Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 14-083128- -00003-0000

Fecha: 2016-04-13 14:13:00 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 400 OPOSIOBSERVTER Folios: 37

DIRECCIÓN DE NUEVAS CREACIONES
PRESENTACIÓN DE OPOSICIÓN

GL

1. Oposición a solicitud de:

X Patente de invención
☐ Registro de Diseño Industrial
☐ Patente de Modelo de Utilidad
☐ Esquema de Trazado de Circuitos Integrados

Número de expediente: **14-083128**

Solicitante: **GENENTECH, INC.**

Apoderado: **JUAN PABLO CADENA SARMIENTO**

Título de la Nueva Creación: **USOS Y ARTICULOS DE FABRICACION QUE INCLUYE EL INHIBIDOR DE LA DIMERIZACIÓN DE HER 2 PERTUZUMAB.**

Gaceta: **749 DEL 15/ENE/2016 PUBLICACION 97**

2. Datos del Opositor

☐ Persona Natural X Persona Jurídica

LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.S.

Nombre o denominación /Razón social completa

TITO NOE PARRA MURILLO
Nombre del representante legal

Documento de identificación: C.C. ☐ C.E ☐ NIT X Otro ☐ Cuál _____

Número **890.301.463-8**

Dirección: **Cra. 1 # 46-84** Ciudad: **Cali - Valle**

Teléfono: **052 6877700** Fax: Correo electrónico tparra@lafrancol.com

☐ **Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico**

3. Datos del representante o apoderado:

PARRA MURILLO TITO NOE	79.579.680	71168 C.S.J.
Apellidos y Nombre	Documento de identificación	Tarjeta profesional

Dirección del representante		
CALLE 108 # 51-45		
Dirección electrónica	No. Fax	Número telefónico
tparra@lafrancol.com	7424092	7422525

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo d-e poder general

TOTAL F. 37

4. Fundamentos de la oposición

Causal: **VER ANEXO**

Justificación: **VER ANEXO**

Prórroga: **SI** X **NO** ☐

5. Anexos

Comprobante de pago de la tasa para la presentación de la oposición No. 16-002496 de 14 MAR 16
Poder, si fuere el caso con el que se acredita la representación .
☐ Anexo indicativo de las clases contra las cuales presenta oposición
☐ Pruebas como sustento de la oposición
Copias para el traslado de la oposición. Se anexan **9** folios (Obligatorio indicar cuántos)
☐ Comprobante de pago de tasa por solicitud de prórroga
Comprobante de pago por invocación de notoriedad
Copia de la solicitud y sus anexos en medio magnético.

6. Firma	
TITO NOE PARRA MURILLO	
Nombre del Firmante	Firma
79.579.680 DE BOGOTA	**71168 C.S.J.**
C.C	Tarjeta Profesional

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
NIT : 800.176.089-2
- / -

Industria y Comercio
SUPERINTENDENCIA

RECIBO DE CAJA

No. 16 - 0024962

Bogotá D.C., Marzo 14 de 2016 - 09:05:04

RECIBIDO DE : LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.S. - LAFRANCOL S.A. NI 890.301.463 RE

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR.PAGO
RECIBO DE CA	SIC ** RECIBO **	999999999	124083	17/11/2015	345.000.00
RECIBO DE CA	SIC ** RECIBO **	999999999	124084	17/11/2015	345.000.00
RECIBO DE CA	SIC ** RECIBO **	999999999	124085	17/11/2015	345.000.00
RECIBO DE CA	SIC ** RECIBO **	999999999	124086	17/11/2015	345.000.00
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RECIBO DE CA	SIC ** RECIBO **	999999999	124088	17/11/2015	345.000.00
RECIBO DE CA	SIC ** RECIBO **	999999999	124089	17/11/2015	345.000.00
RECIBO DE CA	SIC ** RECIBO **	999999999	124090	17/11/2015	345.000.00
RECIBO DE CA	SIC ** RECIBO **	999999999	124091	17/11/2015	345.000.00
RECIBO DE CA	SIC ** RECIBO **	999999999	137781	21/12/2015	6.000.00

*** Conceptos Pagados ***

CANT.	RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
1	50005-01-04 PRESENTACION DE OBSERVACIONES A SOLICITUDES	1632 OPOSICION NUEVA CRACION / PRESENTACION	430.000.00	430.000.00
				$430.000.00

SON: **CUATROCIENTOS TREINTA MIL PESOS MONEDA CORRIENTE***

Responsable: _____
-***-

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
No. 14-083128- -00003-0000
Fecha: 2016-04-13 14:13:00 Dep: 2020 DIR.NUEVASCR
Tra: 11 PATENTEIYII Eve: 379 FASENACIONALII
Act: 400 OPOSIOBSERVTER Folios: 37

3

Señor
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
E.S.D.

REF:	**Expediente: 14-083128**
TÍTULO SOLICITADO:	**USOS Y ARTÍCULO DE FABRICACIÓN QUE INCLUYE EL INHIBIDOR DE LA DIMERIZACIÓN DE HER 2 PERTUZUMAB**
PUBLICACIÓN:	**GACETA 749 del 15 de enero de 2016, Nº de publicación 97.**
SOLICITANTE:	**GENENTECH, INC.**
APODERADO:	**JUAN PABLO CADENA SARMIENTO**
TRÁMITE:	**PRESENTACIÓN DE OPOSICIÓN**

TITO NOÉ PARRA MURILLO, mayor de edad y vecino de Bogotá, identificado como aparece al pie de mi firma, abogado en ejercicio, titular de la tarjeta profesional número 71168 del Consejo Superior de la Judicatura, obrando en calidad de apoderado de la sociedad **LABORATORIO FRANCO COLOMBIANO S.A.S. LAFRANCOL S.A.S.,** sociedad constituida y vigente de conformidad con las leyes colombianas, con domicilio en Cali, Valle, en los términos y para los efectos del poder y del certificado de existencia y representación legal adjuntos, y estando dentro del plazo establecido por el artículo 42 de la Decisión 486 de 2000, muy respetuosamente me permito presentar oposición contra la solicitud de patente de invención indicada en el epígrafe.

I. PETICIÓN

Sírvase, señor Superintendente, proceder a negar el beneficio patentario a la solicitud contenida en el Expediente 14-083128, por incumplir los requisitos materiales de patentabilidad establecidos en el Título II, Capítulo I de la Decisión 486 de 2000 de la Comisión de la Comunidad Andina. Lo anterior con base en los fundamentos que se expondrán a continuación.

II. LEGÍTIMO INTERÉS

Asiste a **LABORATORIO FRANCO COLOMBIANO S.A.S. LAFRANCOL S.A.S.,** legítimo interés para presentar esta oposición, pues la eventual concesión de la solicitud restringiría, en forma contraria al Régimen Común Andino de Propiedad Industrial, el empleo de ciertas sustancias y composiciones farmacéuticas, en detrimento de sus legítimos intereses comerciales y de la libre competencia.

III. FUNDAMENTOS TÉCNICOS DE LA OPOSICIÓN[1]

La solicitud de la referencia incumple los requisitos materiales de patentabilidad establecidos en el Titulo II, Capítulo I de la Decisión 486 de 2000 de la Comisión de la Comunidad Andina, estos son: Novedad, Nivel Inventivo y Aplicación Industrial. Vale recordar que por Novedad se entiende aquella invención que no se encuentra comprendida dentro del estado de la técnica, siendo ésta última la que comprende todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de la patente. Con respecto al Nivel Inventivo, cabe señalar que dicho requisito se cumple cuando a una persona del oficio normalmente versada en la materia técnica correspondiente, no se le hubiese resultado dicha invención obvia o derivada

[1] Informe Técnico elaborado por Q.F. Natalia Soto T., Departamento de Propiedad Industrial – CFR

de manera evidente del estado de la técnica. Por último, se entiende que una aplicación es susceptible de aplicación industrial cuando su objeto puede ser producido o utilizado en cualquier tipo de industria, entendiéndose por industria la referida a cualquier actividad productiva.

a. **Antecedentes Generales de la Solicitud de Patente**

La solicitud de patente relativa al **Expediente Nº 14-083128**, señala la siguiente prioridad: US201161547535P del **14/10/2011**.

Esta solicitud de patente pretende proteger en Colombia, un **método para extender la supervivencia libre de progresión (PFS) en una población de pacientes con cáncer de mama HER2 positivo** en 6 meses o más que comprende administrar **pertuzumab, trastuzumab y quimioterapia** a los pacientes de la población. La quimioterapia comprende taxano, y dicho taxano es **docetaxel**.

La solicitud también reivindica un **método para el tratamiento del cáncer de mama HER2 positivo de etapa temprana** que comprende administrar **pertuzumab, trastuzumab y quimioterapia** a un paciente con cáncer de mama, donde la quimioterapia comprende quimioterapia basada en antraciclina o quimioterapia basada en carboplatino.

Se desea proteger también una **bolsa intravenosa (IV) que contiene una mezcla estable de pertuzumab y trastuzumab** adecuada para la administración a un paciente con cáncer. Dicha bolsa IV contiene una mezcla de alrededor de **420 mg o de alrededor de 840 mg de pertuzumab** y de alrededor de **200 mg a alrededor de 1000 mg de trastuzumab**.

Adicionalmente, la solicitud reivindica un **método para tratar el cáncer gástrico HER2 positivo** en un sujeto humano, que comprende administrarle **pertuzumab, trastuzumab y quimioterapia** al sujeto con cáncer gástrico HER2 positivo. **Pertuzumab se administra a una dosis de 840 mg en todos**

los ciclos de tratamiento. Dicho método también **mejora la supervivencia global (OS)** con respecto a un paciente tratado con trastuzumab y la quimioterapia solamente, o **mejora la supervivencia sin evolución (PFS)** o la tasa de respuesta (RR) con respecto al tratamiento con trastuzumab y quimioterapia solamente.

Finalmente, la solicitud pretende proteger un **método para tratar el cáncer de mama recurrente a nivel local o metastásico HER2 positivo** en un paciente, que comprende administrarle **pertuzumab, trastuzumab y taxoide** al paciente, donde **el paciente se ha tratado previamente con trastuzumab y/o lapatinib como tratamiento adyuvante o neoadyuvante.** Se indica que dicho taxoide es **docetaxel**, paclitaxel o nab-paclitaxel.

Todo lo publicado antes del **14/10/2011** es parte del estado de la técnica.

b. **Antecedentes que respaldan nuestra oposición a la Solicitud de patente relativa al Expediente N° 14-083128.**

1. US 2008/102069 del 01/05/2008.

"Tumor therapy with a combination of anti-HER2 antibodies".

Prioridad: EP20060019317 del **15/09/2006.**

Esta solicitud de patente, que es parte del estado de la técnica, pretende proteger un **método para tratar metástasis o cáncer HER2 positivo** en pacientes que no responden a la monoterapia con trastuzumab o pertuzumab, o a sucesivas monoterapias con trastuzumab y pertuzumab, que comprende **coadministrar a dicho paciente una cantidad terapéuticamente eficaz de trastuzumab y pertuzumab simultánea o secuencialmente**.

Dicha coadministración de trastuzumab y pertuzumab comprende la administración de una dosis inicial de trastuzumab o pertuzumab en una cantidad de aproximadamente 1µg/kg a aproximadamente 50 mg/kg.

Se señala que dicho cáncer HER2 positivo se selecciona de: **cáncer de mama**, cáncer de pulmón, cáncer de colon, y cáncer de próstata. Más específicamente se indica que **el cáncer HER2 positivo es cáncer de mama**.

Se desea proteger también un **kit que comprende trastuzumab y pertuzumab** y un paquete de inserto instruyendo al usuario a coadministrar trastuzumab y pertuzumab a un paciente que sufre de cáncer HER2 positivo que no responde a la monoterapia con trastuzumab o pertuzumab.

Este antecedente **le resta nivel inventivo** a la solicitud con **Expediente Nº 14-083128.**

2. WO 2009/154651 del 23/12/2009.

"Treatment of metastic breast cancer by a combination of two anti-her2 antibodies and a taxane".

Prioridad: US20080061962P del **16/06/2008.**

Esta solicitud de patente, que es parte del estado de la técnica, pretende proteger un **método de tratamiento de cáncer de mama**, que comprende administrar a un paciente con **cáncer de mama HER2 positivo** una cantidad eficaz de **un anticuerpo inhibidor del crecimiento de HER2, un anticuerpo inhibidor de la dimerización de HER2, y un taxano**, en donde el paciente no recibió quimioterapia o terapia biológica previa.

El anticuerpo inhibidor del crecimiento de HER2 es trastuzumab, o un fragmento del mismo, **el anticuerpo inhibidor de la dimerización de HER2 es pertuzumab**, o un fragmento del mismo, y **el taxano es docetaxel**.

Se señala que dichos anticuerpos monoclonales y dicho taxano **se administran simultánea o consecutivamente**, en cualquier orden. Y que la administración de **pertuzumab, trastuzumab y docetaxel resulta en un efecto sinérgico.**

La administración de pertuzumab, trastuzumab y docetaxel **extiende la supervivencia del paciente** humano relativo al tratamiento en ausencia de al menos uno de pertuzumab, trastuzumab y docetaxel. Específicamente, **extiende la supervivencia libre de progresión (PFS).**

Este antecedente **le resta nivel inventivo** a la solicitud con **Expediente Nº 14-083128.**

3. **Portera C., Walshe J, Rosing D, *et al.*:** "Cardiac Toxicity and Efficacy of Trastuzumab Combined with Pertuzumab in Patients with Trastuzumab-Insensitive Human Epidermal Growth Factor Receptor 2- Positive Metastatic Breast Cancer", Clinical Cancer Research 2008, vol. 14, N°9, pages 2710-2716.

Esta publicación, que es parte del estado de la técnica, señala que el anticuerpo monoclonal humanizado **trasatuzumab (Herceptin, Genentech Inc.) tiene como blanco el dominio extracelular del receptor 2 del factor de crecimiento epidérmico humano (HER2).** El trastuzumab en combinación con la quimioterapia mejora la supervivencia en un 50% en pacientes con cáncer de mama temprano metastásico HER2+. **Aunque algunos pacientes con cáncer de mama metastásico HER2+ se benefician del tratamiento inicial con trastuzumab solo, la progresión del tumor se produce con el tiempo**. La resistencia a trastuzumab es un problema difícil en el manejo del cáncer de mama HER2+.

Se indica además que trastuzumab se une al dominio IV extracellular de HER2 pero no bloquea significativamente la formación de heterodímeros entre HER2 y HER3. Se ha demostrado que los ligandos del receptor HER pueden superar la detención del ciclo celular en G1 inducida por trastuzumab y la inhibición de la fosforilación de tirosina de los receptores HER. **Estos resultados sugieren que trastuzumab por sí solo no es suficiente para suprimir la señalización de HER2** debido a la naturaleza compleja de las interacciones receptor-ligando

de la familia HER. **Varios estudios preclínicos han demostrado que la combinación de anticuerpos de varios epítopos de HER2 fue más efectiva que los anticuerpos individuales en la inducción de la citotoxicidad dependiente de anticuerpos, en la aceleración de receptor baja regulación, interfiriendo la señalización del receptor de la familia HER, e inhibiendo el crecimiento de células tumorales.**

Pertuzumab (Genentech), un anticuerpo monoclonal humanizado contra una región del dominio II extracelular de HER2, bloquea la heterodimerización de HER2 y la señalización activada por ligando de los heterodímeros HER2/HER1 y HER2/HER3.

Se realizó un estudio clínico para evaluar la toxicidad y la eficacia de trastuzumab más pertuzumab en el tratamiento de pacientes con cáncer de mama metastásico HER2+ que progresaron a través de terapias a base de trastuzumab. Basándose en los resultados de los estudios preclínicos, se propuso como hipótesis que **la combinación de los dos anticuerpos anti-HER2 sería más eficiente en el control de crecimiento del tumor y en la superación de la resistencia a trastuzumab.** Nos enfocamos en la evaluación cardíaca para garantizar la seguridad de los pacientes que toman este tratamiento combinado.

El diseño experimental consistió en que los **pacientes con cáncer de mama metastásico HER2+ medible,** regímenes basados en trastuzumab V3, y fracción de eyección ventricular izquierda (FEVI) z55% **recibieron 8 o 6 mg/kg de trastuzumab y 840 mg de pertuzumab por vía intravenosa seguido de 6 mg/kg de trastuzumab y 420 mg de pertuzumab cada 3 semanas.** Se evaluó la función cardíaca y la respuesta del tumor cada 3 y 6 semanas, respectivamente.

Este antecedente **le resta nivel inventivo** a la solicitud con **Expediente Nº 14-083128.**

4. **Yamashita-Kashima Y, Iijima S, Yorozu K, *et al.*:** "Pertuzumab in combination with trastuzumab shows significantly enhanced antitumor activity in HER2-positive human gastric cancer xenograft models." Clinical Cancer Research, 2011Aug 1, vol.17, N°15, pages 5060-5070.

Esta publicación, que es parte del estado de la técnica, investiga la **actividad antitumoral de la combinación de dos anticuerpos monoclonales humanizados del factor de crecimiento epidérmico humano (HER2) diferentes, pertuzumab y trastuzumab, para el cáncer gástrico.**

La publicación plantea que **pertuzumab y trastuzumab en combinación pueden proporcionar una actividad antitumoral más eficaz que cualquiera de los agentes solos para los tumores HER2 positivos, incluyendo el cáncer gástrico,** debido a sus diferentes mecanismos de inhibición de la señal HER2. Esta terapia combinada podría proporcionar beneficios clínicos para los pacientes con cáncer gástrico HER2-positivo.

Se señala que en el presente estudio, **se investigó la eficacia antitumoral de pertuzumab en combinación con trastuzumab**, así como su mecanismo de acción como una **nueva terapia para el cáncer gástrico** usando modelos modelos de xenoinjertos de ratón que sobreexpresan **cáncer gástrico humano HER 2.**

Los resultados demostraron que **pertuzumab en combinación con trastuzumab presenta una significativa actividad antitumoral comparada con cada monoterapia** en NCI-N87, un modelo de xenoinjerto de cáncer gástrico humano HER2 positivo. La eficacia fue más fuerte que la de la dosis máxima eficaz con cada monoterapia.

Finalmente la publicación concluye que, **comparado con el tratamiento con un solo fármaco, pertuzumab en combinación con trastuzumab mostró una actividad antitumoral significativamente más fuerte** en modelos de xenoinjerto de ratón de **cáncer gástrico humano HER2 positivos** a través de

la mejora de la inhibición directa del crecimiento celular, de la citotoxicidad celular dependiente de anticuerpo (ADCC) y de la actividad anti-angiogénesis. **La terapia combinada de pertuzumab y trastuzumab conviene examinarla como una nueva terapia para el cáncer gástrico HER2-positivo.**

Este antecedente **le resta nivel inventivo** a la solicitud con **Expediente Nº 14-083128.**

c. **Conclusiones**

Por todos los antecedentes expuestos (se adjunta una copia de todos los documentos citados en este informe), es evidente que **la solicitud del Expediente N° 14-083128 carece de nivel inventivo**, considerando que los usos y artículo de fabricación que incluye el inhibidor de la dimerización de HER 2 pertuzumab, **se desprenden del arte previo.**

Siendo, los usos y artículo de fabricación que incluye el inhibidor de la dimerización de HER 2 pertuzumab, según lo indicado en la solicitud relativa al Expediente Nº 14-083128, algo que se desprende del estado del arte, se reafirma el fundamento de la carencia de nivel inventivo, pues **el objeto de la invención y los elementos que la componen son conocidos y forman parte del arte previo.**

Por consiguiente, podemos afirmar en forma categórica, que esta solicitud de patente **debe ser denegada**, debido a que **no presenta nivel inventivo** para gozar de un privilegio industrial en Colombia, ya que los usos y artículo de fabricación que incluye el inhibidor de la dimerización de HER 2 pertuzumab, según lo indicado en esta solicitud de patente, **se desprenden del estado de la técnica.**

IV. FUNDAMENTOS DE DERECHO

Son fundamentos de derecho los artículos 16, 18 y 20 de la Decisión 486 de 2000.

V. ANEXOS

Para efectos del trámite correspondiente, me permito anexar al presente escrito los siguientes anexos:

1. Poder para actuar.
2. Certificado de existencia y representación legal de LAFRANCOL S.A.S.
3. Constancia de pago de las tasas de tramitación de oposición.
4. Copia para correr traslado al solicitante.
5. Los antecedentes mencionados en los fundamentos técnicos.

VI. NOTIFICACIONES

Mi representada y el suscrito las recibiremos en la Secretaría de su Despacho, o en la sede de LAFRANCOL ubicada en la Calle 108 No. 51-45 de Bogotá D.C.

Señor Superintendente,

Atentamente,

TITO NOÉ PARRA MURILLO
C.C. 79.579.680 DE BOGOTÁ.
T.P. 71168 C.S.J.

