C., Colombia

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Folio 361

2020
Bogotá D.C.,

Doctor(a)
CADENA SARMIENTO JUAN PABLO
Apoderado(a) y/o Representante de
GENENTECH INC.

REFERENCIA	Radicación No.	7 80736
	Solicitud internacional	PCT/US2006/004926
	Fecha inicio fase nacional	09/09/07
	Trámite	11
	Evento	378
	Actuación	416
	Oficio No.	3986
	Folios	1

Teniendo en cuenta que la solicitud de privilegio de patente que se tramita bajo el expediente indicado en la referencia, reúne los requisitos de forma establecidos en la Decisión 486 de la Comisión de la Comunidad Andina, el Tratado de Cooperación en Materia de Patentes (PCT), el Reglamento y la Circular Única de la Superintendencia de Industria y Comercio, se envía el extracto de la solicitud a la Oficina de Comunicaciones para efecto de su publicación en la Gaceta de Propiedad Industrial.

Cúmplase
Dado en Bogotá DC, 02 OCT. 2007

ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la división de Nuevas Creaciones

jfernand