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

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International Bureau

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A61K 45/06 (2006.01)

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PCT/US2012/059683

(22) International Filing Date:
11 October 2012 (11.10.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

61/547,535	14 October 2011 (14.10.2011)	US
61/567,015	5 December 2011 (05.12.2011)	US
61/657,669	8 June 2012 (08.06.2012)	US
61/682,037	10 August 2012 (10.08.2012)	US
61/694,584	29 August 2012 (29.08.2012)	US

(71) Applicants: **GENENTECH, INC.** [US/US]; 1 DNA Way, South San Francisco, California 94080 (US). **F. HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacherstrasse 124, CH-4070 Basel (CH). **ROCHE PRODUCTS LIMITED** [GB/GB]; 6 Falcon Way, Shire Park Hexagon Place, Welwyn Garden City Hertfordshire AL7 1TW (GB).

(72) Inventors; and

(71) Applicants : **ALAVATTAM, Sreedhara** [US/US]; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US). **AMLER, Lukas C.** [DE/US]; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US). **BENYUNES, Mark C.** [US/US]; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US). **CLARK, Emma L.** [GB/GB]; c/o Roche Products Limited, 6 Falcon Way, Shire Park Hexagon Place, Welwyn Garden City Hertfordshire AL7 1TW (GB). **DE TOLEDO PELIZON, Christina H.** [BR/CA]; c/o F. Hoffmann-La Roche AG, Grenzacherstrasse 124, CH-4070 Basel (CH). **KWONG GLOVER, Zephania W.** [US/US]; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US). **MITCHELL, Lada** [GB/CH]; c/o F. Hoffmann-La Roche AG, Grenzacherstrasse 124, CH-4070 Basel (CH). **RATNAYAKE, Jayantha** [GB/GB]; c/o Roche Products Limited, 6 Falcon Way, Shire Park Hexagon Place, Welwyn Garden City Hertfordshire AL7 1TW (GB). **ROSS, Graham A.** [GB/GB]; c/o Roche Products Limited, 6 Falcon Way, Shire Park Hexagon Place, Welwyn Garden City Hertfordshire AL7 1TW (GB). **WALKER, Ru-Amir** [US/US]; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US).

(74) Agents: **LEE, Wendy M.** et al.; Genentech, Inc., 1 DNA Way, MS 49, South San Francisco, California 94080 (US).

[Continued on next page]

(54) Title: USES FOR AND ARTICLE OF MANUFACTURE INCLUDING HER2 DIMERIZATION INHIBITOR PERTUZUMAB

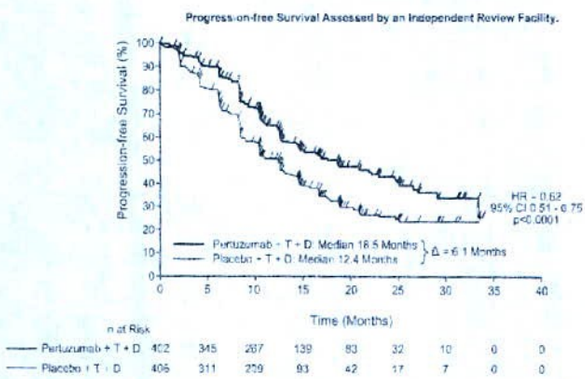


FIG. 8

(57) Abstract: The present application describes uses for and articles of manufacture including Pertuzumab, a first-in-class HER2 dimerization inhibitor. In particular, the application describes methods for extending progression free survival in a HER2-positive breast cancer patient population; combining two HER2 antibodies to treat HER2-positive cancer without increasing cardiac toxicity; treating early-stage HER2-positive breast cancer; treating HER2-positive cancer by co-administering a mixture of Pertuzumab and Trastuzumab from the same intravenous bag; treating HER2-positive metastatic gastric cancer; treating HER2-positive breast cancer with Pertuzumab, Trastuzumab and Vinorelbine; treating HER2-positive breast cancer with Pertuzumab, Trastuzumab and aromatase inhibitor; and treating low HER3 ovarian, primary peritoneal, or fallopian tube cancer. It also describes an article of manufacture comprising a vial with Pertuzumab therein and a package insert providing safety and/or efficacy data thereon; a method of making the article of manufacture; and a method of ensuring safe and effective use of Pertuzumab related thereto. In addition the application describes an intravenous (IV) bag containing a stable mixture of Pertuzumab and Trastuzumab suitable for administration to a cancer patient.

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(54) **Title**: TREATMENT OF METASTIC BREAST CANCER BY A COMBINATION OF TWO ANTI-HER2 ANTIBODIES AND A TAXENE

(57) **Abstract**: The present invention concerns treatment of previously untreated HER2-positive metastatic breast cancer with a combination of a growth inhibitory HER2 antibody, a HER2 dimerization inhibitor antibody and a taxane. In particular, the invention concerns the treatment of HER2-positiv metastatic breast cancer in patients who did not receive prior chemotherapy or biologic therapy with a HER2 antibody binding essentially to epitope 2C4, a HER2 antibody binding essentially to epitope 4D5, and a taxane. The invention further comprises extending survival of such patients by the combination therapy of the present invention. In a preferred embodiment, the treatment involves administration of trastuzumab, pertuzumab and docetaxel.

internal to the patient, the treatment is called brachytherapy (BT). Radioactive atoms for use in the context of this invention can be selected from the group including, but not limited to, radium, cesium-137, iridium-192, americium-241, gold-198, cobalt-57, copper-67, technetium-99, iodine-123, iodine-131, and indium-111. Where the EGFR kinase inhibitor according to this invention is an antibody, it is also possible to label the antibody with such radioactive isotopes. Preferably the trastuzumab and pertuzumab combination treatment is used without such ionizing radiation.

[0087] Radiation therapy is a standard treatment for controlling unresectable or inoperable tumors and/or tumor metastases. Improved results have been seen when radiation therapy has been combined with chemotherapy. Radiation therapy is based on the principle that high-dose radiation delivered to a target area will result in the death of reproductive cells in both tumor and normal tissues. The radiation dosage regimen is generally defined in terms of radiation absorbed dose (Gy), time and fractionation, and must be carefully defined by the oncologist. The amount of radiation a patient receives will depend on various considerations, but the two most important are the location of the tumor in relation to other critical structures or organs of the body, and the extent to which the tumor has spread. A typical course of treatment for a patient undergoing radiation therapy will be a treatment schedule over a 1 to 6 week period, with a total dose of between 10 and 80 Gy administered to the patient in a single daily fraction of about 1.8 to 2.0 Gy, 5 days a week. In a preferred embodiment of this invention there is synergy when tumors in human patients are treated with the combination treatment of the invention and radiation. In other words, the inhibition of tumor growth by means of the agents comprising the combination of the invention is enhanced when combined with radiation, optionally with additional chemotherapeutic or anticancer agents. Parameters of adjuvant radiation therapies are, for example, contained in International Patent Publication WO 99/60023.

[0088] The antibodies are administered to a patient according to known methods, by intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, or intrathecal routes. Intravenous or subcutaneous administration of the antibodies is preferred.

[0089] The invention further comprises a kit comprising trastuzumab and pertuzumab, either in the form of one single or two separate formulations, and a package insert instructing the user to administer trastuzumab and pertuzumab to a patient suffering from HER2 positive cancer, who does not respond to a monotherapy with trastuzumab or a monotherapy with pertuzumab. In an embodiment of the present invention, the patient does not respond to a first-line monotherapy with trastuzumab.

[0090] The term "package insert" refers to instructions customarily included in commercial packages of therapeutic products, which may include information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

[0091] In a preferred embodiment, the aforementioned kit may further include a pharmaceutically-acceptable carrier. The kit may further include a sterile diluent.

[0092] As used herein, a "pharmaceutically acceptable carrier" is intended to include any and all material compatible with pharmaceutical administration including solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and other materials and compounds compatible with pharmaceutical administration. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Pharmaceutical Formulations

[0093] Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody 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). Preferred lyophilized HER2 antibody formulations are described in WO 97/04801.

[0094] The formulations according to the invention may be two separate formulations for each of the antibodies trastuzumab and pertuzumab. Alternatively the formulation herein may also contain both antibodies trastuzumab and pertuzumab in one formulation.

[0095] Additionally, the composition may further comprise a chemotherapeutic agent, cytotoxic agent, cytokine, growth inhibitory agent, anti-hormonal agent, EGFR-targeted drug, anti-angiogenic agent, and/or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

[0096] The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interracial 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).

[0097] 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,

therapy with trastuzumab, or to successive monotherapies with trastuzumab and pertuzumab. Preferably the trastuzumab and pertuzumab combination treatment is used without such additional cytotoxic, chemotherapeutic or anti-cancer agents, or compounds that enhance the effects of such agents.

[0082] Such agents include, for example: alkylating agents or agents with an alkylating action, such as cyclophosphamide (CTX; e.g. cytoxan®), chlorambucil (CHL; e.g. leukeran®), cisplatin (CisP; e.g. platinol®) busulfan (e.g. myleran®), melphalan, carmustine (BCNU), streptozotocin, triethylenemelamine (TEM), mitomycin C, and the like; antimetabolites, such as methotrexate (MTX), etoposide (VP16; e.g. vepesid®), 6-mercaptopurine (6 MP), 6-thioguanine (6TG), cytarabine (Ara-C), 5-fluorouracil (5-FU), capecitabine (e.g. Xeloda®), dacarbazine (DTIC), and the like; antibiotics, such as actinomycin D, doxorubicin (DXR; e.g. adriamycin®), daunorubicin (daunomycin), bleomycin, mithramycin and the like; alkaloids, such as vinca alkaloids such as vincristine (VCR), vinblastine, and the like; and other antitumor agents, such as paclitaxel (e.g. taxol®) and paclitaxel derivatives, the cytostatic agents, glucocorticoids such as dexamethasone (DEX; e.g. decadron®) and corticosteroids such as prednisone, nucleoside enzyme inhibitors such as hydroxyurea, amino acid depleting enzymes such as asparaginase, leucovorin and other folic acid derivatives, and similar, diverse antitumor agents. The following agents may also be used as additional agents: amifostine (e.g. ethyol®), dactinomycin, mechlorethamine (nitrogen mustard), streptozocin, cyclophosphamide, lomustine (CCNU), doxorubicin lipo (e.g. doxil®), gemcitabine (e.g. gemzar®), daunorubicin lipo (e.g. daunoxome®), procarbazine, mitomycin, docetaxel (e.g. taxotere®), aldesleukin, carboplatin, oxaliplatin, cladribine, camptothecin, CPT 11 (irinotecan), 10-hydroxy 7-ethyl-camptothecin (SN38), floxuridine, fludarabine, ifosfamide, idarubicin, mesna, interferon beta, interferon alpha, mitoxantrone, topotecan, leuprolide, megestrol, melphalan, mercaptopurine, plicamycin, mitotane, pegaspargase, pentostatin, pipobroman, plicamycin, tamoxifen, teniposide, testolactone, thioguanine, thiotepa, uracil mustard, vinorelbine, chlorambucil. Preferably the trastuzumab and pertuzumab combination treatment is used without such additional agents.

[0083] In the context of this invention, an anti-hormonal agent may be used in the trastuzumab and pertuzumab combination treatment of HER2 positive cancer or metastasis of HER2 positive cancer in a patient, who does not respond to a monotherapy with trastuzumab or pertuzumab, preferably a first-line monotherapy with trastuzumab, or to successive monotherapies with trastuzumab and pertuzumab. As used herein, the term "anti-hormonal agent" includes natural or synthetic organic or peptidic compounds that act to regulate or inhibit hormone action on tumors. Antihormonal agents include, for example: steroid receptor antagonists, anti-estrogens such as tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, other aromatase inhibitors, 42-hydroxytamoxifen, trioxifene, keoxifene, LY 117018, onapristone, and toremifene (e.g. Fareston®); anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above; agonists and/or antagonists of glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH) and LHRH (luteinizing hormone-releasing hormone); the LHRH agonist goserelin acetate, commercially available as Zoladex® (AstraZeneca); the LHRH antagonist D-alaninamide N-acetyl-3-(2-naphthalenyl)-D-alanyl-4-chloro-D-phenylalanyl-3-(3-pyridinyl)-D-alanyl-L-seryl-N-6-(3-pyridinylcarbonyl)-L-lysyl-N-6-(3-pyridinyl-carbonyl)-D-lysyl-L-leucyl-N-6-(1-methylethyl)-L-lysyl-L-proline (e.g. Antide®, Ares-Serono); the LHRH antagonist ganirelix acetate; the steroidal anti-androgens cyproterone acetate (CPA) and megestrol acetate, commercially available as Megace® (Bristol-Myers Oncology); the nonsteroidal anti-androgen flutamide(2-methyl-N-[4,20-nitro-3-(trifluoromethyl)phenylpropanamide), commercially available as Eulexin® (Schering Corp.); the non-steroidal anti-androgen nilutamide, (5,5-dimethyl-3-[4-nitro-3-(trifluoromethyl-4'-nitrophenyl)-4,4-dimethyl-imidazolidine-dione); and antagonists for other non-permissive receptors, such as antagonists for RAR (retinoic acid receptor), RXR (retinoid X receptor), TR (thyroid receptor), VDR (vitamin-D receptor), and the like. Preferably the trastuzumab and pertuzumab combination treatment is used without such additional antihormonal agent. The use of the cytotoxic and other anticancer agents described above in chemotherapeutic regimens is generally well characterized in the cancer therapy arts, and their use herein falls under the same considerations for monitoring tolerance and effectiveness and for controlling administration routes and dosages, with some adjustments. For example, the actual dosages of the cytotoxic agents may vary depending upon the patient's cultured cell response determined by using histoculture methods. Generally, the dosage will be reduced compared to the amount used in the absence of additional other agents.

[0084] Typical dosages of an effective cytotoxic agent can be in the ranges recommended by the manufacturer, and where indicated by in vitro responses or responses in animal models, can be reduced by up to about one order of magnitude concentration or amount. Thus, the actual dosage will depend upon the judgment of the physician, the condition of the patient, and the effectiveness of the therapeutic method based on the in vitro responsiveness of the primary cultured malignant cells or histocultured tissue sample, or the responses observed in the appropriate animal models.

[0085] In the context of this invention, additional antiproliferative agents may be used in the trastuzumab and pertuzumab combination treatment of HER2 positive cancer or metastasis of HER2 positive cancer in a patient, who does not respond to a monotherapy with trastuzumab or pertuzumab, preferably a first-line monotherapy with trastuzumab, or to successive monotherapies with trastuzumab and pertuzumab, including, for example: Inhibitors of the enzyme farnesyl protein transferase and inhibitors of the receptor tyrosine kinase PDGFR, including the compounds disclosed and claimed in U.S. Pat. Nos. 6,080,769, 6,194,438, 6,258,824, 6,586,447, 6,071,935, 6,495,564, 6,150,377, 6,596,735 and 6,479,513, and International Patent Publication WO 01/40217. Preferably the trastuzumab and pertuzumab combination treatment is used without such additional antiproliferative agents.

[0086] In the context of this invention, an effective amount of ionizing radiation may be carried out and/or a radiopharmaceutical may be used in addition to the trastuzumab and pertuzumab combination treatment of HER2 positive cancer or metastasis of HER2 positive cancer in a patient, who does not respond to a monotherapy with trastuzumab or pertuzumab, preferably a first-line monotherapy with trastuzumab, or to successive monotherapies with trastuzumab and pertuzumab. The source of radiation can be either external or internal to the patient being treated. When the source is external to the patient, the therapy is known as external beam radiation therapy (EBRT). When the source of radiation is

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(54) TUMOR THERAPY WITH A COMBINATION OF ANTI-HER2 ANTIBODIES

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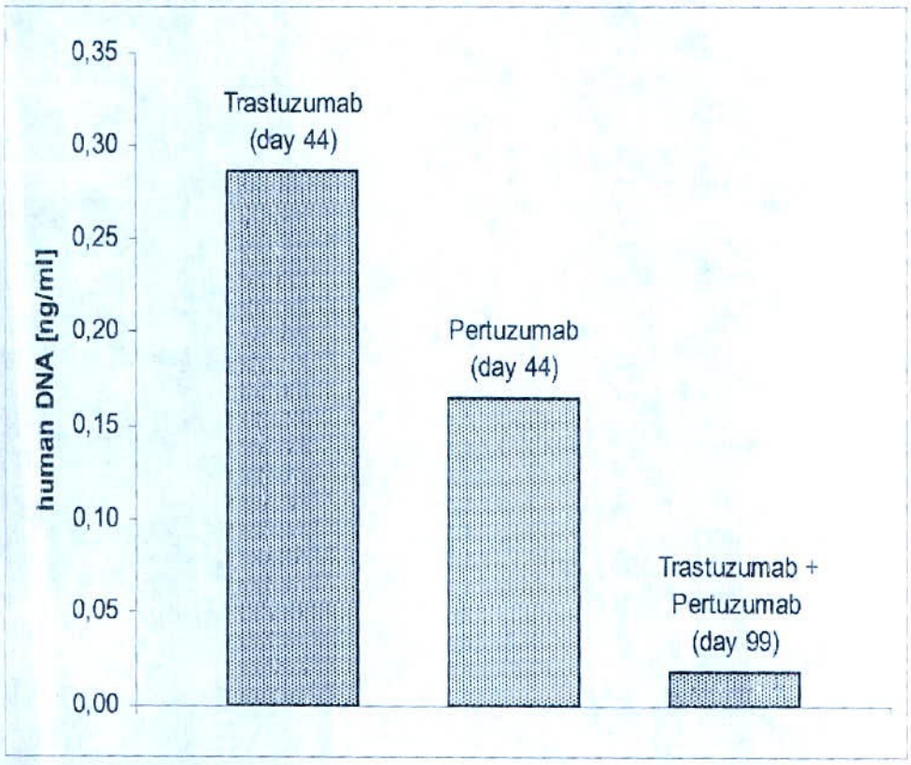
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(57) ABSTRACT

The present invention provides a method for treating HER2 positive cancer or metastasis of HER2 positive cancer in a patient, who does not respond to a monotherapy with trastuzumab or a monotherapy with pertuzumab or to successive monotherapies with trastuzumab and pertuzumab, comprising co-administering to said patient a therapeutically-effective amount of trastuzumab and pertuzumab simultaneously or sequentially. The invention also provides A kit which comprises trastuzumab and pertuzumab and a package insert instructing the user to co-administer trastuzumab and pertuzumab to a patient suffering from HER2 positive cancer who does not respond to a monotherapy with trastuzumab or a monotherapy with pertuzumab.



39. The method of claim 38 wherein Pertuzumab, Trastuzumab, cisplatin, and capecitabine or 5-FU are administered.

40. The method of any one of claims 35 to 39 wherein Pertuzumab is administered at a dose of 840 mg in all treatment cycles.

41. The method of any one of claims 35 to 40 which improves overall survival (OS) relative to a patient treated with Trastuzumab and the chemotherapy only, or improves progression free survival (PFS) or response rate (RR) relative to treatment with Trastuzumab and chemotherapy only.

42. A method of treating gastric cancer in a human subject comprising administering Pertuzumab to the subject with gastric cancer, wherein Pertuzumab is administered at a dose of 840 mg in all treatment cycles.

43. The method of claim 42 wherein Pertuzumab is administered at the dose of 840 mg for six treatment cycles.

44. The method of claim 42 or claim 43 which maintains Pertuzumab trough levels above about 20 μg/mL in the subject.

45. The method of any one of claims 42 to 44 further comprising administering Trastuzumab and chemotherapy to the subject.

46. A method of treating HER2-positive non-resectable or metastatic adenocarcinoma of the stomach or gastroesophageal junction in a human patient who did not receive prior chemotherapy for metastatic disease, except prior adjuvant or neoadjuvant therapy completed more than six months before the current treatment, comprising administering Pertuzumab, Trastuzumab, cisplatin, and capecitabine or fluorouracil (5-FU) to the patient in an amount to improve progression free survival (PFS) and/or overall survival (OS), wherein the patient has an Eastern Cooperative Oncology Group Performance Status Scale (ECOG PS) of 0-1.

47. A method of improving progression free survival (PFS) in a human patient with HER2-positive non-resectable or metastatic adenocarcinoma of the stomach or gastroesophageal junction comprising administering Pertuzumab to the patient in combination with Trastuzumab and chemotherapy.

48. A method of treating HER2-positive breast cancer in a patient comprising administering Pertuzumab, Trastuzumab and vinorelbine to the patient.

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27. The method of claim 26 which further comprises administering chemotherapy to the patient.

28. An intravenous (IV) bag containing a stable mixture of Pertuzumab and Trastuzumab suitable for administration to a cancer patient.

29. The IV bag of claim 28 wherein the mixture is in saline solution.

30. The IV bag of claim 29 wherein the saline solution comprises about 0.9% NaCl or about 0.45% NaCl.

31. The IV bag of any one of claims 28 to 30 which is a 250mL 0.9% saline polyolefin or polyvinyl chloride infusion bag.

32. The IV bag of any one of claims 28 to 31 which contains a mixture of about 420mg or about 840mg of Pertuzumab and from about 200mg to about 1000mg of Trastuzumab.

33. The IV bag of any one of claims 28 to 32 wherein the mixture is stable for up to 24 hours at 5°C or 30°C.

34. The IV bag of any one of claims 28 to 33 wherein stability has been evaluated by an assay selected from the group consisting of: color, appearance and clarity (CAC), concentration and turbidity analysis, particulate analysis, size exclusion chromatography (SEC), ion-exchange chromatography (IEC), capillary zone electrophoresis (CZE), image capillary isoelectric focusing (iCIEF), and potency assay.

35. A method of treating HER2-positive gastric cancer in a human subject comprising administering Pertuzumab, Trastuzumab, and chemotherapy to the subject with HER2-positive gastric cancer.

36. The method of claim 35 wherein the gastric cancer comprises non-resectable locally advanced gastric cancer, or metastatic gastric cancer, or advanced, post-operatively recurrent gastric cancer, which may not be amenable to curative therapy by known methods, or adenocarcinoma of the stomach or gastroesophageal junction.

37. The method of claim 35 or claim 36 wherein the patient has not received prior anti-cancer treatment for metastatic gastric cancer, has an Eastern Cooperative Oncology Group Performance Status Scale (ECOG PS) of 0-1, or has a HER2-positive status of IHC 3+ or IHC2+/ISH+.

38. The method of any one of claims 35 to 37 wherein the chemotherapy comprises platin (cisplatin) and/or fluoropyrimidine (capecitabine or 5-fluorouracil (5-FU)).

14. An article of manufacture comprising a vial with Pertuzumab therein and a package insert, wherein the package insert provides the safety data in Table 3 or Table 4.

15. The article of manufacture of claim 14 wherein the package insert provides the efficacy data in Table 2, Table 5, Figure 8, or Figure 10.

16. The article of manufacture of claim 14 or claim 15 wherein the vial is a single-dose vial containing about 420mg of Pertuzumab.

17. A method for making an article of manufacture comprising packaging together a vial with Pertuzumab therein and a package insert, wherein the package insert provides the safety data in Table 3 or Table 4.

18. A method of ensuring safe and effective use of Pertuzumab comprising packaging together a vial with Pertuzumab therein and a package insert, wherein the package insert provides the safety data in Table 3 or Table 4 and the efficacy data in Table 2, Table 5, Figure 8, or Figure 10.

19. A method of treating early-stage HER2-positive breast cancer comprising administering Pertuzumab, Trastuzumab, and chemotherapy to a patient with the breast cancer, wherein the chemotherapy comprises anthracycline-based chemotherapy, or carboplatin-based chemotherapy.

20. The method of claim 19 wherein chemotherapy comprises anthracycline-based chemotherapy which comprises 5-FU, epirubicin, and cyclophosphamide (FEC).

21. The method of claim 20 wherein the Pertuzmab is administered concurrently with the anthracycline-based chemotherapy.

22. The method of claim 19 wherein the chemotherapy comprises carboplatin-based chemotherapy which comprises Docetaxel and Carboplatin.

23. The method of claim 22 wherein the Pertuzmab is administered concurrently with the carboplatin-based chemotherapy.

24. The method of any one of claims 19 to 23 wherein Pertuzumab administration does not increase cardiac toxicity relative to the treatment without Pertuzumab.

25. The method of any one of claims 19 to 24 which comprises neoadjuvant or adjuvant therapy.

26. A method of treating HER2-positive cancer in a patient comprising co-administering a mixture of Pertuzumab and Trastuzumab from the same intravenous bag to the patient.

WHAT IS CLAIMED IS:

1. A method for extending progression free survival in a HER2-positive breast cancer patient population by 6 months or more comprising administering Pertuzumab, Trastuzumab and chemotherapy to the patients in the population.

2. The method of claim 1 which results in an objective response rate of 80% or more in the patients in the population.

3. The method of claim 1 or claim 2 wherein the chemotherapy comprises taxane.

4. The method of claim 3 wherein the taxane is Docetaxel.

5. The method of any one of claims 1 to 4 wherein the breast cancer is metastatic or locally recurrent, unresectable breast cancer, or *de novo* Stage IV disease.

6. The method of any one of claims 1 to 5 wherein the patients in the population have not received previous treatment or have relapsed after adjuvant therapy.

7. The method of any one of claims 1 to 6 wherein the HER2-positive breast cancer is defined as immunohistochemistry (IHC) 3+ and/or fluorescence *in situ* hybridization (FISH) amplification ratio ≥2.0.

8. The method of any one of claims 1 to 7 wherein the patients in the population have a left ventricular ejection fraction (LVEF) of ≥50% at baseline.

9. The method of any one of claims 1 to 8 wherein the patients in the population have an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0 or 1.

10. A method of combining two HER2 antibodies to treat HER2-positive cancer without increasing cardiac toxicity in a HER2-positive cancer patient population, comprising administering Pertuzumab, Trastuzumab, and chemotherapy to the patients in the population.

11. The method of claim 10 wherein cardiac toxicity in the patient population is monitored for incidence of symptomatic left ventricular systolic dysfunction (LVSD) or congestive heart failure (CHF), or decrease in left ventricular ejection fraction (LVEF).

12. The method of claim 10 or claim 11 wherein the HER2-positive cancer is breast cancer.

13. The method of any one of claims 10 to 12 wherein the breast cancer is metastatic or locally recurrent, unresectable breast cancer, or *de novo* Stage IV disease.

PCR assay and IHC. Such assessment of HER receptor mRNA/protein expression will occur after obtaining the patient's informed consent at any time after the primary surgery and prior to screening.

It is anticipated that Pertuzumab in combination with topotecan or paclitaxel will be safe and effective in patients with epithelial ovarian, primary peritoneal, or fallopian tube cancer.

In addition, it is anticipated that Pertuzumab plus chemotherapy (topotecan, paclitaxel, or gemcitabine) will be superior to placebo plus chemotherapy in patients with epithelial ovarian, primary peritoneal, or fallopian tube cancer where efficacy is measured by PFS.

medication should be implemented according to local practices and the chosen chemotherapy.

Topotecan Dosage and Administration

Topotecan should be administered 1.25 mg/m² as a 30-minute intravenous infusion daily on Days 1–5 every 3 weeks, as per the directions in the summary of product characteristics.

Paclitaxel Dosage and Administration

Paclitaxel should be administered 80 mg/m2 as a 1-hour i.v. infusion on Days 1, 8, 15 and 22. Pharmacists should follow the summary of product characteristics for information regarding the preparation and administration of the 80 mg/m2 dose.

Gemcitabine Dosage and Administration

Gemcitabine (Part 2 of the study only) should be administered 1000 mg/m2 as a 30-minute intravenous infusion on Days 1 and 8 every 3 weeks as per the directions described in the summary of product characteristics.

HER3 mRNA Expression

Patients will be asked to specifically consent to the collection and testing of primary tumor tissue samples to assess HER3 mRNA level, including mRNA and protein levels of other HER family receptors e.g. HER2, before they provide consent to participate in the trial. Only patients who have tumors expressing low levels of HER3 mRNA will be eligible to participate in the trial.

During the initial screening for HER3 mRNA levels, other receptors of the HER family (e.g. EGFR, HER2, or HER4) will be assessed at the mRNA level and/or protein level in parallel to the HER3 assessment, in order to obtain a more complete picture of the status of HER family receptors by mRNA level.

The cut-off defined for study eligibility is defined as a concentration ratio of ≤2.81 as assessed by qRT-PCR on a COBAS z480® instrument using the "COBAS® HER2 & HER3 (qRT-PCR) mRNA expression assay" provided by Roche Molecular Diagnostics. The rationale for cut off definition is based on a cut off modeling in previous studies as well as on a transformation function that had to be introduced since the assay was switched to a new instrument; the COBAS z480®. It is anticipated that 40-50% of screened patients will have HER3 mRNA levels below the cutoff of 2.81 and that 30% of patients expressing low levels of HER3 mRNA will be ineligible for enrollment owing to other inclusion/exclusion criteria.

Submission of a formalin-fixed, paraffin-embedded tumor specimen of the primary tumor from the original surgery will be required for all patients prior to screening; cytology specimens are not acceptable replacements. Patients will be assessed for HER3 mRNA expression level, as well as mRNA expression and protein expression levels of other HER family receptors by the use of a qRT-

Determination of Sample Size

At least 50 ECG-evaluable patients will be enrolled to this substudy. The sample size for this substudy has been primarily chosen to provide an estimate of key PK and ECG parameters. No formal statistical hypothesis testing is planned. Assuming an equal rate of participation between treatment arms (25 patients per treatment arm) *and an estimated standard deviation of 20 ms, the two-sided 90% confidence interval for the baseline-adjusted difference in QTcF between treatment arms (ddQTcF) will be within 10 ms of the observed difference.*

It is expected that at least 40 patients enrolled to this substudy will be PK-evaluable. A PK-evaluable patient is defined as a patient who has had complete PK samples collected at Cycle 1 and Cycle 3. With a sample size of 40 evaluable patients and an inter-patient coefficient of variation in AUC of 30%, the 90% confidence interval for the ratio of the geometric mean docetaxel concentrations between treatment arms will be (86%, 117%) if the observed geometric mean AUCs for both treatment arms are identical.

An ECG-evaluable patient is defined as a patient who has an interpretable baseline ECG as well as an interpretable ECG at Cycle 3 Day 1 immediately post-pertuzumab/placebo infusion (steady-state C_{max}). With this sample size of 40 evaluable patients and an estimated standard deviation of 20 ms, a 95% confidence interval for the difference between treatment arms in mean change in QTcF from baseline to Cycle 3 will be ± 12.4 ms from the observed mean change.

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WHAT IS CLAIMED IS:

1. A method for the treatment of breast cancer, comprising administering to a HER2 positive metastatic breast cancer patient an effective amount of a growth inhibitory HER2 antibody, a HER2 dimerization inhibitor antibody, and a taxane, wherein the patient did not receive prior chemotherapy or biologic therapy.

2. The method of claim 1 wherein the growth inhibitory HER2 antibody binds to an epitope within Domain IV (SEQ ID NO: 17) of the HER2 amino acid sequence.

3. The method of claim 2 wherein the growth inhibitory HER2 antibody binds essentially to epitope 4D5 of HER2.

4. The method of claim 1 wherein the HER2 dimerization inhibitor antibody binds HER2 at the junction of domains I, II and III (SEQ ID NOs: 14, 15, and 16).

5. The method of claim 4 wherein the HER2 dimerization inhibitor antibody binds essentially to epitope 2C4.

6. The method of claim 1 wherein the growth inhibitory and/or the HER2 dimerization inhibitor antibody is an antibody fragment.

7. The method of claim 1 wherein the growth inhibitory and/or the HER2 dimerization inhibitor antibody is chimeric, humanized, or human.

8. The method of claim 1 wherein the growth inhibitory antibody is trastuzumab, or a fragrement thereof, the HER2 dimerization antibody is pertuzumab, or a fragment thereof, and the taxane is docetaxel.

9. A method for the treatment of breast cancer, comprising administering to a HER2 positive metastatic breast cancer patient an effective amount of a first HER2 antibody binding essentially to epitope 2C4, a second HER2 antibody binding essentially to epitope 4D5, and a taxane, wherein the patient did not receive prior chemotherapy or biologic therapy.

10. The method of claim 9 wherein the patient is a human patient.

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11. The method of claim 10 wherein said first and second antibodies are monoclonal antibodies.

12. The method of claim 10 wherein at least one of said first and said second antibody is an antibody fragment.

13. The method of claim 11 wherein at least one of said first and said second antibody is chimeric humanized, or human.

14. The method of claim 10 wherein said first antibody is pertuzumab.

15. The method of claim 10 or claim 14 wherein said second antibody is trastuzumab.

16. The method of claim 14 wherein said taxane is docetaxel.

17. The method of claim 15 wherein said taxane is docetaxel.

18. The method of claim 10 wherein said first and second antibodies and said taxane are administered concurrently.

19. The method of claim 10 wherein said first and second antibodies and said taxane are administered consecutively, in any order.

20. The method of claim 10 wherein administration of the first antibody precedes administration of the second antibody and the taxane.

21. The method of claim 16 wherein at least one of the pertuzumab and the transtuzumab is a naked antibody.

22. The method of claim 16 wherein at least one of the pertuzumab and the transtuzumab is an intact antibody.

23. The method of claim 16 wherein administration of the pertuzumab, trastuzumab and docetaxel results in a synergistic effect.

24. The method of claim 16 wherein administration of the pertuzumab, trastuzumab and docetaxel extends survival of the human patient relative to treatment in the absence of at least one of

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pertuzumab, trastuzumab and docetaxel.

25. The method of claim 24 wherein progression free survival (PFS) is extended.

26. The method of claim 24 wherein overall survival (OS) is extended.

27. The method of claim 10 further comprising the administration of a further therapeutic agent selected from the group consisting of chemotherapeutic agent, a different HER antibody, antibody directed against a tumor associated antigen, anti-hormonal compound, cardioprotectant, cytokine, EGFR-targeted drug, anti-angiogenic agent, tyrosine kinase inhibitor, COX inhibitor, non-steroidal anti-inflammatory drug, farnesyl transferase inhibitor, antibody that binds oncofetal protein CA 125, HER2 vaccine, HER targeting therapy, Raf or ras inhibitor, liposomal doxorubicin, topotecan, taxane, dual tyrosine kinase inhibitor, TLK286, EMD-7200, a medicament that treats nausea, a medicament that prevents or treats skin rash or standard acne therapy, a medicament that treats or prevents diarrhea, a body temperature-reducing medicament, and a hematopoietic growth factor.

28. A kit comprising a first HER2 antibody binding essentially to epitope 2C4, a second HER2 antibody binding essentially to epitope 4D5, and a taxane, and a package insert or label with directions to treat a HER2 positive metastatic breast cancer patient, who did not receive prior chemotherapy or biologic therapy.

29. A method of promoting pertuzumab for the treatment of a HER2 positive metastatic breast cancer patient who did not receive prior chemotherapy or biologic therapy, in combination with trastuzumab and a taxane.

30. The method of claim 29 wherein the taxane is docetaxel.

31. A method of promoting trastuzumab for the treatment of a HER2 positive metastatic breast cancer patient who did not receive prior chemotherapy or biologic therapy, in combination with pertuzumab and a taxane.

32. The method of claim 31 wherein the taxane is docetaxel.

33. A method for promoting a taxane for the treatment of a HER2 positive metastatic breast cancer patient who did not receive prior chemotherapy or biologic therapy, in combination

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Clinical Cancer Research

Pertuzumab in Combination with Trastuzumab Shows Significantly Enhanced Antitumor Activity in HER2-Positive Human Gastric Cancer Xenograft Models

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Abstract

Purpose: We investigated the antitumor activity of the combination of two different humanized monoclonal human epidermal growth factor receptor (HER) 2 antibodies, pertuzumab and trastuzumab, for gastric cancer.

Experimental Design: Tumor mouse xenograft models were used to examine antitumor activity. Cell proliferation was examined using crystal violet staining. HER family proteins' expression was analyzed by ELISA and immunohistochemistry. Phosphorylated proteins and heterodimers were detected by Western blotting and *in situ* proximity ligation assay (PLA), respectively. Apoptosis activity was examined by caspase 3/7 activity. Antibody-dependent cellular cytotoxicity (ADCC) activity was detected by xCELLigence. Microvessel density was examined by CD31 staining.

Results: Pertuzumab in combination with trastuzumab showed significant antitumor activity compared with each monotherapy in NCI-N87, an HER2-positive human gastric cancer xenograft model. The efficacy was stronger than that of the maximum effective dose with each monotherapy. Similar antitumor activity was shown in 4-1ST, another HER2-positive gastric cancer model, but not in MKN-28, an HER2-negative model. Combining pertuzumab with trastuzumab enhanced cell growth inhibition and apoptosis activity by inhibiting EGFR-HER2 heterodimerization and the phosphorylation of these receptors and their downstream factors. This effect was also seen in HER2-HER3 signaling. Furthermore, pertuzumab in combination with trastuzumab potentiated the ADCC activity of those antibodies and reduced tumor microvessel density.

Conclusions: We showed the significantly enhanced efficacy of pertuzumab combining with trastuzumab for HER2 overexpressing gastric cancer through the potentiation of cell growth inhibition, apoptosis activity, cell killing activity by ADCC, and antiangiogenic activity. This study suggests the clinical benefit of combination therapy with pertuzumab and trastuzumab for patients with HER2-positive gastric cancers. *Clin Cancer Res; 17(15); 5060–70. ©2011 AACR.*

Introduction

Gastric cancer shows a poor prognosis and is the second leading cause of cancer-related deaths. Its incidence is estimated at 934,000 cases, 56% of new cases are in Eastern Asia, 41% in China, and 11% in Japan (1). Although fluoropyrimidine- and platinum-based combination chemotherapy is the most widely accepted in the world at present, its benefit does not translate into higher overall survival rates. Therefore, more effective therapies for gastric cancer are required.

The human epidermal growth factor receptor (HER) family is composed of epidermal growth factor receptor (EGFR), HER2, HER3, and HER4. They regulate cell proliferation, differentiation, and apoptosis through the activation of their signal transduction by forming homodimers or heterodimers (2). The overexpression of HER family protein is often related to tumor malignancy. In gastric cancer, EGFR, HER2, and HER3 overexpression has been identified and a relationship with prognosis is suggested (3–7). Therefore, inhibiting the signal transduction through heterodimers including HER2 possibly provides more benefit to patients with gastric cancer. Recently, the ToGA trial [a phase III study of trastuzumab (Herceptin) in HER2-positive advanced and inoperable gastric cancer] showed a survival benefit

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Translational Relevance

Gastric cancer shows a poor prognosis. Although various therapies including fluoropyrimidine- and platinum-based combination chemotherapy have been used for gastric cancer, these therapies have not improved the rate of overall survival. Recently, the effectiveness of trastuzumab for gastric cancer has been shown in a clinical phase III study, ToGA, revealing how meaningful anti-HER2 therapy is for gastric cancer. This is the first report showing the usefulness of a new HER2 antibody, pertuzumab, for HER2 overexpressing gastric cancer *in vivo* and *in vitro*. These results were a scientific rationale for developing pertuzumab for HER2-positive gastric cancer.

when trastuzumab was added to chemotherapy in HER2-overexpressing gastric cancer patients (8) and the Food and Drug Administration has approved trastuzumab for HER2-positive metastatic gastric and gastroesophageal junction cancer. Thus, anti-HER2 therapy has been identified to be of clinical significance.

Trastuzumab is a humanized anti-HER2 antibody and is suggested to show its antitumor activity through inhibiting ligand-independent HER2 signaling, enhancing antibody-dependent cellular cytotoxicity (ADCC) activity (9, 10), blocking formation of p95HER2, a constitutively active form of HER2 (11), and suppressing tumor angiogenesis (12). Trastuzumab has shown survival benefit in the treatment of HER2-overexpressing metastatic breast cancer and in the adjuvant therapy of HER2-overexpressing breast cancer (13–15). For gastric cancer, our previous study showed that trastuzumab significantly inhibited tumor growth in HER2-overexpressing human gastric cancer mouse xenograft models (16).

Pertuzumab, which is a new humanized anti-HER2 antibody, is thought to exert antitumor activity in a different manner from trastuzumab. Pertuzumab binds to domain II of HER2, the region of dimer formation, whereas trastuzumab binds to domain IV of HER2. Thus, pertuzumab could inhibit the dimerization of HER2 with other HER family proteins and prevent ligand-dependent HER2 signaling (17). In HER2-positive breast cancer, the usefulness of pertuzumab and trastuzumab was shown in preclinical and clinical reports (18, 19). To investigate the effect of pertuzumab and trastuzumab for patients with previously untreated HER2-positive metastatic breast cancer, a phase III trial of pertuzumab in combination with trastuzumab and docetaxel is underway (20). Clinical benefit of combination therapy of pertuzumab and trastuzumab is expected.

Pertuzumab and trastuzumab in combination might provide more effective antitumor activity than either single agent for HER2-positive tumors including gastric cancer because of their different mechanisms of HER2 signal inhibition. This combination therapy could provide clinical benefits for HER2-positive gastric cancer patients. In the present study, we investigated the antitumor efficacy of pertuzumab in combination with trastuzumab as well as its mechanism of action as a new therapy for gastric cancer by using HER2-overexpressing human gastric cancer mouse xenograft models.

Materials and Methods

Molecular modeling of trastuzumab/pertuzumab/HER2 ternary complex

The binding position of the Fab of pertuzumab or trastuzumab to HER2 was assigned using the crystallographic structure of the Fab (pertuzumab)/HER2 complex [Protein Data Bank (PDB) ID: 1s78; ref. 17] or the Fab (trastuzumab)/HER2 complex (PDB ID: 1n8z; ref. 21). We used the three-dimensional (3D) structure of IgG1 monoclonal antibody (PDB ID: 1IGY; ref. 22) as a surrogate for that of each whole antibody because the 3D structures of the whole antibodies are not known. The position of pertuzumab or trastuzumab whole antibody was estimated by superposing the 3D structure of IgG1 (PDB ID: 1IGY) onto that of the Fab (pertuzumab or trastuzumab)/HER2 complex (PDB ID: 1s78 or 1n8z, respectively). The superposition of the Fab of IgG1 and the Fab of pertuzumab or trastuzumab was done using the software PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC). Finally, the pertuzumab whole antibody/HER2 complex model and trastuzumab whole antibody/HER2 complex model were merged by superposing the 3D structures of HER2 of the 2 models.

Construction of the transfectants of NK-92 expressed human FcγRIIIa-158V allotypes

CD16-negative NK-92 tumor line was purchased from the American Type Culture Collection (ATCC). The cDNA encoding FcγRIIIa was amplified by PCR from the fetal spleen cDNA Library (Clontech) using a specific primer set (CD16F1: TAA GAA TTC CCA CCA TGT GGC AGC TGC TCC TCC C, CD16F2: TAA GCG GCC GCT TAT CAT TTG TCT TGA GGG TCC TTT GTC C). The cDNA encoding FcγRIIIa was subcloned into pGEM T-Easy vector (Promega). Because cDNA sequencing revealed that the obtained FcγRIIIa were all 158F allotype, the FcγRIIIa-158V allotype sequence was generated by site-directed mutagenesis (QuickChange; Stratagene). The cDNA encoding FcγRIIIa-158V allotype was cloned at the *Eco*RI and *Not*I sites of the pCXND3 expression vector, which is a derivative of pCXN (23) containing a CAG promoter and a neomycin-resistant gene. The resulting plasmid was designated pCXND3/CD16(V). To establish transfectants of NK-92 cells that produce human FcγRIIIa-158V allotype, pCXND3/CD16(V) was transfected by the electroporation method using GENE-PULSER II (Bio-Rad) under the conditions of 1.5 kV and 25 μFD. Flow cytometry–based screening was conducted using αCD16-FITC (Beckman Coulter), and ^{51}Cr-release assay was conducted to confirm the ADCC activity in pCXND3/CD16(V)-transfected NK-92 cells. The resulting FcγRIIIa-158V–positive NK-92 cells

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exerting ADCC activity were designated CD16(158V)/NK-92 cells.

Test agents

Trastuzumab and pertuzumab were provided by F. Hoffmann (La Roche) as a fine powder and a liquid, respectively. Trastuzumab was dissolved in purified water. Both antibodies were diluted with saline or culture medium in *in vivo* or *in vitro* experiments. Human immunoglobulin G (HulgG) was purchased from MP Biomedicals, Inc. and was reconstituted with water and diluted with saline.

Animals

Male, 5-week-old BALB-*nu/nu* mice (CAnN.Cg-Foxn1<nu>/CrlCrlj nu/nu) were obtained from Charles River Japan. All animals were allowed to acclimatize and recover from shipping-related stress for 1 week prior to the study. The health of the mice was monitored by daily observation. Chlorinated water and irradiated food were provided *ad libitum*, and the animals were kept under a controlled light–dark cycle (12–12 hours). All the animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee.

Cell lines and culture

Three human gastric cancer cell lines were used in the present study. NCI-N87 cells were purchased from ATCC. MKN-28 cells were purchased from Immuno-Biochemical Laboratories Co., Ltd. NCI-N87 and MKN-28 were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS at 37°C under 5% CO_2. 4-1ST cells were purchased from the Central Institute for Experimental Animals and maintained in BALB-*nu/nu* mice by subcutaneous inoculation of pieces of tumor. CD16(158V)/NK-92 was maintained in α-minimal essential medium (MEMα; Wako) supplemented with 12.5% FBS, 12.5% horse serum, 0.02 mmol/L folic acid, 0.1 mmol/L 2-mercaptoethanol, 0.2 mmol/L inositol, and 0.5 mg/mL G418, and 20 ng/mL recombinant human interleukin (IL) 2 at 37°C under 5% CO_2.

Immunohistochemistry of EGFR and HER2

Tumor xenograft tissues were resected and processed as formalin-fixed, paraffin-embedded specimen sections. These were examined for the expression of EGFR or HER2 protein by immunohistochemistry (IHC) using EGFR pharmDx kit and HercepTest.

In vivo tumor growth inhibition studies

Each mouse was inoculated subcutaneously into the right flank with either 5×10^6 cells per mouse of human gastric cancer cell lines MKN-28 or NCI-N87, or an 8-mm^3 piece of 4-1ST tumor tissue. Several weeks after tumor inoculation, mice were randomly allocated to control and treatment groups. The administration of anticancer agents was started when the tumor volumes reached approximately 0.2 to 0.3 cm^3. To evaluate the antitumor activity of the test agents, tumor volume was measured twice a week. The tumor volumes (V) were estimated and the percentage of tumor growth inhibition (TGI%) was calculated as described previously (24). Trastuzumab and pertuzumab were administered intraperitoneally once a week for 3 weeks.

In vitro antiproliferation assays

NCI-N87 cells were seeded on 96-well plates at 5×10^3 cells per well and precultured for 24 hours. The cells were treated with pertuzumab, trastuzumab, or both and cultured for 3 days. The cells were fixed with 10% formalin neutral buffer solution. Crystal violet staining and extraction were conducted as described previously (16). Their absorbance was measured at 595 nm. The cells after 24-hour preculture were also detected by crystal violet staining as the absorbance of the precultured well. The percentage of cell proliferation inhibition (% inhibition) was calculated as follows: % Inhibition = [1 − (absorbance of treatment well − absorbance of precultured well)/(absorbance of nontreatment well − absorbance of precultured well)] × 100. In case of ligand-dependent proliferation, cells were precultured in RPMI 1640 with 0.1% FBS and treated with pertuzumab, trastuzumab or both 30 min before EGF or HRGα stimulation. The percentage of cell proliferation (% proliferation) was calculated as follows: % Proliferation = (absorbance of treatment well − absorbance of precultured well)/(absorbance of unstimulated well − absorbance of precultured well) × 100.

Apoptosis assay

NCI-N87 cells were seeded on 96-well plates at 1×10^4 cells per well and cultured in the same way as the ligand-dependent antiproliferation assay (see above). Twenty-four hours after treatment, Caspase-Glo 3/7 Assay (Promega) was used to measure caspase 3/7 activity. Caspase 3/7 activity was calculated as follows: Caspase 3/7 activity = (luminescent unit of treatment well − luminescent unit of blank well)/(mean luminescent unit of control well).

Cell stimulations

Cells were precultured for 1 day after seeding. After that the cells were starved in RPMI 1640 supplemented with 0.1% FBS for 20 to 24 hours and treated with pertuzumab, trastuzumab or both. After 3.5 hours of incubation, cells were exposed to EGF or heregulin (HRG) α for 5 minutes.

Western blotting

Cells were washed with ice-cold PBS and lysed with Cell Lysis Buffer (Cell Signaling Technology) with 10 mmol/L NaF, 1 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonylfluoride. After centrifugation (4°C, 14,000 × g, 5 minutes), the resultant supernatant was used for the assays. Protein concentration of the supernatant was quantified using the DC Protein Assay Kit (Bio-Rad). Cell lysates (20 μg protein per lane) were electrophoresed on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in SuperBlock T20 (TBS) Blocking Buffer (Thermo Scientific) and probed with

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each antibody against HER2 (sc-31153), HER3 (sc-285; Santa Cruz Biotechnology), EGFR (#2963), pEGFR (Tyr1068; #2236), pHER2 (Tyr1248; #2247), pHER3 (Tyr1289; #4791), Akt (#2920), pAkt (Ser473; #4058), ERK1/2 (#9102), pERK1/2 (Thr202/Tyr204; #9106; Cell Signaling Technology), and actin (A2228; Sigma-Aldrich) as the first antibodies. These proteins were detected by horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology). The bands were visualized using ECL plus (GE Healthcare Life Sciences).

ELISA

Tumor samples were taken when the tumors had reached a volume of approximately 0.3 to 0.5 cm^3 and were immediately frozen in liquid nitrogen and stored at −80°C. Tumor samples were homogenized in PBS containing 0.05% Tween 20 and centrifuged (4°C, 10,000 × *g*, 20 minutes). The resultant supernatant was used for the assays. We used Duo Set IC (R&D Systems) to detect human total EGFR, HER2, and HER3. Quantikine (R&D systems) was used to detect human VEGF. Total protein levels in the tumor tissue samples using a DC protein assay kit (Bio-Rad).

In situ proximity ligation assay

In situ proximity ligation assay (PLA) was done to detect EGFR-HER2 heterodimer. We used Duolink *in situ* PLA (Olink Bioscience). In the assay, oligonucleotide-conjugated "PLA probe" antibodies are directed against primary antibodies for EGFR or HER2. Annealing of the "PLA probes" occurs when EGFR and HER2 are in close proximity, which initiates the amplification of repeat sequences recognized by the fluorescently labeled oligonucleotide probe. Cells were seeded on 8-well chamber slides at 1×10^4 cells per well and stimulated as described above. Cells were washed with ice-cold PBS and fixed with 4% (w/v) paraformaldehyde PBS for 30 minutes at room temperature. The cells were then washed with ice-cold PBS and permeabilized with 0.2% Triton X-100 PBS for 5 minutes at room temperature. After that, the cells were washed with ice-cold PBS and assayed with Duolink *in situ* PLA. Anti-HER2 Ab (OP15; Calbiochem) and anti-EGFR Ab (sc-03; Santa Cruz Biotechnology) were used as primary antibodies. PLA probe anti-Mouse MINUS and PLA probe anti-Rabbit PLUS were used as 2 PLA probes. In a negative control, primary antibodies were not included. For detection, Duolink detection kit 563 was used. ProLong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) was used as the mounting medium. The specimens were observed using a fluorescence microscope (BX50; Olympus).

Immunoprecipitation

Streptavidin-coupled magnetic beads (Invitrogen) were incubated with biotinylated HER2 antibody (ab79205; Abcam) at 4°C for 1 hour. Cell lysates (1 mg) were added for 2 hours at 4°C. Beads were washed 3 times with lysis buffer (20 mmol/L HEPES, 15 mmol/L NaCl, 2 mmol/L EDTA, 10 mmol/L NaF, 0.5% NP-40, and 0.1 mmol/L Na_3VO_4 supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail; Sigma-Aldrich), resuspended in SDS sample buffer, and boiled. The supernatant was used for Western blotting.

ADCC assays

We used RTCA (Real-time Cell Analyzer; xCELLigence, Roche Diagnostics K. K.) to monitor ADCC activities in real time. The system measures electrical impedance on the bottom of the tissue culture E-Plates, which contain interdigitated electrodes, as the cell index. NCI-N87 cells were seeded on E-plates at 5×10^3 cells per well and were precultured for 24 hours. CD16(158V)/NK-92 were added as the effector at a target ratio of 1:1. Cells were treated with pertuzumab, trastuzumab, or both at the same time. The cell index was measured for 3 days after treatment. The normalized cell index was calculated as follows: (cell index at each point)/(cell index at the point of pertuzumab and trastuzumab treatment).

Measurement of microvessel density in tumor tissues

Microvessel density in tumor tissue was evaluated with immunohistochemical staining of CD31. Tumor samples were collected 96 hours after administration of trastuzumab, pertuzumab, or both. Immunohistochemical staining was conducted as described previously (25). Microvessel density (%) was calculated from the ratio of the CD31-positive staining area to the total observation area in the viable region. Three to 6 fields per section (0.4856 mm^2 each) were randomly analyzed, excluding necrotic areas. Positive staining areas were calculated using imaging analysis software (WinROOF; Mitani Corporation).

Results

Pertuzumab in combination with trastuzumab inhibits tumor cell growth more than each does as a single agent

Pertuzumab has a different binding site from trastuzumab and could bind to HER2 without competing with trastuzumab (Fig. 1A). Therefore, we hypothesized that pertuzumab would show significant antitumor activity in combination with trastuzumab. To examine whether pertuzumab could provide efficacy in combination with trastuzumab, we first analyzed tumor cell growth inhibition of pertuzumab in combination with trastuzumab *in vitro*. Pertuzumab alone inhibited the growth of NCI-N87, the HER2-positive human gastric cancer cell line (Fig. 1B, open rhombus). In combination with trastuzumab, pertuzumab could potentiate its antiproliferation activity (Fig. 1B, filled rhombus). Trastuzumab alone at 20 μg/mL inhibited the growth of the cells to 55.2% ± 1.3% (mean ± SD, $n = 3$).

Pertuzumab has antitumor activity as a single agent in a mouse xenograft model

We examined the efficacy of pertuzumab in NCI-N87 tumor xenograft. On day 22 (21 days after starting

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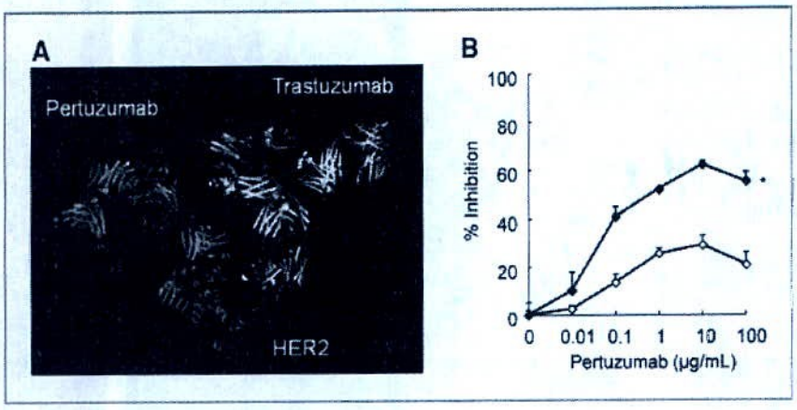


Figure 1. A, 3D structure of the trastuzumab/pertuzumab/HER2 ternary complex. B, antiproliferative activity of pertuzumab in combination with trastuzumab. Cells were treated with pertuzumab (0.1–100 μg/mL), with or without trastuzumab (20 μg/mL), for 3 days in the culture medium. Open rhombus: without trastuzumab; and filled rhombus: with trastuzumab. Data points are mean ± SD ($n = 3$). The 0% point was defined as the point of 0 μg/mL of pertuzumab of both open and filled rhombuses. Statistically significant differences are shown as *, $P < 0.05$ by the 2-way ANOVA.

treatment), tumor growth inhibition rates (TGI%) were 18%, 31%, 46%, and 41% at doses of 10, 20, 40, and 80 mg/kg, respectively. Pertuzumab showed significant antitumor activity at 40 and 80 mg/kg (Fig. 2A). The maximum effective dose was 40 mg/kg.

Pertuzumab in combination with trastuzumab enhances antitumor activity

Next, we investigated the antitumor activity of pertuzumab in combination with trastuzumab in NCI-N87 tumor xenograft. The combination of pertuzumab and trastuzumab dramatically enhanced the antitumor activity (TGI% = 145%) compared with pertuzumab or trastuzumab alone (TGI% = 31% and 52%, respectively; Fig. 2B). We also examined the efficacy of pertuzumab and trastuzumab in 2 other human gastric cancer xenografts, HER2-positive 4-1ST and HER2-negative MKN-28. The antitumor activity of pertuzumab in combination with trastuzumab in 4-1ST was also significantly higher than either pertuzumab or trastuzumab as single agents (TGI% = 22%, 57%, and 109%, respectively; Fig. 2C). On the other hand, no combined effect was seen in MKN-28, (TGI% = −2% and 27% in the pertuzumab treatment group and the combination treatment group, respectively; Fig. 2D). MKN-28 was insensitive for trastuzumab in a separate experiment (16). We examined the expression levels of HER family protein in these gastric tumor tissues of mice xenograft models by ELISA and IHC (Table 1). All cell lines expressed EGFR and HER3 by ELISA. All cell lines were also identified as EGFR-positive cells by IHC according to clinical diagnosis. We

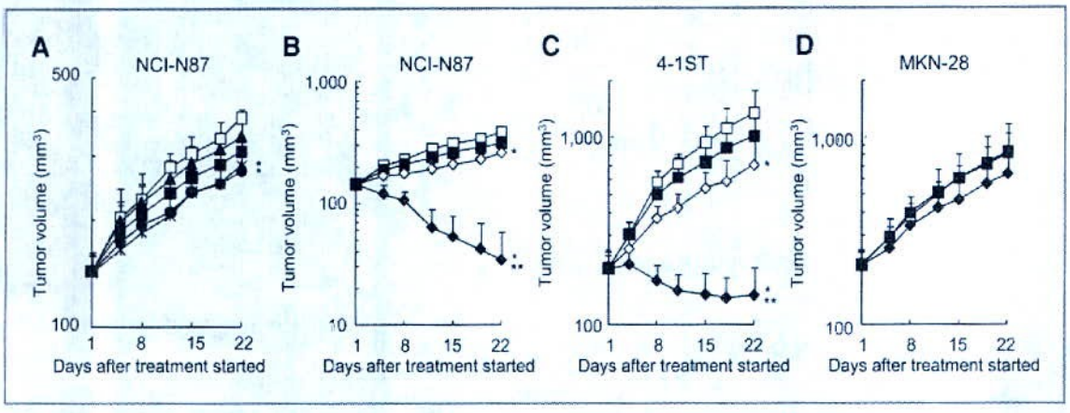


Figure 2. Antitumor activity of pertuzumab as a single agent and in combination with trastuzumab. A, mice bearing NCI-N87 tumors were randomly divided into 5 groups ($n = 6$ per group) and treated with HulgG or pertuzumab once a week for 3 weeks. Open square: HulgG; filled triangle: pertuzumab 10 mg/kg; filled square: pertuzumab 20 mg/kg; filled circle: pertuzumab 40 mg/kg and *, pertuzumab 80 mg/kg. B–D, tumor-bearing mice were randomly divided into 4 groups (B and C) or 2 groups (D; $n = 6$ per group) and treated with HulgG, pertuzumab, trastuzumab, or both pertuzumab and trastuzumab once a week for 3 weeks. B, NCI-N87 tumors (HER2 positive); C, 4-1ST tumors (HER2 positive); and D, MKN-28 tumors (HER2-negative). Open square: HulgG; filled square: pertuzumab at 20 mg/kg (B) or 40 mg/kg (C and D); open rhombus: trastuzumab at 20 mg/kg and filled rhombus: combination of pertuzumab and trastuzumab at 20 mg/kg and 20 mg/kg (B) or 40 mg/kg and 20 mg/kg (C and D, respectively). Data points are mean ± SD of the tumor volume (mm^3). Statistically significant differences are shown as *, $P < 0.05$ versus the control group; **, $P < 0.05$ versus the singe-agent groups by the Wilcoxon test.

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Table 1. HER family protein expression levels in human gastric tumor tissues

Xenograft	ELISA,[a] ng/mg protein			IHC[b]	
	EGFR	HER2	HER3	EGFR	HER2
NCI-N87	9.0 ± 1.2	180 ± 76	0.88 ± 0.084	+	+
4-1ST	0.94 ± 0.10	330 ± 72	1.9 ± 0.49	+	+
MKN-28	3.5 ± 0.37	1.7 ± 0.39	0.70 ± 0.20	+	−

[a]Means ± SD ($n = 4$).
[b]IHC was according to clinical diagnosis (EGFR pharmDx kit and HercepTest).

also stained HER3 immunohistochemically; however, HER3 was only slightly detected on the plasma membrane in all examined cell lines (data not shown). The expression level of HER2, but not EGFR or HER3, was considered to be a key factor of the efficacy.

Pertuzumab in combination with trastuzumab strongly reduces EGFR-HER2 signaling

To examine the mechanism of combination efficacy of pertuzumab and trastuzumab, we next analyzed HER2 signal transduction. We focused on the EGFR-HER2 signaling because HER3 expression level was very low in our gastric cancer models. First, we examined the phosphorylation of HER2 under serum starvation or under EGFR stimulation to evaluate ligand-independent or ligand-dependent signals, respectively. In the absence of EGF, phosphorylation of HER2 and the downstream factor Akt and extracellular signal–regulated kinase (ERK) 1/2 were strongly reduced by combination of pertuzumab and trastuzumab. In the presence of EGF, pHER2 was also more attenuated by the combination than by either antibody alone. Phosphorylation of EGFR, Akt, and ERK1/2 were increased by EGF stimulation, and the combination of pertuzumab and trastuzumab strongly reduced these phosphorylations (Fig. 3A). Next, we examined whether pertuzumab in combination with trastuzumab downregulated the phosphorylation of EGFR and HER2 by inhibiting their heterodimerization. We evaluated the dimerization level of EGFR-HER2 induced by EGF in the presence of pertuzumab, trastuzumab, or both by *in situ* PLA. EGFR-HER2 heterodimers were slightly decreased by pertuzumab. However, pertuzumab and trastuzumab in combination remarkably reduced the EGFR-HER2 heterodimers on the plasma membrane (Fig. 3B). The inhibition of EGFR-HER2 heterodimerization by the combination was also detected by immunoprecipitation (Fig. 3B). We also examined the cell proliferation and apoptosis activity under EGF stimulation. The cells proliferated EGF dependently and both pertuzumab and trastuzumab inhibited this EGF-dependent cell growth. The combination of pertuzumab and trastuzumab significantly enhanced the cell growth inhibition (Fig. 3C). In addition, the combination of pertuzumab and trastuzumab significantly enhanced apoptosis activity (Fig. 3D). These results suggest that pertuzumab in combination with trastuzumab inhibits EGFR-HER2 dimerization and its downstream signaling, although neither pertuzumab nor trastuzumab does it sufficiently alone.

Pertuzumab in combination with trastuzumab enhances the ADCC activity

Both pertuzumab and trastuzumab are reported to have ADCC activity. We investigated the ADCC activity induced by pertuzumab and trastuzumab as another mechanism of the combined effect by using RTCA. We observed the cell index of NCI-N87 for 3 days after the addition of pertuzumab, trastuzumab, and CD16(158V)/NK-92 cells. The cell index was normalized at the point of pertuzumab and trastuzumab addition. Normalized cell index after natural killer cells were added was significantly reduced by the addition of pertuzumab and trastuzumab compared with the addition of each single agent alone. No reduction of the normalized cell index was seen after the addition of pertuzumab and trastuzumab without natural killer cells (Fig. 4A). These results suggest that combination therapy of pertuzumab and trastuzumab has more potent antitumor activity through the enhancement of ADCC activity.

Pertuzumab in combination with trastuzumab shows antiangiogenic activity

There are some reports on the antiangiogenic activity of trastuzumab (12, 26). As another combination mechanism, we examined whether pertuzumab enhances the antiangiogenic activity of trastuzumab. In our study, neither pertuzumab nor trastuzumab could significantly decrease microvessel density. However, a significant reduction in microvessel density was seen if the tumor tissue was treated with pertuzumab in combination with trastuzumab (Fig. 4B and C). We also quantified the VEGF protein levels in those tumor tissues. Trastuzumab reduced VEGF levels in tumor tissues significantly. Pertuzumab also tended to reduce the VEGF levels, although it was not significant. When mice were treated with pertuzumab in combination with trastuzumab, the VEGF levels decreased more than they did with pertuzumab alone or trastuzumab alone (Fig. 4D). These results show that pertuzumab in combination with trastuzumab has an effect not only by inhibition of EGFR-HER2 signaling

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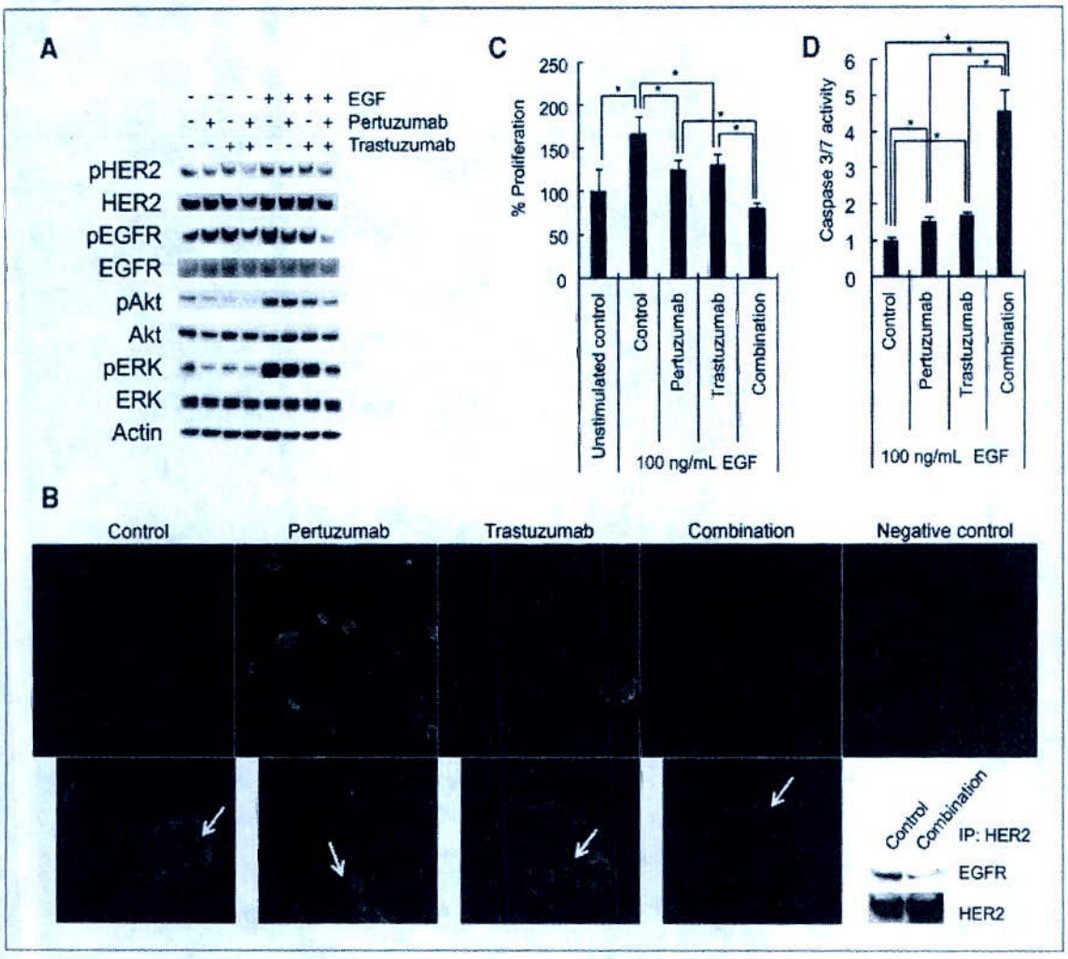


Figure 3. Effect of pertuzumab in combination with trastuzumab on the signal transduction of HER family proteins. A, NCI-N87 cells were treated with pertuzumab (40 μg/mL), trastuzumab (40 μg/mL), or both for 3.5 hours followed by EGF (100 ng/mL) stimulation for 5 minutes. The pHER2, HER2, pEGFR, EGFR, pAkt, Akt, pERK1/2, ERK1/2, and actin were detected by Western blotting. B, EGFR-HER2 heterodimer was detected by Duolink *in situ* PLA and immunoprecipitation (IP). Red, EGFR-HER2 heterodimer; blue, nuclear. EGFR-HER2 heterodimers were slightly decreased by pertuzumab and strongly decreased by the combination of pertuzumab and trastuzumab (white arrows). Objective 20×. C, the cell growth inhibition in the presence of EGF was examined. NCI-N87 cells were treated with pertuzumab (20 μg/mL), trastuzumab (20 μg/mL), or both 30 minutes before the addition of EGF (100 ng/mL). Control was untreated cells. The cell proliferation was examined 3 days after the treatment. Data points are mean ± SD ($n = 3$). Statistically significant differences are shown as *, $P < 0.05$ by the Student's *t* test. D, the caspase 3/7 activity of pertuzumab and trastuzumab combination was measured. NCI-N87 cells were treated with pertuzumab (20 μg/mL), trastuzumab (20 μg/mL), or both 30 minutes before the addition of EGF (100 ng/mL). Control was untreated cells. The caspase 3/7 activity was measured 24 hours after treatment. Data points are mean ± SD ($n = 5$). Statistically significant differences are shown as *, $P < 0.05$ by the Student's *t* test.

and enhancement of ADCC activity but also by inhibition of tumor angiogenesis.

Pertuzumab in combination with trastuzumab strongly reduces HER2-HER3 signaling

Finally, we also examined the signal transduction of HER2-HER3 in NCI-N87. Although NCI-N87 showed low expression levels of HER3 by ELISA and immunohistochemical assay, it has been reported that HER3 triggered strong signal transduction by dimer formation with HER2. Therefore, it is possible that a few HER3 proteins in NCI-N87 still play a role as mediators of the growth signal. We examined the phosphorylation levels of HER2, HER3, Akt, and ERK1/2 under HRGα stimulation after treatment with pertuzumab and trastuzumab. HRGα increased pHER3 and pAkt, and the combination of trastuzumab and pertuzumab reduced them as well as pHER2 (Fig. 5A). We also examined the contribution of HRGα to cell proliferation. The cells proliferated HRGα dependently, and both pertuzumab and trastuzumab inhibited the HRGα-dependent

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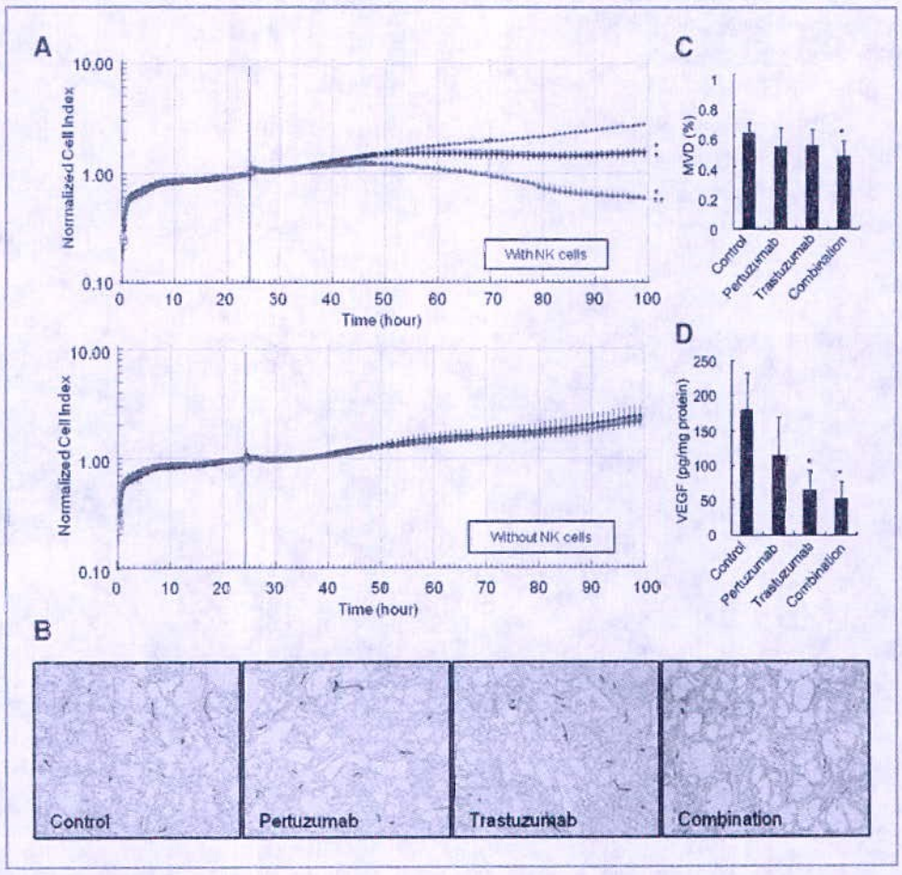


Figure 4. Effect of pertuzumab in combination with trastuzumab on ADCC activity and antiangiogenic activity. A, ADCC activity was examined with RTCA. NCI-N87 cells were precultured in the culture medium for 24 hours and treated with pertuzumab (10 ng/mL), trastuzumab (2 ng/mL), or both. Natural killer (NK) cells were added as the effector at target ratios of 1:1 simultaneously. Cell indices were measured every 5 minutes for the first 4 hours, every 15 minutes for the next 44 hours, and every hour thereafter. Cell indices were normalized at the treatment point. Red, control (nontreatment) group; green, pertuzumab group; blue, trastuzumab group; magenta, combination group. Data points are mean ± SD ($n = 3$). Statistically significant differences are shown as *, $P < 0.05$ versus the control group; **, $P < 0.05$ versus the single-agent groups by the Student's t test. B, tumor microvessel was stained immunohistochemically with anti-CD31 antibody at 96 hours after the treatment with HulgG (40 mg/kg; as a control), pertuzumab (20 mg/kg), trastuzumab (20 mg/kg), or both ($n = 5$–6 per group). C, the microvessel density of NCI-N87 was determined as described above. D, the VEGF protein levels of each treatment groups at 96 hours were quantified by ELISA ($n = 5$–6 per group). Data points are mean ± SD of tumor volume (mm^3). Statistically significant differences are shown as *, $P < 0.05$ versus control group by the Wilcoxon test.

cell growth. The combination of pertuzumab and trastuzumab significantly enhanced the cell growth inhibition. (Fig. 5B). The apoptosis activity of pertuzumab and trastuzumab in combination also significantly increased compared with that of pertuzumab or trastuzumab alone (Fig. 5C). Therefore, HER3 would also play a role in showing the efficacy of the combination even though the HER3 protein expression is low.

Discussion

It is reported that pertuzumab and trastuzumab bind to different domains of HER2. A previous study showed that pertuzumab and trastuzumab were oriented at different angles with respect to HER2 after binding to HER2, by using a 3D structure model of the Fab region of pertuzumab and trastuzumab and p185HER2 (27). As shown in Figure 1A, binding of whole IgG to HER2 ECD protein, pertuzumab would not interfere with the binding of trastuzumab on HER2, even though both antibodies are displayed in their whole IgG conformation. This result indicates that pertuzumab and trastuzumab could have combination effects on antitumor activity.

We examined the efficacy of pertuzumab and trastuzumab *in vivo*. The reason for *in vivo* study is that the *in vivo* models indicate the efficacy of this antitumor activity more accurately because trastuzumab or pertuzumab have a mechanism through ADCC activity. We used NCI-N87

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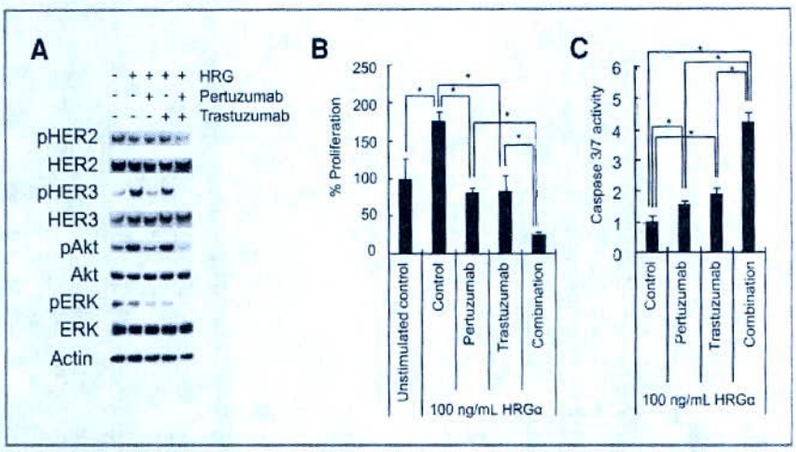


Figure 5. Effect of pertuzumab in combination with trastuzumab on the signal transduction of HER2 and HER3. A, NCI-N87 cells were treated with pertuzumab (20 μg/mL), trastuzumab (20 μg/mL), or both for 3.5 hours followed by HRGα (100 ng/mL) stimulation for 5 minutes. The phosphorylation levels of HER2 and HER3 in the cell lysates were detected by Western blotting. B, the cell growth inhibition in the presence of HRGα was examined. NCI-N87 cells were treated with pertuzumab (20 μg/mL), trastuzumab (20 μg/mL), or both 30 minutes before the addition of HRGα (100 ng/mL). Control was untreated cells. The cell proliferation was examined 3 days after treatment. Data points are mean ± SD ($n = 3$). Statistically significant differences are shown as *, $P < 0.05$ by the Student's t test. C, the caspase 3/7 activity of pertuzumab and trastuzumab combination was measured. NCI-N87 cells were treated with pertuzumab (20 μg/mL), trastuzumab (20 μg/mL), or both 30 minutes before the addition of HRGα (100 ng/mL). Control was untreated cells. The caspase 3/7 activity was measured 24 hours after treatment. Data points are mean ± SD ($n = 5$). Statistically significant differences are shown as *, $P < 0.05$ by the Student's t test.

and 4-1ST as HER2-positive models. NCI-N87 and 4-1ST were determined as HER2 2+ and 3+, respectively by IHC (HercepTest) and FISH (Pathvysion) in our previous study (16). MKN-28 was determined to be an HER2-negative cell line (16), so we used it as a negative control model. At first, we examined the dose dependent activity of pertuzumab on tumor growth in the xenograft model to determine the combination dose. In the NCI-N87 model, 3-week treatment with pertuzumab showed a significant antitumor activity at 40 and 80 mg/kg. The maximum effective dose of pertuzumab was determined as 40 mg/kg because the efficacy of 40 mg/kg of pertuzumab was the same as that of 80 mg/kg (TGI% = 46% and 41%, respectively). Next, we examined the antitumor activity of pertuzumab in combination with trastuzumab. In the NCI-N87 HER2-positive xenograft model, remarkable tumor regression was observed. The antitumor activity of the combination group was more potent than that of the maximum effective dose of both antibodies as single agents (the TGI% of our current combinational study was 145% and the maximum TGI% of pertuzumab and trastuzumab were 46% in our current study and 89% in a separate experiment, respectively; ref. 16). Another HER2-positive model 4-1ST also showed a potent effect of the pertuzumab and trastuzumab combination. Meanwhile, in MKN-28, an HER2-negative tumor model expressing HER1 or HER3, an effect of the combination was not shown. Therefore, the usefulness of this combination would only be observed in HER2-positive tumors. We could not examine the safety profile except for body weight in the present study because of species difference. No significant loss of body weight was observed. In the present study, we used subcutaneous xenograft models. However, orthotopic models might reflect actual tumors better and it would be interesting to know whether the same efficacy would be shown in the orthotopic models.

To explain the strongly enhanced antitumor efficacy of pertuzumab and trastuzumab combination treatment, we hypothesized 2 mechanisms direct action on tumor cells and a specific mechanism caused by the tumor environment. Actually, pertuzumab showed more potent cell growth inhibition and apoptosis activity in combination with trastuzumab than either pertuzumab or trastuzumab monotherapy showed *in vitro*, and the efficacy of the combination in mouse xenograft models was more remarkable than *in vitro*. As the direct action on tumor cells, we examined whether pertuzumab in combination with trastuzumab enhanced the inhibition of HER2 signal transduction. In the analysis of inhibition of the signaling pathways through HER1 and HER2, downregulation of pHER1, pHER2, pAkt, and pERK1/2 was observed in the combination group compared with the single agents. We also showed that pertuzumab in combination with trastuzumab reduced EGFR-HER2 heterodimers with Duolink *in situ* PLA, which was reported to detect heterodimers (28, 29). Others reported that pertuzumab could enhance the endocytic downregulation of EGFR by counteracting the EGFR-HER2 heterodimerization (30). In our HER2-positive gastric cancer model, only a little decrease in EGFR-HER2 heterodimers was detected by pertuzumab alone. Anyways, we consider that one mechanism of the effect of the combination of pertuzumab and trastuzumab is the reduction of EGFR-HER2 heterodimers and their signal

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transduction. In NCI-N87, HER3 expression was low and we considered that EGFR and HER2 were mainly functional. However, HER2-HER3 heterodimer is considered the most active HER signaling dimer (20, 31) and there are some reports suggesting that pertuzumab showed antitumor activity through HER2-HER3 signal inhibition in non–small-cell lung cancer and breast cancer (32, 33). In fact, HRGα induced the phosphorylation of HER3 and Akt in NCI-N87, and pertuzumab in combination with trastuzumab reduced them strongly; therefore, it is possible that low expression of HER3 was functional in this model and that pertuzumab in combination with trastuzumab also showed antitumor activity by inhibiting HER2-HER3 signaling as well as EGFR-HER2 signaling. In our study, total HER2 level of the combination was decreased. It is reported that anti-HER2 antibodies like trastuzumab led to degradation through the c-Cbl–regulated proteolytic pathway (34, 35). We could not examine the signal inhibition in 4-1ST model because it was an *in vivo* maintained cell line. In this study, it was suggested that both the EGFR-HER2 and the HER2-HER3 signaling were involved in the tumor growth inhibition of the combination of pertuzumab and trastuzumab. Comparing the degree of cell growth, the HER3-dependent cell growth was inhibited more strongly than the EGFR-dependent cell growth. However, it should not be concluded that HER3 is more important than EGFR for the clinical therapy of HER2-positive gastric cancer, and the contributing rate of EGFR and HER3 to HER signaling might be different between types of cancer (e.g., gastric cancer and breast cancer). In an HER2-positive breast cancer cell line, KPL-4, where pertuzumab in combination with trastuzumab showed strongly enhanced antitumor activity (19), EGF did not stimulate phosphorylation of EGFR but HRGα did phosphorylate HER3 (data not shown). It is necessary to examine exhaustively whether EGFR or HER3 is valuable as a secondary biomarker in clinical research from many points of view such as the expression level of ligands or HER family proteins.

As an *in vivo* specific mechanism caused by the tumor environment, we considered the potentiation of ADCC activity and antiangiogenic activity. ADCC activity is one of the key mechanisms of the antitumor activity of trastuzumab (36). Moreover, recent reports showed that pertuzumab also had ADCC activity (19, 37). Antiangiogenic activity is also shown as the mechanism of antitumor activity of trastuzumab (12). We examined the potentiation of ADCC because both trastuzumab and pertuzumab showed antitumor activity through ADCC activity. ADCC was measured using RTCA, reported as a new method by which it is possible to observe cell adhesion areas according to time (38). The degree of ADCC measured with RTCA was equivalent to that of measured with ^{51}Cr-release assay. We tried to use CD16-transfected NK-92 as the effector cell. The ADCC of trastuzumab was reported using the NK-92 cell, a human natural killer cell line (39). However, our NK-92 cell did not show ADCC activity to the target cell NCI-N87. Using this CD16-transfected cell line, the ADCC activity of pertuzumab or trastuzumab on NCI-N87 was observed and that of the combination group was significantly more potent. In a breast cancer model, another report showed that pertuzumab in combination with trastuzumab did not enhance ADCC activity of either antibody alone (19). This difference may be due to the different materials and methods. Second, we tried to clarify the antiangiogenic activity of these antibodies. In the combination group, a significant decrease in microvessel density and the VEGF protein in tumor tissue was observed. VEGF has been reported as a key angiogenic factor in tumors (40). Therefore, it was considered that pertuzumab in combination with trastuzumab decreased microvessel density by reduction of VEGF as a result of HER2 signal inhibition. On the basis of these results, the potent activity of the combination was due to enhancement of the ADCC activity and antiangiogenic activity in addition to direct cell growth inhibition through HER2 signal inhibition.

In conclusion, compared with single-agent treatment, pertuzumab in combination with trastuzumab showed significantly stronger antitumor activity in HER2-positive human gastric cancer mouse xenograft models through the enhancement of direct cell growth inhibition, ADCC activity, and antiangiogenesis activity. The combination therapy of pertuzumab and trastuzumab is worth examining as a new therapy for HER2-positive gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Pertuzumab in Combination with Trastuzumab Shows Significantly Enhanced Antitumor Activity in HER2-Positive Human Gastric Cancer Xenograft Models

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Cardiac Toxicity and Efficacy of Trastuzumab Combined with Pertuzumab in Patients with Trastuzumab-Insensitive Human Epidermal Growth Factor Receptor 2–Positive Metastatic Breast Cancer

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Abstract

Purpose: To evaluate safety and efficacy of trastuzumab with pertuzumab in patients with human epidermal growth factor receptor 2 (HER2)–positive metastatic breast cancer who had progressive disease on trastuzumab-based therapy.

Experimental Design: Patients with measurable HER2$^+$ metastatic breast cancer, ≤3 trastuzumab-based regimens, and left ventricular ejection fraction (LVEF) ≥55% received 8 or 6 mg/kg trastuzumab and 840 mg pertuzumab i.v. followed by 6 mg/kg trastuzumab and 420 mg pertuzumab every 3 weeks. Cardiac evaluation and tumor response were assessed every 3 and 6 weeks, respectively.

Results: Eleven patients received 64 cycles of trastuzumab plus pertuzumab. A total of 92 echocardiograms and 8 cardiac magnetic resonance imaging studies were done. With the lower limit of normal LVEF 55%, left ventricular systolic dysfunction was observed in six patients, three grade 1, two grade 2, and one grade 3 according to the National Cancer Institute Common Terminology Criteria for Adverse Events. The objective response rate was 18%. Two patients had partial responses, three had stable disease, and six had progressive disease. The median time to progression was 6 weeks. In baseline tumors from formalin-fixed paraffin-embedded primary and/or metastatic tumor biopsies, pHER2-Y1248 trended toward an increase in patients with partial response compared with those with stable disease/progressive disease ($P = 0.095$).

Conclusion: Trastuzumab plus pertuzumab may have clinical benefit in selected patients who have previously been treated with trastuzumab. Cardiac toxicity, although asymptomatic in most cases, was associated with this treatment. Further evaluation of efficacy of this combination is required to define the overall risks and benefits.

The humanized monoclonal antibody trastuzumab (Herceptin, Genentech, Inc.) targets the extracellular domain of the human epidermal growth factor receptor 2 (HER2) protein. Trastuzumab in combination with chemotherapy improves survival by 50% in patients with early and metastatic HER2$^+$ breast cancer (1–3). Although some patients with HER2$^+$ metastatic breast cancers benefit from the initial trastuzumab treatment alone, tumor progression eventually occurs (4–6). Resistance to trastuzumab is a challenging problem in management of HER2$^+$ breast cancer.

Mechanisms of resistance to trastuzumab are likely multifactorial as recently reviewed (7). Unlike other HER family receptors that require ligand binding for activation, HER2 always presents an active open conformation and is the preferred receptor for heterodimerization with other HER family receptors through regions of extracellular domains II and IV (8, 9). Trastuzumab binds to the extracellular domain IV of HER2 but does not significantly block the heterodimer formation between HER2 and HER3 (10). It has been shown that HER receptor ligands can overcome trastuzumab-induced G_1 cell cycle arrest and inhibition of tyrosine phosphorylation of the HER receptors (11). These results suggest that trastuzumab alone is not sufficient to abolish HER2 signaling due to the complex nature of the HER family receptor-ligand interactions. Several preclinical studies have shown that combination of antibodies to various epitopes of HER2 were more effective

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than individual antibodies in inducing antibody-dependent cytotoxicity, accelerating receptor down-regulation, interfering HER family receptor signaling, and inhibiting tumor cell growth (12–15).

Pertuzumab (Genentech), a humanized monoclonal antibody against a region of HER2 extracellular domain domain II, blocks HER2 heterodimerization and ligand-activated signaling from HER2/HER1 and HER2/HER3 heterodimers (10, 16, 17). Pertuzumab in combination with trastuzumab synergistically inhibited growth and induced apoptosis of tumor cells *in vitro* through disruption of HER2 heterodimerization and downstream signaling (18). Phase I study of pertuzumab showed acceptable toxicity profiles in treatment of solid tumors (19).

Among the toxicities of HER2 targeted therapy, cardiac dysfunction has a potential serious consequence. HER family members play a crucial role in normal cardiac development. Mice with HER2 or HER4 knockout had malformation of cardiac ventricles, which were embryonically lethal (20, 21). Cardiac-restricted deletion of HER2 led to dilated cardiomyopathy and reduced tolerance to fluid overload in mice (22, 23). It has been shown that heregulin stimulated proliferation and inhibited apoptosis of neonatal and adult cardiac myocytes that persistently expressed HER2 and HER4 (24). Thus, targeting HER2 may alter the signals necessary for survival of cardiac myocytes leading to cardiac toxicity.

We conducted a clinical study evaluating the toxicity and efficacy of trastuzumab plus pertuzumab in the treatment of patients with HER2$^+$ metastatic breast cancer who progressed through trastuzumab-based therapies. Based on the results of preclinical studies, we hypothesized that combination of the two anti-HER2 antibodies would be more efficient in controlling tumor growth and in overcoming trastuzumab resistance. We focused on the cardiac evaluation to ensure safety of the patients taking this combination treatment.

Table 1. Patient demographics

Characteristic	*n* (%)
No. patients	11 (100)
Age, y	
Median (range)	53 (36-68)
Race/ethnicity	
White	6 (54.5)
Black	3 (27.3)
Asian	1 (9)
Hispanic	1 (9)
Stage of disease	
Metastatic	11 (100)
Visceral only	3 (27.3)
Visceral and nonvisceral	3 (27.3)
Nonviscera: only	5 (45.4)
Hormone receptor status	
ER, PR, or both positive	3 (27.3)
ER and PR negative	8 (72.7)
HER2 positive (IHC combined with FISH)	11 (100)
Previous therapy	
Anthracyclines	9 (81.8)
Taxanes	10 (91)
Trastuzumab as adjuvant therapy	4 (36.4)
Trastuzumab for metastatic disease	11 (100)
Cumulative duration of prior trastuzumab (wk)	
Median (range)	82 (22-136)
Time from last cycle trastuzumab to initiation of trastuzumab and pertuzumab (wk)	
Median (range)	13 (4-96)

Abbreviations: ER, estrogen receptor; PR, progestin receptor; IHC, immunohistochemistry; FISH, fluorescent *in situ* hybridization.

Materials and Methods

Patient population. Patient eligibility criteria included measurable metastatic breast cancer; ≤3 prior trastuzumab-based treatments; age ≥18 y; HER2 overexpression (3+ by immunohistochemistry or gene amplification by fluorescent *in situ* hybridization); an Eastern Cooperative Oncology Group performance status ≤1; recovery from prior chemotherapy- and/or radiation-related toxicities to grade ≤1 (excluding alopecia); left ventricular ejection fraction (LVEF) ≥55% with no clinical signs or symptoms of congestive heart failure; and adequate hematologic, renal, and hepatic organ functions. Patients with brain and/or leptomeningeal metastases were required to have asymptomatic stable disease after standard surgery and/or radiation and could not be receiving corticosteroids for management of neurologic symptoms. Patients were excluded if they had uncontrolled hypertension, significant valvular disease, uncontrolled cardiac arrhythmias, prior myocardial infarction or angina pectoris requiring antianginal medication, poorly controlled diabetes mellitus, or prior exposure to >360 mg/m^2 doxorubicin and/or liposomal doxorubicin.

All patients gave written informed consent. The National Cancer Institute (NCI) Institutional Review Board approved the protocol and consent form.

Treatment schedule. Trastuzumab and pertuzumab were provided by Genentech directly to the Pharmaceutical Development Service of NCI for trial purposes only. Patients received 8 mg/kg trastuzumab i.v. on cycle 1 day 1 if they had not had trastuzumab within 1 mo, or 6 mg/kg trastuzumab if they received trastuzumab within 1 mo. On cycle 1 day 2, patients received 840 mg pertuzumab i.v. On day 1 of each of the subsequent 3-wk cycles, patients received 6 mg/kg trastuzumab i.v. plus 420 mg pertuzumab i.v. If patients developed hypersensitivity during any treatment cycle, they received antihistamine before subsequent cycles of monoclonal antibody infusion.

Tumor response. Tumor burden was evaluated with physical examination at baseline and before each cycle of treatment and imaging studies including computed tomography and bone scans at baseline and computed tomography after every 2 cycles plus bone scan if bone metastases were present. Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (25).

Safety assessments and cardiac evaluation. Clinical examination and laboratory assessments were done on day 1 of each cycle. Adverse events were graded according to the NCI Common Terminology Criteria for Adverse Events (NCI-CTCAE), version 3.0.[7]

Cardiac function was monitored with electrocardiogram and echocardiogram at the baseline and every 3 wk before treatment. Cardiac magnetic resonance imaging was done at the baseline for patients who enrolled later in the trial and for those who had a reduction in LVEF by echocardiogram. All cardiac studies during the trial were done at the NCI facilities. Efforts were made to have the same technician carry out the echocardiogram studies for each patient using the same machine throughout the trial. Each echocardiogram and electrocardiogram were read by one of three cardiologists. The lower limit of normal LVEF is 55% for our institution. Left ventricular systolic dysfunction (LVSD) was graded based on changes in LVEF according to the NCI-CTCAE version 3.0: grade 1 (LVEF 55-50%), grade 2 (LVEF 50-40%), and grade 3 (LVEF 40-20%). Trastuzumab and pertuzumab

[7] http://ctep.cancer.gov

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Table 2. Observed toxicity events for patients receiving trastuzumab plus pertuzumab

Toxicity	CTC grade			
	1	2	3	4
	n (%)			
Hematologic				
Neutropenia	0 (0)	0 (0)	0 (0)	0 (0)
Hemoglobin	2 (18)	0 (0)	0 (0)	0 (0)
Thrombocytopenia	2 (18)	0 (0)	0 (0)	0 (0)
Nonhematologic				
Diarrhea	2 (18)	3 (27)	0 (0)	0 (0)
Allergic reaction	2 (18)	0 (0)	1 (9)	0 (0)
Rash	1 (9)	0 (0)	0 (0)	0 (0)
Nail changes	2 (18)	1 (9)	0 (0)	0 (0)
LVSD	3 (27)	2 (18)	1 (9)	0 (0)

NOTE: *n*, number of patients.

were discontinued for symptomatic cardiac toxicities. For asymptomatic cardiac toxicities, trastuzumab and pertuzumab were held and cardiac function was reevaluated. Treatment was resumed if the LVEF was ≥55% within 1 to 3 wk. If the LVEF was <55% after 3 wk of holding therapy, trastuzumab and pertuzumab were permanently discontinued.

Correlative studies. Immunohistochemistry for HER2, pHER2-Y877, pHER2-Y1248, phoshorylated mitogen-activated protein kinase (pMAPK), pAkt, and Ki67 was done on formalin-fixed paraffin-embedded tumors. Core or punch biopsies of tumors were obtained before and after one cycle of trastuzumab plus pertuzumab as baseline and posttreatment, respectively. If a tumor biopsy was not feasible before treatment, tissue blocks from prior tumor biopsies were obtained and used as baseline. Antibodies used in this study were HER2 (CB11, mouse monoclonal; Biogenex), pHER2-Y877 (rabbit polyclonal; Cell Signaling Technology), pHER2-Y1248 (rabbit polyclonal; Cell Signaling Technology), Ki67 (MIB-1, mouse monoclonal; DAKO Corp.), pMAPK (pTEpY, rabbit polyclonal; Promega), and pAkt (S473, rabbit polyclonal; Cell Signaling Technology; refs. 26–30). These antibodies were validated with proper positive and negative controls before applying to the tumor samples. Immunohistochemistry was done according to the methods and conditions established in our laboratory (29, 30). The HER2 pathway markers were scored quantitatively with the Automated Cellular Imaging System (DAKO, ref. 29). The immunohistochemical staining indices of HER2, pHER2-Y877, pHER2-Y1248, pMAPK, and pAkt, and Ki67 labeling percentage were determined as described in our previous publications (29, 30).

Statistical considerations. The primary end points of this study were safety and response rate of the combination treatment. The secondary end points were time to progression and correlative studies of HER2 signaling. Time to progression is defined as time from registration date to the date of documented disease progression or death on study, whichever occurs first. The planned sample size was 37 based on Simon two-stage optimal design target, a 20% response rate, and to rule out an unacceptably low 5% response rate ($P = 0.05$; ref. 31). The trial would stop accrual early in the event that 2 or more of the initial 12 patients developed symptomatic congestive heart failure or any other serious toxicity.

An exact Wilcoxon rank sum test was used to determine the statistical significance of the difference in the levels of various proteins determined with immunohistochemical staining of the tumors from patients who had a partial response (PR) compared with stable disease (SD) or progressive disease (PD).

Results

Patients. A total of 11 patients were enrolled in this study. The study did not reach the planned accrual because of safety concerns as discussed in the toxicities section and logistical factors. Patient characteristics are summarized in Table 1. HER2 amplification was confirmed by fluorescent *in situ* hybridization in 10 patients. One patient had a tumor with HER2 3+ by immunohistochemistry but was negative by fluorescent *in situ* hybridization. Nine patients had received anthracyclines (range, 240-360; median, 240 mg/m^2), and four received trastuzumab as part of their adjuvant therapy. All patients had received trastuzumab for treatment of metastatic disease. The median cumulative duration of prior trastuzumab was 82 weeks (range, 22-136 weeks). The median time from last cycle of trastuzumab to initiation of trastuzumab and pertuzumab was 13 weeks (range, 4-96 weeks).

Toxicities. A total of 64 cycles of trastuzumab and pertuzumab were given. The major adverse events are summarized in

Table 3. Characteristics of patients experiencing cardiac toxicity on trastuzumab plus pertuzumab

Characteristic	Pt3	Pt6	Pt7	Pt8	Pt9	Pt11
Age	52	46	53	58	54	36
Baseline EF, % (method)	61 (Echo)	60 (Echo)	60 (Echo)	56 (Echo)	60 (Echo)	69 (Echo)
Lowest EF, % (method)	51 (Echo)	51 (Echo)	48 (MRI)	48 (Echo)	26 (MRI)	54 (Echo)
Absolute change in EF, %	10	9	12	8	34	15
LVSD (grade)	1	1	2	2	3	1
Cycles of T/P when LVSD occurred	18	2	2	1	2	1
Cumulative duration of prior T, wk	136	52	82	22	112	92
LSVD while on prior T	No	Yes	Yes	No	No	No
Cumulative dose of doxorubicin, mg/m^2	240	240	240	240	240	360
History of HTN	Yes	Yes	No	No	No	No
XRT to chest wall (side)	Yes (left)	No	No	Yes (right)	Yes (left)	Yes (right)
Extensive chest wall disease	No	No	No	No	Yes	No
Resolution of LVSD (time of follow-up)	Yes (3 wk)	Yes (3 mo)	Yes (3 mo)	No (4 mo)	No*	Yes (1 wk)

Abbreviations: Pt, patient; EF, ejection fraction; T, trastuzumab; P, pertuzumab; HTN, hypertension; XRT, radiation treatment; Echo, echocardiogram; MRI, magnetic resonance imaging.
*The patient died 2 mo after the onset of LVSD.

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Table 2. Grade 2 diarrhea was seen in 3 (27%) patients. Two patients had grade 1 and one patient had grade 3 allergic reaction related to infusion and symptoms resolved after antihistamine treatment. These patients received antihistamine before subsequent monoclonal antibody infusion.

Cardiac toxicities. A total of 92 echocardiogram and 8 cardiac magnetic resonance imaging studies were done. Six (54%) patients experienced a reduction in LVEF. Three patients had grade 1, two had grade 2, and one had grade 3 LVSD. The patient with the grade 3 LVSD experienced symptomatic congestive heart failure and required medical management. Grade 2/3 LVSD were associated with global hypokinesis. Two patients had a LVEF reduction of ≥15%. No specific electrocardiogram changes related to acute cardiac dysfunction were observed when cardiac toxicities occurred throughout the trial.

Table 3 lists the characteristics of patients who experienced cardiac toxicity. Two patients had a history of reduced LVEF during prior adjuvant trastuzumab-based treatment but tolerated subsequent rechallenge with trastuzumab for treatment of metastatic disease before receiving trastuzumab and pertuzumab. A reduced LVEF appeared within one to two cycles in most patients. Figure 1 shows LVEF in all patients and in patients who experienced cardiac toxicity. LVEF returned to normal in four patients within 1 week to 3 months after withholding treatment. One patient had persistent grade 2 LVSD 4 months after treatment discontinuation. The patient with congestive heart failure had a history of left chest wall radiation, baseline tachycardia, and extensive chest wall disease including an 8-cm mass extending through the chest wall. She had the greatest decrease in and the lowest absolute LVEF. She died of PD with congestive heart failure 2 months after treatment termination.

Efficacy. Two (18%) patients achieved a PR, 3 (27%) had SD for ≥18 weeks (range, 18-36 weeks), and 6 (55%) had PD while on treatment for ≤6 weeks. The median time to

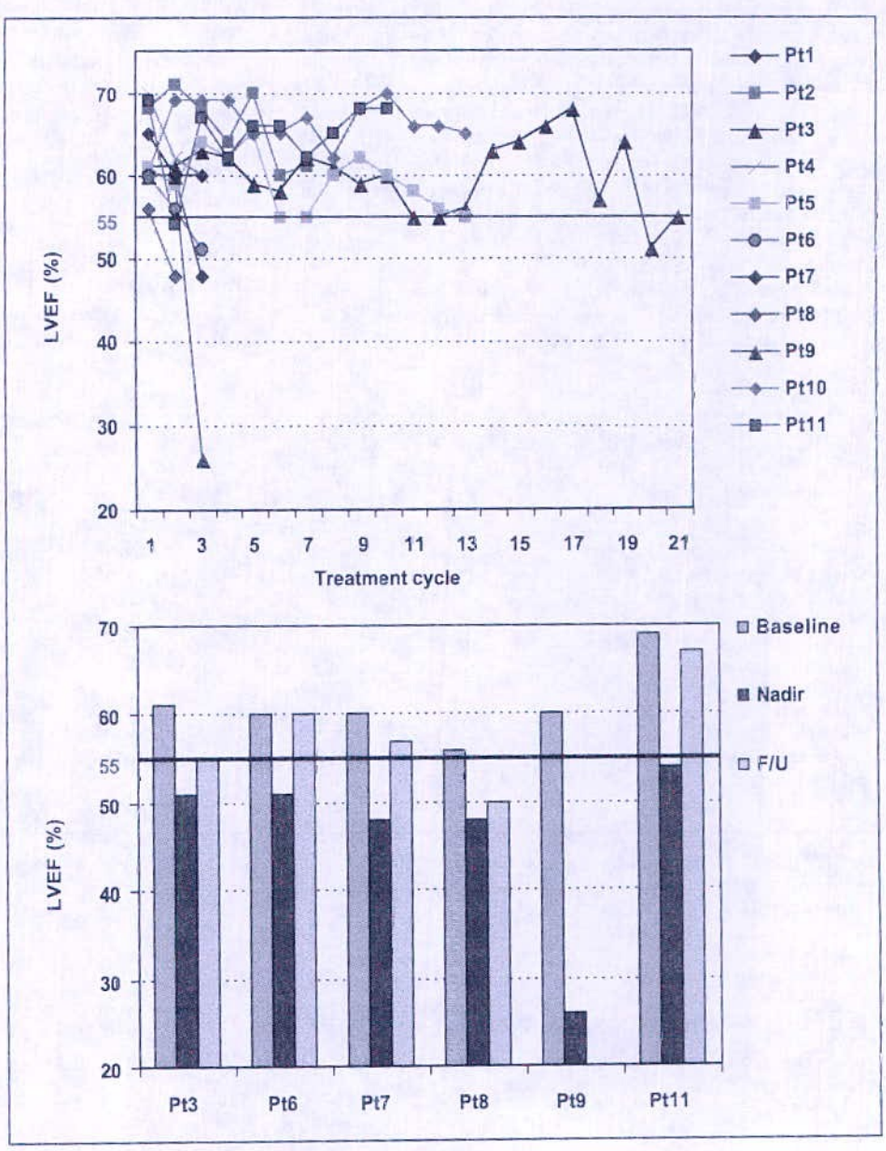


Fig. 1. *A*, LVEF in all patients treated with trastuzumab combined with pertuzumab. *B*, LVEF in patients treated with LVSD. Pt, patient; C, cycle of treatment; F/U, follow-up.

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Table 4. Immunohistochemical staining indices of the HER2 pathway markers and percentages of the Ki67-positive cells in the baseline tumors

Markers	PR (n = 2)	SD/PD (n = 6)
	Median (range)	
pHER2-Y1248	54.3-57.7	3.7 (0-44.0)*
pHER2-Y877	38.8 (33.4-44.1)	3.3 (0-44.5)
pMAPK	56.6 (42.4-70.8)	33.7 (12.9-90.1)
pAkt	13.5 (4.2-22.7)	1.6 (0-36.8)
HER2	64.3 (61.6-67.1)	74.6 (18.7-91.5)
Ki67 †	49.4 (37.2-61.6)	57.4 (18.6-95.3)

NOTE: Immunohistochemical staining index = (staining intensity of a sample - staining intensity of negative control) × percentage of stained cells / 100 (ref. 30).
*P = 0.095.
† Percentages of immunohistochemically stained cells.

progression was 6 weeks (range, 3-54 weeks). The two patients who achieved PR had nonvisceral disease only. One patient had received prior trastuzumab for 136 weeks, started trastuzumab and pertuzumab 96 weeks from the prior trastuzumab treatment, and achieved PR after 32 weeks. She remained in PR at 54 weeks and elected to discontinue treatment. The second responder had prior trastuzumab for 120 weeks, came on study 4 weeks from the prior trastuzumab treatment, and achieved PR after 12 weeks of treatment. Her disease progressed after 36 weeks.

Tumor proliferative state and HER2 pathway evaluation. Correlative studies were conducted on baseline tumors and tumors after trastuzumab and pertuzumab treatment. Eight baseline patient tumor samples (three from the metastatic and five from the primary tumors) and one paired pre- and post-trastuzumab/pertuzumab samples were evaluated for Ki67, HER2, pHER2-Y1248, pHER2-Y877, pMAPK, and pAkt by immunohistochemistry. Ki67-positive tumor cells ranged from 18.6% to 95.3% in the baseline tumors. Table 4 summarizes the immunohistochemical staining indices of the markers and the percentages of Ki67-positive cells. There were no statistical differences in the levels of Ki67 or HER2 pathway markers in the baseline tumors for patients with PR compared with those with SD or PD as determined by an exact Wilcoxon rank sum test. However, there was a trend toward higher levels of pHER2-Y1248 in the baseline tumors from patients with PR (54.3 and 57.7) compared with tumors from patients with SD or PD (median, 3.7; range, 0-44; P = 0.095). Interestingly, the levels of pHER2-Y1248, pHER2-Y877, pMAPK, pAkt, and Ki67 increased, whereas HER2 levels decreased, after one cycle of trastuzumab and pertuzumab in a patient who had PD (Fig. 2).

Discussion

In this study, the response rate to trastuzumab plus pertuzumab was 18%, which was consistent with an ongoing study of similar patient population with the same combination treatment (32). Due to the small size of this study, the definitive clinical efficacy of trastuzumab plus pertuzumab in the studied patient population cannot be determined. Overall, the combination trastuzumab and pertuzumab was well tolerated. However, a higher incidence of cardiac toxicity was observed.

Because of the known cardiac toxicity of trastuzumab and unknown cardiac effects of trastuzumab plus pertuzumab, we applied stringent cardiac evaluation criteria during this trial. In our study, 6 of 11 patients had a reduction in LVEF <55%, 3 had LVEF <50%, and 1 had symptomatic congestive heart failure with an LVEF of 26%. All patients had prior exposure to anthracyclines, the majority had exposure to adjuvant trastuzumab, and all of them had trastuzumab for treatment of metastatic disease. As cardiac safety was a major concern, accrual of this study was stopped before the planned enrollment of 37 patients.

Cardiac toxicity of trastuzumab was reported in clinical trials when trastuzumab was used alone or in combination with

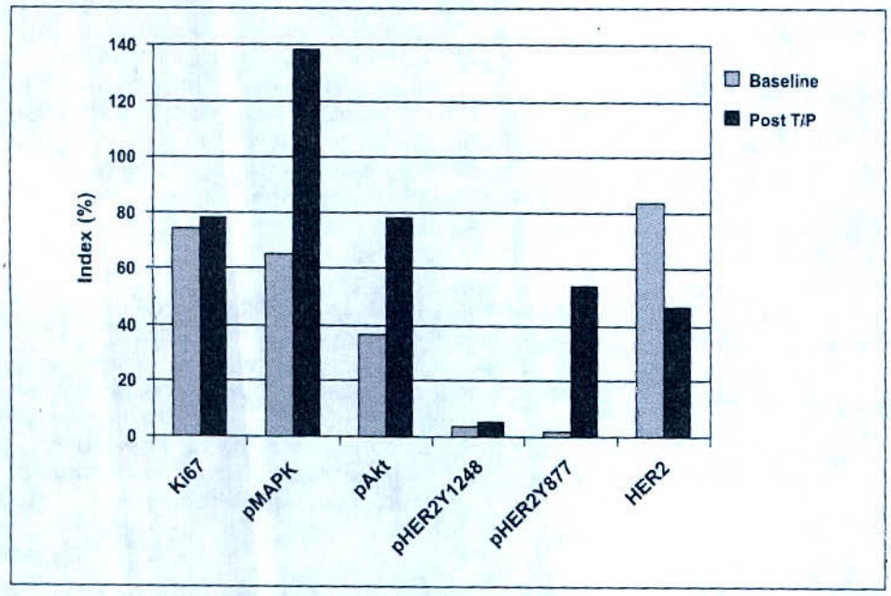


Fig. 2. Immunohistochemical staining indices of HER2 pathway markers and the percentage of Ki67-positive cells of a patient with progressive disease. IHC, immunohistochemistry; T/P, trastuzumab/pertuzumab.

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chemotherapy for treatment of HER2$^+$ metastatic breast cancer. Cardiac dysfunction occurred in 4.7% patients in one clinical trial and 2% in another when patients were treated with weekly trastuzumab alone (4, 5). When trastuzumab was given every 3 weeks, 16.4% patients had a reduction in LVEF of ≥15%, and 13.4% had LVEF <50%. Prior anthracycline exposure was associated with more frequent and worse cardiac dysfunction (6). It is known that anthracyclines cause direct damage to cardiac myocytes and long-term clinical adverse cardiac consequences (33, 34). It is conceivable that prior anthracycline exposure leads to a compromised myocardium and a reduced adaptive capacity to damages caused by trastuzumab and pertuzumab.

Interestingly, the cardiac events of trastuzumab-based treatment in the adjuvant setting ranged from 0% to 4.1%, as recently reviewed (35). These rates seemed to be lower than what was reported in metastatic breast cancer patients treated with trastuzumab. In the pivotal phase III clinical trial of the metastatic breast cancer patients, 27% and 13% had cardiac dysfunction after treatment with concurrent trastuzumab and anthracycline or trastuzumab plus paclitaxel, respectively (1). These observations support the hypothesis that trastuzumab-related cardiac toxicity may increase in the heavily pretreated population with more advanced disease, which could also account for the observed high incidences of cardiac toxicity in our study.

Cardiac toxicity due to pertuzumab has been documented in several studies. The phase I clinical study of pertuzumab reported that 2 of the 21 patients had reduced LVEF of ≤50% from normal baseline values and one of them developed congestive heart failure after two cycles of pertuzumab. It was not clear if these patients had prior exposure to trastuzumab or anthracyclines (19). In the study of pertuzumab for treatment of advanced ovarian cancer patients, 5 of 123 patients had a reduction in LVEF to <50% but only one was centrally confirmed (36). After two cycles of pertuzumab, 6 of the 67 hormone-refractory prostate patients experienced a decrease of >10% to a LVEF <50% (37). A recently published phase II study of pertuzumab in treatment of 41 advanced prostate cancer patients observed a 26.8% asymptomatic grade 1/2 reduction in LVEF, 4.9% asymptomatic LVEF <50%, and no congestive heart failure (38). Based on the tumor types, it is likely that few patients on the above discussed studies had prior exposure to anthracyclines or trastuzumab.

The majority of the cardiac toxicity observed in our study was asymptomatic and reversible. Although trastuzumab-induced cardiac dysfunction is reversible in most patients, the long-term cardiac effects of trastuzumab are still unknown (39). Similarly, the long-term cardiac effects of trastuzumab plus pertuzumab are yet to be determined. Furthermore, the NCI-CTCAE criteria have not been uniformly used for all the clinical trials. Therefore, the differences in the criteria used to evaluate cardiac toxicity must be taken into consideration.

The evaluation of HER2 signaling was exploratory and was limited due to the small numbers of tumor samples and yet yielded some interesting results. There was a trend toward increased levels of pHER2-Y1248 in baseline tumors from patients with PR after trastuzumab and pertuzumab treatment. Increased levels of pHER2 were associated with increased response to pertuzumab and improved progression-free survival, although not statistically significant, in ovarian cancer patients (36). More studies are needed to confirm these observations because pHER2 may have the potential to be used to select patients who would respond to pertuzumab-based treatment.

The tyrosine residues Y877 and Y1248 of HER2, residing in the activation loop and cytoplasmic tail, respectively, are autophosphorylated on HER2 receptor activation. Whereas pHER2Y877 regulates HER2 kinase activity, pHER2Y1248 serves as a docking site for cytoplasmic proteins of HER2 signal transduction cascade (40, 41). Both trastuzumab and pertuzumab reduced phosphorylated receptor tyrosine levels and inhibited HER2 signaling in the preclinical models (10, 18). We observed increased levels of pHER2Y877, pHER2Y1248, pMAPK, and pAkt, suggesting activation of the HER2 pathway in one patient with PD after trastuzumab/pertuzumab treatment. It has been reported that monoclonal antibodies against HER2 can either be growth inhibitory or stimulatory depending on the intracellular or extracellular localization of the monoclonal antibodies (42). Further clinical and laboratory studies are required to confirm this observation and to elucidate the potential mechanisms.

This observation is also consistent with the *in vitro* studies that showed incomplete blockade of the HER2 signaling and/or compensatory activation of other pathways accounting for the failure of HER2-targeted treatment. It has been shown that insulin-like growth factor type I receptor levels increased in trastuzumab-resistant tumor cells, and antibody to insulin-like growth factor type I receptor restored the sensitivity to trastuzumab (43, 44). A recent study showed that the combination of lapatinib, a HER1/HER2 tyrosine kinase inhibitor, with trastuzumab or with a polyclonal antibody against HER2 significantly down-regulated survivin and enhanced apoptosis of the tumor cells *in vitro* (45). Triple combination of trastuzumab, pertuzumab, and gefitinib effectively blocked signals from HER homodimerization and heterodimerization and inhibited the growth of HER2$^+$ breast cancer xenografts more effectively than single agents or dual combinations (46). Targeting other HER2 downstream kinases such as mammalian target of rapamycin is also being tested in clinical trials (47). Based on the available information, it is apparent that complete blockade of HER receptor signaling at multiple levels may be required for effective growth inhibition of HER2$^+$ tumors.

In conclusion, more studies are needed to define the efficacy of trastuzumab plus pertuzumab and to identify patients who could benefit from this treatment with minimal cardiac toxicity. Although stringent criteria for evaluation of cardiac toxicity and advanced disease may have contributed to the observed high rate of cardiac toxicity, cardiac safety is an issue of concern. Prior exposure to anthracyclines and prior left ventricular dysfunction with trastuzumab treatment may be potential risk factors of cardiac toxicity for patients who are treated with trastuzumab plus pertuzumab. Cardiac status should be closely monitored in future clinical trials using this combination treatment.

Disclosure of Potential Conflicts of Interest

S.M. Swain: travel costs defrayed by Genentech. The other authors disclosed no potential conflicts of interest.

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EL SUSCRITO SECRETARIO DE LA CAMARA DE COMERCIO DE CALI

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DIRECCION DOMICILIO PRINCIPAL:CRA. 1 NRO. 46 84
DIRECCION NOTIFICACION JUDICIAL:CRA. 1 NRO. 46 84
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CERTIFICA

QUE POR ESCRITURA NRO. 1434 DEL 29 DE SEPTIEMBRE DE 1958 NOTARIA CUARTA DE CALI ,INSCRITA EN LA CAMARA DE COMERCIO EL 02 DE OCTUBRE DE 1958 BAJO EL NRO. 18319 DEL LIBRO IX ,SE CONSTITUYO LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.

CERTIFICA

QUE POR ESCRITURA NRO. 3672 DEL 24 DE AGOSTO DE 2009 NOTARIA TERCERA DE CALI ,INSCRITA EN LA CAMARA DE COMERCIO EL 27 DE AGOSTO DE 2009 BAJO EL NRO. 9876 DEL LIBRO IX ,CAMBIO SU NOMBRE DE LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A. . POR EL DE LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A. SIGLA: LAFRANCOL S.A. .

CERTIFICA

QUE POR ACTA NRO. 124 DEL 30 DE MARZO DE 2012 ASAMBLEA GENERAL ,INSCRITA EN LA CAMARA DE COMERCIO EL 31 DE MAYO DE 2012 BAJO EL NRO. 6645 DEL LIBRO IX ,SE TRANSFORMO DE SOCIEDAD ANÓNIMA EN SOCIEDAD POR ACCIONES SIMPLIFICADA BAJO EL NOMBRE DE LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.S. SIGLA: LAFRANCOL S.A.S. .

CERTIFICA

QUE POR ACTA NRO. 124 DEL 30 DE MARZO DE 2012 ASAMBLEA DE ACCIONISTAS ,INSCRITA EN LA CAMARA DE COMERCIO EL 03 DE DICIEMBRE DE 2012 BAJO EL NRO. 14136 DEL LIBRO IX ,SE APROBO LA ESCISION ENTRE (ESCINDENTE) AMERICAN GENERICS S.A.S. Y (BENEFICIARIA(S)) LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.S. .

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ACT 135	02/10/2014	ASAMBLEA DE ACCIONISTAS	17/10/2014 13928	IX

CERTIFICA

VIGENCIA: INDEFINIDA

CERTIFICA

OBJETO SOCIAL. LA SOCIEDAD EN DESARROLLO DE SU OBJETO SOCIAL PODRÁ REALIZAR, ENTRE OTROS, LAS SIGUIENTES ACTIVIDADES COMERCIALES Y EMPRESARIALES: 2.1) FABRICACIÓN, DISTRIBUCIÓN Y VENTA EN EL TERRITORIO DE LA REPÚBLICA DE COLOMBIA O EN EL EXTRANJERO DE CUALESQUIERA TIPOS DE VACUNAS, PRODUCTOS QUÍMICOS, FARMACÉUTICOS, ALIMENTICIOS FUNCIONALES, COSMÉTICOS Y FISIOLÓGICOS, REACTIVOS, ANTIBIÓTICOS PARA USO HUMANO Y VETERINARIO, PRODUCTOS DIETÉTICOS, COLORANTES, DE PERFUMERÍA, DISPOSITIVOS MÉDICOS Y AFINES. 2.2) LA REPRESENTACIÓN DE CASAS FABRICANTES NACIONALES O EXTRANJERAS DE ESTOS MISMOS PRODUCTOS. 2.3) FABRICACIÓN, IMPORTACIÓN, DISTRIBUCIÓN Y VENTA DE CUALESQUIERA TIPO DE PRODUCTOS VETERINARIOS. 2.4) LA REALIZACIÓN DE TODOS LOS ACTOS, CONTRATOS, CONVENIOS, DIRECTAMENTE RELACIONADOS CON LOS FINES ANTES INDICADOS. 2.5) IMPORTAR CUALESQUIERA TIPOS DE VACUNAS, PRODUCTOS QUÍMICOS, FARMACÉUTICOS, ALIMENTICIOS FUNCIONALES, COSMÉTICOS Y FISIOLÓGICOS, REACTIVOS, ANTIBIÓTICOS PARA USO HUMANO Y

VETERINARIO, PRODUCTOS DIETÉTICOS, COLORANTES, DE PERFUMERÍA, DISPOSITIVOS MÉDICOS Y AFINES Y/O PRODUCTOS INTERMEDIOS O A GRANEL. PARÁGRAFO UNO: EN DESARROLLO DE SU OBJETO SOCIAL PODRÁ LA SOCIEDAD ADQUIRIR Y VENDER BIENES RAÍCES Y MUEBLES, DARLOS Y TOMARLOS EN ARRENDAMIENTO, GARANTIZAR CON HIPOTECA SOBRE LOS BIENES RAÍCES O CON PRENDA SOBRE LOS BIENES MUEBLES, OBLIGACIONES A CARGO DE LA SOCIEDAD; DAR Y RECIBIR EN MUTUO DINERO Y CELEBRAR LOS CONTRATOS COMERCIALES DE CAMBIO EN TODAS SUS MANIFESTACIONES, FUNDAR EMPRESAS QUE TENGAN O NO LA MISMA FINALIDAD SOCIAL O ASOCIARSE CON ELLAS, O CUALESQUIERA OTRAS EMPRESAS DE OTROS OBJETOS SOCIALES. PARÁGRAFO DOS: ADEMÁS DE LAS ACTIVIDADES REFERIDAS EN ESTE ARTICULO, Y LAS ENUNCIADAS EN EL PARÁGRAFO UNO, COMO SOCIEDAD POR ACCIONES SIMPLIFICADA, PODRÁ REALIZAR CUALQUIER ACTIVIDAD LICITA DE COMERCIO EN EL PAÍS O EN EL EXTERIOR, POR EJEMPLO, Y DE MANERA ILUSTRATIVA MÁS NO LIMITATIVA, GESTIÓN DE LICENCIAS, REGISTROS DE EXPORTACIÓN O IMPORTACIÓN, CONVENIOS RELATIVOS A ESAS ACTIVIDADES Y LOS ACTOS RELACIONADOS CON EL MISMO. PARÁGRAFO TRES: POR RAZÓN DE SU OBJETO SOCIAL, LA SOCIEDAD NO PODRÁ CONSTITUIRSE GARANTE NI FIADORA DE OBLIGACIONES DISTINTAS DE LAS PROPIAS NI GARANTIZAR CON SUS BIENES OBLIGACIONES DE TERCEROS, A MENOS QUE LO AUTORICE EN CADA CASO LA ASAMBLEA GENERAL DE ACCIONISTAS CON EL VOTO DE LA TOTALIDAD DE LOS MIEMBROS PRESENTES EN LA REUNIÓN CORRESPONDIENTE.

CERTIFICA

DIRECCIÓN Y ADMINISTRACIÓN. LA DIRECCIÓN Y ADMINISTRACIÓN DE LA SOCIEDAD ESTARÁ A CARGO DE: A) LA ASAMBLEA GENERAL DE ACCIONISTAS; Y B) LOS REPRESENTANTES LEGALES COMO SE DESCRIBEN EN SUS CARGOS Y FUNCIONES EN LOS PRESENTES ESTATUTOS.

REPRESENTACIÓN LEGAL. ADMINISTRACIÓN. LA ADMINISTRACIÓN DE LA SOCIEDAD CORRESPONDE AL REPRESENTANTE LEGAL, QUE SE DENOMINARÁ GERENTE, QUIEN TENDRÁ HASTA CINCO SUPLENTES. EL GERENTE Y SUS SUPLENTES SERÁN LOS REPRESENTANTES LEGALES QUIENES SERÁN DESIGNADOS POR LA ASAMBLEA GENERAL DE ACCIONISTAS POR UN TÉRMINO INDEFINIDO Y SE MANTENDRÁN VIGENTES HASTA TANTO ESTOS NO SEAN REMOVIDOS DE SUS CARGOS POR LA ASAMBLEA GENERAL DE ACCIONISTAS. LOS SUPLENTES REEMPLAZARÁN AL GERENTE, DURANTE SUS AUSENCIAS TEMPORALES, ABSOLUTAS O ACCIDENTALES.

FUNCIONES DEL REPRESENTANTE LEGAL.: SON FUNCIONES DEL GERENTE: (A) REPRESENTAR A LA SOCIEDAD ANTE LOS ACCIONISTAS, ANTE TERCEROS Y ANTE AUTORIDADES DE LOS ÓRDENES ADMINISTRATIVO O JURISDICCIONAL, CON FACULTADES PARA TRANSIGIR, COMPROMETER Y DESISTIR Y PARA COMPARECER EN JUICIO; (B) SUPERVISAR EL COMPORTAMIENTO DE LOS EMPLEADOS DE LA SOCIEDAD PARA ASEGURAR EL CUMPLIMIENTO DE SUS OBLIGACIONES. (C) CUMPLIR Y HACER CUMPLIR LAS DETERMINACIONES DE LA ASAMBLEA GENERAL DE ACCIONISTAS. (D) PRESENTAR, EN LAS REUNIONES ORDINARIAS DE LA ASAMBLEA GENERAL DE ACCIONISTAS, EL BALANCE GENERAL DE LOS NEGOCIOS DEL RESPECTIVO EJERCICIO, JUNTO CON EL ESTADO DE RESULTADOS, UN PROYECTO SOBRE DISTRIBUCIÓN DE UTILIDADES O SOBRE CANCELACIÓN DE PÉRDIDAS, JUNTO CON UN INFORME SOBRE EL ESTADO DE LOS NEGOCIOS DE LA SOCIEDAD. (E) SUMINISTRAR A LA ASAMBLEA GENERAL DE ACCIONISTAS LOS INFORMES QUE ÉSTAS LE SOLICITEN. (F) OTORGAR LOS PODERES QUE SEAN NECESARIOS PARA LA DEFENSA O REPRESENTACIÓN DE LA SOCIEDAD. (G) SOMETER LAS DIFERENCIAS QUE PUEDAN SURGIR ENTRE LA SOCIEDAD Y TERCEROS A LA DECISIÓN DE ÁRBITROS Y CONCURRIR A LA DESIGNACIÓN DE DICHOS ÁRBITROS Y DEL APODERADO QUE HAYA DE REPRESENTARLA ANTE EL RESPECTIVO TRIBUNAL. (H) EJERCER LIBREMENTE TODOS LOS ACTOS Y ACUERDOS APROPIADOS Y NECESARIOS PARA EL CUMPLIMIENTO DEL OBJETO SOCIAL, Y (I) EJERCER LAS DEMÁS ATRIBUCIONES QUE LE CONFIEREN LAS LEYES Y LOS ESTATUTOS SOCIALES Y LAS DEMÁS QUE LE CORRESPONDAN EN RAZÓN DE LA NATURALEZA DE SU CARGO.

EL GERENTE REQUIERE AUTORIZACIÓN PREVIA DE LA ASAMBLEA GENERAL DE ACCIONISTAS, PARA LLEVAR A CABO LOS SIGUIENTES ACTOS:

1. CELEBRAR TODA CLASE DE CONTRATOS CUYO OBJETO SEA LA APERTURA DE SUCURSALES, AGENCIAS

O DEPENDENCIAS, O LA EMISIÓN DE BONOS DE LA SOCIEDAD.

2. APROBAR, CUALQUIERA SEA SU NATURALEZA, TODOS LOS ACTOS QUE IMPLIQUEN RENUNCIA A DERECHOS DE LA SOCIEDAD, TALES COMO TRANSACCIONES, DESISTIMIENTOS, CONCILIACIONES O ACTOS DE SIMILAR NATURALEZA, CUYA CUANTÍA ABSOLUTA SEA SUPERIOR AL EQUIVALENTE EN MONEDA LEGAL COLOMBIANA A LA SUMA DE QUINIENTOS MIL DÓLARES DE LOS ESTADOS UNIDOS DE AMÉRICA (USD $500.000.00). EN LOS ASUNTOS DE NATURALEZA LABORAL ESTE REQUISITO SE EXIGIRÁ CUANDO LA REMUNERACIÓN ANUAL DEL RESPECTIVO TRABAJADOR SEA SUPERIOR A SEISCIENTOS CINCUENTA SALARIOS MÍNIMOS LEGALES MENSUALES VIGENTES (650 SMLMV) DE LA REPÚBLICA DE COLOMBIA.

3. INICIAR CUALQUIER ACCIÓN JUDICIAL O ADMINISTRATIVA CUYA CUANTÍA ABSOLUTA SEA SUPERIOR AL EQUIVALENTE EN MONEDA LEGAL COLOMBIANA A LA SUMA DE QUINIENTOS MIL DÓLARES DE LOS ESTADOS UNIDOS DE AMÉRICA (USD $500.000.00).

4. SUSCRIBIR O REALIZAR CUALQUIER CONTRATO, ACUERDO, ACTO U OPERACIÓN PARA LA ADQUISICIÓN, VENTA, CESIÓN, TRASPASO O DISPOSICIÓN DE ACTIVOS FIJOS DE LA SOCIEDAD CUYO VALOR EN PESOS COLOMBIANOS, O EN CUALQUIER OTRA MONEDA EXCEDA DE UN MILLÓN DE DÓLARES DE LOS ESTADOS UNIDOS DE AMÉRICA (USD $1.000.000).

5. GRAVAR O ASUMIR CUALQUIER GRAVAMEN SOBRE LOS ACTIVOS FIJOS DE LA SOCIEDAD, CUALQUIERA QUE SEA SU VALOR.

6. OTORGAR GARANTÍAS POR PARTE DE LA SOCIEDAD PARA GARANTIZAR OBLIGACIONES DE TERCEROS, SALVO POR/AS OBLIGACIONES DE COMPAÑÍAS FINALES DE LA SOCIEDAD.

7. LAS OPERACIONES FINANCIERAS Y BANCARIAS DEBEN REALIZARSE DE ACUERDO CON LAS POLÍTICAS ESTABLECIDAS POR LA ASAMBLEA GENERAL DE ACCIONISTAS, QUIEN CONFORMARÁ UN COMITÉ DE FINANZAS, PARA ESTE FIN.

PARA CALCULAR EL EQUIVALENTE DE LOS MONTOS ANTERIORES EN PESOS COLOMBIANOS, SERÁ USADA LA TASA REPRESENTATIVA DEL MERCADO (TRM) CERTIFICADA POR LA SUPERINTENDENCIA FINANCIERA O LA ENTIDAD COMPETENTE PARA EL DÍA EN QUE SE SUSCRIBA O REALICE LA TRANSACCIÓN.

CERTIFICA

DOCUMENTO: ACTA No. 134 DEL 26 DE SEPTIEMBRE DE 2014
ORIGEN: ASAMBLEA DE ACCIONISTAS
INSCRIPCION: 17 DE OCTUBRE DE 2014 No. 13926 DEL LIBRO IX

FUE(RON) NOMBRADO(S):

GERENTE
MANUEL CAMILO CAMACHO PEREZ
C.C.80504680

PRIMER SUPLENTE DEL GERENTE
MARIO FRANCISCO PEREZ ANZOLA
C.C.79656956

SEGUNDO SUPLENTE DEL GERENTE
MARIA ISABEL RIVAS PARRA
C.C.52251884

Cámara de Comercio de Cali

CODIGO DE VERIFICACION: 0816HP38BU
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CERTIFICA

DOCUMENTO: ACTA No. 135 DEL 02 DE OCTUBRE DE 2014
ORIGEN: ASAMBLEA DE ACCIONISTAS
INSCRIPCION: 17 DE OCTUBRE DE 2014 No. 13929 DEL LIBRO IX

FUE(RON) NOMBRADO(S):

TERCER SUPLENTE DEL GERENTE
FRANCISCO JAVIER PICART LAURENZ
C.E.399661

CUARTO SUPLENTE DEL GERENTE
TITO NOE PARRA MURILLO
C.C.79579680

CERTIFICA

DOCUMENTO: ACTA No. 139 DEL 23 DE NOVIEMBRE DE 2015
ORIGEN: ASAMBLEA DE ACCIONISTAS
INSCRIPCION: 07 DE DICIEMBRE DE 2015 No. 23664 DEL LIBRO IX

FUE(RON) NOMBRADO(S):

REVISOR FISCAL
ERNST & YOUNG AUDIT S.A.S.
NIT.860008890-5

CERTIFICA

DOCUMENTO: DOCUMENTO PRIVADO DEL 02 DE DICIEMBRE DE 2015
ORIGEN: ERNST & YOUNG AUDIT S.A.S
INSCRIPCION: 07 DE DICIEMBRE DE 2015 No. 23665 DEL LIBRO IX

FUE(RON) NOMBRADO(S):

REVISOR FISCAL PRINCIPAL
YENCI VIVIANA SARMIENTO GRACIA
C.C.38669429

REVISOR FISCAL SUPLENTE
MANUEL ANTONIC RAMIREZ YANTEN
C.C.1117496898

CERTIFICA

CAPITAL AUTORIZADO: $40,000,000,224
NUMERO DE ACCIONES: 3,333,333,352
VALOR NOMINAL: $12
CAPITAL SUSCRITO: $24,140,480,940
NUMERO DE ACCIONES: 2,011,706,745
VALOR NOMINAL: $12
CAPITAL PAGADO: $24,140,480,940
NUMERO DE ACCIONES: 2,011,706,745
VALOR NOMINAL: $12

CERTIFICA

CERTIFICA ESPECIAL POR VOLUNTAD DEL USUARIO:

DOCUMENTO: DOCUMENTO PRIVADO DEL 22 ENERO DEL 2015
INSCRIPCIÓN: 11 DE FEBRERO DE 2015 NRO. 1943 DEL LIBRO IX

CONSTA EL GRUPO EMPRESARIAL :

MATRIZ: ABBOTT INVESTMENTS LUXEMBOURG S.A.R.L.
DOMICILIO: LUXEMBURGO
NACIONALIDAD: LUXEMBURGUESA
ACTIVIDAD: PARTICIPAR DE CUALQUIER FORMA EN CUALQUIER ACTIVIDAD DE TIPO COMERCIAL, INDUSTRIAL, FINANCIERA U OTRA, EN LUXEMBURGO O EN CUALQUIER COMPAÑÍA O EMPRESA EXTRANJERA.

SUBORDINADA: ABBOTT LABORATORIES DE COLOMBIA S.A
NIT: 860002134-8
DOMICILIO: BOGOTA D.C.
NACIONALIDAD: COLOMBIANA.
ACTIVIDAD: IMPORTACION, EXPORTACION, DISTRIBUICION, COMPRA Y VENTA DE TODA CLASE DE MEDICAMENTOS Y PRODUCTOS QUIMICOS Y FARMACÉUTICOS Y ESPECIALIDADES MEDICINALES PARA USO HUMANO Y VETERINARIO.

SUBORDINADA: LABORATORIOS SYNTHESIS S.A.S
NIT; 860000760-1
DOMICILIO: BOGOTA D.C
NACIONALIDAD: COLOMBIANA.
ACTIVIDAD: IMPORTACION, EXPORTACION, FABRICACIÓN, COMERCIALIZACION, DISTRIBUCION Y VENTA DE PRODUCTOS FARMACÉUTICOS, HIGIENICOS, VETERINARIOS E IMPLEMENTOS MEDICOS.

SUBORDINADA: LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.S.
NIT.890301463-8
DOMICILIO: CALI
NACIONALIDAD: COLOMBIANA
ACTIVIDAD: LA FABRICACIÓN, DISTRIBUCIÓN Y VENTA EN EL TERRITORIO DE LA REPÚBLICA DE COLOMBIA O EN EL EXTRANJERO, DE VACUNAS, PRODUCTOS QUÍMICOS, FARMACÉUTICOS, ALIMENTICIOS FUNCIONALES, COSMÉTICOS Y FISIOLÓGICOS, REACTIVOS, ANTIBIÓTICOS PARA USO HUMANO Y VETERINARIO

SUBORDINADA: AMERICAN GENERICS S A S

NIT. 800223214-9
DOMICILIO: CALI
NACIONALIDAD: COLOMBIANA
ACTIVIDAD: FABRICAR, COMPRAR, VENDER, ADQUIRIR, MANTENER, IMPORTAR, EXPORTAR, DISTRIBUIR Y ENAJENAR A CUALQUIER TITULO PRODUCTOS, INSUMOS, ARTÍCULOS O MERCANCÍAS DE USO HUMANO Y/O VETERINARIO Y PRESTAR LOS SERVICIOS CORRESPONDIENTES CON DICHOS INSUMOS, ARTÍCULOS, ETC.

SUBORDINADA: LABORATORIOS NATURMEDIK S.A.S.
NIT. 800226363-1
DOMICILIO: CALI
NACIONALIDAD: COLOMBIANA
ACTIVIDAD: FABRICAR, COMPRAR, VENDER, ADQUIRIR, MANTENER, IMPORTAR, EXPORTAR, DISTRIBUIR Y ENAJENAR A CUALQUIER TITULO PRODUCTOS, INSUMOS, ARTÍCULOS O MERCANCÍAS DE USO HUMANO Y/O VETERINARIO Y PRESTAR LOS SERVICIOS CORRESPONDIENTES CON DICHOS INSUMOS, ARTÍCULOS, ETC.

SUBORDINADA: LABORATORIOS PAULY PHARMACEUTICAL S.A.S.
NIT: 800.097.402-6
DOMICILIO: CALI, VALLE
NACIONALIDAD: COLOMBIANA
ACTIVIDAD: FABRICAR, COMPRAR, VENDER, ADQUIRIR, MANTENER, IMPORTAR, EXPORTAR, DISTRIBUIR Y ENAJENAR A CUALQUIER TÍTULO PRODUCTOS, INSUMOS, ARTÍCULOS O MERCANCÍAS DE USO HUMANO Y/O VETERINARIO Y PRESTAR LOS SERVICIOS CORRESPONDIENTES CON DICHOS INSUMOS, ARTÍCULOS ETC.

SUBORDINADA: DISTRIBUCIONES UQUIFA S.A.S.
NIT: 800182021-7
DOMICILIO: CALI, VALLE
NACIONALIDAD: COLOMBIANA
ACTIVIDAD: FABRICAR, COMPRAR, VENDER, ADQUIRIR, MANTENER, IMPORTAR, EXPORTAR, DISTRIBUIR Y ENAJENAR A CUALQUIER TÍTULO PRODUCTOS, INSUMOS, ARTÍCULOS O MERCANCÍAS DE USO HUMANO Y/O VETERINARIO Y PRESTAR LOS SERVICIOS CORRESPONDIENTES CON DICHOS INSUMOS, ARTÍCULOS ETC.

SUBORDINADA: FOCUS PHARMACEUTICAL S.A.S
NIT: 900.104.555-8
DOMICILIO: CALI, VALLE
NACIONALIDAD: COLOMBIANA
ACTIVIDAD: FABRICAR, COMPRAR, VENDER, ADQUIRIR, MANTENER, IMPORTAR, EXPORTAR, DISTRIBUIR Y ENAJENAR A CUALQUIER TÍTULO PRODUCTOS, INSUMOS, ARTÍCULOS O MERCANCÍAS DE USO HUMANO Y/O VETERINARIO Y PRESTAR LOS SERVICIOS CORRESPONDIENTES CON DICHOS INSUMOS, ARTÍCULOS ETC.

SUBORDINADA: LAFRANCOL PERU S.R.L.
DOMICILIO:LIMA
NACIONALIDAD: PERUANA
ACTIVIDAD: DISTRIBUCION Y COMERCIALIZACION DE PRODUCTOS FARMACEUTICOS DE USO HUMANO.

SUBORDINADA: LAFRANCOL DOMINICANA S.A.S.
DOMICILIO: SANTO DOMINGO
NACIONALIDAD: REPUBLICA DOMINICANA
ACTIVIDAD: FABRICACION DE PRODUCTOS FARMACEUTICOS.

SUBORBINADA: LAFRANCOL GUATEMALA S.A.
DOMICILIO: GUATEMALA

NACIONALIDAD: GUATEMALTECA
ACTIVIDAD: PRODUCCION, FABRICACION, EXPORTACION Y DISTRIBUCION DE PRODUCTOS FARMACEUTICOS Y/O ALIMENTICIOS Y NATURALES DE CONSUMO HUMANO Y LA COMERCIALIZACION DE TODA CLASE DE MERCANCIAS POR CUENTA PROPIA O EN NOMBRE DE TERCEROS.

PRESUPUESTO DE CONTROL: ABBOTT INVESTMENTS LUXEMBOURG S.A. R.L ADQUIRIÓ EL 26 DE SEPTIEMBRE DE 2014 UNA PARTICIPACIÓN INDIRECTA DE MAS DEL 50% DEL CAPITAL DE LABORATORIO FRANCO COLOMBIANO LAFRANCOL SAS.
UNA PARTICIPACIÓN INDIRECTA EN EL CAPITAL DE LAS SOCIEDADES ACTUALMENTE REGISTRADAS COMO SUBORDINADAS DE LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.S EN COLOMBIA Y EN EL EXTERIOR (LAS SOCIEDADES SUBORDINADAS A SABER: AMERICAN GENERICS S A S, LABORATORIOS NATURMEDIK S.A.S., LABORATORIOS PAULY PHARMACEUTICAL S.A.S., DISTRIBUCIONES UQUIFA S.A.S., FOCUS PHARMACEUTICAL S.A.S. LAFRANCOL PERU SRL, LAFRANCOL DOMINICANA S.A.S, LAFRANCOL GUATEMALA S.A.)
UNA PARTICIPACIÓN INDIRECTA DE MAS DEL 50% DEL CAPITAL DE LABORATORIOS SYNTHESIS S.A.S, CON DOMICILIO EN BOGOTA E IDENTIFICADA CON NIT: 860000760-1
ABBOTT INVESTMENTE LUXEMBOURG S.A.R.L. TIENE UNA PARTICIPACIÓN DIRECTA DEL MAS DEL 50% EN EL CAPITAL DE ABBOT LABORATORIES DE COLOMBIA S.A SITUACION DE CONTROL QUE FUE REGISTRADA MEDIANTE DOCUMENTO PRIVADO DEL 15 DE OCTUBRE DE 2013, INSCRITO EL 22 DE OCTUBRE DEL MISMO AÑO.
ADEMÁS DEL VÍNCULO DE SUBORDINACIÓN POR PARTICIPACIÓN QUE EXISTE ENTRE ABBOTT INVESTMENTS LUXEMBOURG S.A. R.L, (SOCIEDAD CONTROLANTE) Y LAS SOCIEDADES COLOMBIANAS ABBOTT LABORATORIES DE COLOMBIA S.A., LABORATORIOS SYNTHESIS S.A.S., LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.S, Y LAS SOCIEDADES SUBORDINADAS, EN LOS TÉRMINOS DEL NUMERAL 1 DEL ARTÍCULO 261 DEL CÓDIGO DE COMERCIO; EXISTE ENTRE DICHAS SOCIEDADES UNIDAD DE PROPÓSITO Y DIRECCIÓN, SEGÚN LO DISPUESTO EN EL ARTÍCULO 28 DE LA LEY 222 DE 1995. POR LO TANTO, SE CONFIGURA UN GRUPO EMPRESARIAL A PARTIR DEL 26 DE SEPTIEMBRE DE 2014.

CERTIFICA

QUE A NOMBRE DE LA SOCIEDAD FIGURA MATRICULADO EN LA CAMARA DE COMERCIO BAJO EL NRO.5234-2 ESTABLECIMIENTO DE COMERCIO: LABORATORIO FRANCO COLOMBIANO LAFRANCOL
UBICADO EN: ---K 1 46 84 DE CALI
RENOVO : POR EL AÑO 2016

CERTIFICA

QUE A NOMBRE DE LA SOCIEDAD FIGURA MATRICULADO EN LA CAMARA DE COMERCIO BAJO EL NRO.870576-2 ESTABLECIMIENTO DE COMERCIO: LABORATORIOS FRANCO COLOMBIANO LAFRANCOL SAS
UBICADO EN: CL. 36 A NRO. 4 03 DE CALI
FECHA MATRICULA : 02 DE MAYO DE 2013
RENOVO : POR EL AÑO 2016

CERTIFICA

QUE LA SOCIEDAD EFECTUO LA RENOVACION DE SU MATRICULA MERCANTIL EL 30 DE MARZO DE 2016
.

CERTIFICA

QUE NO FIGURAN OTRAS INSCRIPCIONES QUE MODIFIQUEN TOTAL O PARCIALMENTE EL PRESENTE CERTIFICADO.
LOS ACTOS ADMINISTRATIVOS DE REGISTRO QUEDAN EN FIRME DIEZ (10) DIAS HABILES DESPUES DE LA FECHA DE SU INSCRIPCION, SIEMPRE Y CUANDO DENTRO DE DICHO TERMINO NO SEAN OBJETO DE

Cámara de Comercio de Cali

CODIGO DE VERIFICACION: 0816HP38BU
NUMERO DE RADICACION: 20160259264-INT
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RECURSOS.

LA PERSONA O ENTIDAD A LA QUE USTED LE VA A ENTREGAR EL CERTIFICADO PUEDE VERIFICAR, POR UNA SOLA VEZ, SU CONTENIDO INGRESANDO A http://www.ccc.org.co/registraya/ Y DIGITANDO EL CODIGO DE VERIFICACION QUE SE ENCUENTRA EN EL ENCABEZADO DEL PRESENTE DOCUMENTO.
EL CERTIFICADO A VALIDAR CORRESPONDE A LA IMAGEN Y CONTENIDO DEL CERTIFICADO CREADO EN EL MOMENTO EN QUE SE GENERO EN LAS SEDES O A TRAVES DE LA PLATAFORMA VIRTUAL DE LA CAMARA.

ESTE CERTIFICADO CUENTA CON PLENA VALIDEZ JURIDICA SEGUN LO DISPUESTO EN LA LEY 527 DE 1999. EN EL SE INCORPORAN TANTO LA FIRMA MECANICA QUE ES UNA REPRESENTACION GRAFICA DE LA FIRMA DEL SECRETARIO DE LA CAMARA DE COMERCIO DE CALI, COMO LA FIRMA DIGITAL , LAS CUALES PODRA VERIFICAR A TRAVES DE SU APLICATIVO VISOR DE DOCUMENTOS PDF.
LA PERSONA O ENTIDAD A LA QUE USTED LE VA A ENTREGAR EL CERTIFICADO PUEDE VERIFICAR, POR UNA SOLA VEZ, SU CONTENIDO INGRESANDO A http://www.ccc.org.co/registraya/ Y DIGITANDO EL CODIGO DE VERIFICACION QUE SE ENCUENTRA EN EL ENCABEZADO DEL PRESENTE DOCUMENTO.
EL CERTIFICADO A VALIDAR CORRESPONDE A LA IMAGEN Y CONTENIDO DEL CERTIFICADO CREADO EN EL MOMENTO EN QUE SE GENERO EN LAS SEDES O A TRAVES DE LA PLATAFORMA VIRTUAL DE LA CAMARA.

DADO EN CALI A LOS 09 DIAS DEL MES DE ABRIL DEL AÑO 2016 HORA: 05:09:08 PM