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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SEÑOR

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

E.

S.

D.

REF:

Expediente 09 018.558

TÍTULO SOLICITADO: TERAPIA TUMORAL CON UN ANTICUERPO ANTI-VEGF.

PUBLICACIÓN: Gaceta 615 de 20 de abril de 2010, N° de publicación 193.

PRIORIDAD: EP 06017330.9 de 21/08/2006.

SOLICITANTE: F. HOFFMANN-LA ROCHE AG.

APODERADO: ALICIA LLORED A RICAURTE.

TRÁMITE: SUSTENTACION DE OPOSICIÓN

JOSÉ LUIS REYES VILLAMIZAR, mayor de edad, identificado como aparece al pie de mi firma, abogado en ejercicio, titular de la tarjeta profesional número 44655 del Consejo Superior de la Judicatura, obrando en calidad de apoderado de la sociedad **LABORATORIO FRANCO-COLOMBIANO, LAFRANCOL S.A.**, dentro del trámite de la referencia, en ejercicio de lo dispuesto por el artículo 42 inciso 2° de la Decisión 486 de 2000, por medio del presente escrito, procedo a sustentar la oposición presentada oportunamente ante ese Despacho, en los siguientes términos:

I. COMPLEMENTO DE INFORMACIÓN

Comendidamente me permito presentar los argumentos que demuestran que la solicitud de la referencia incumple los requisitos de patentabilidad establecidos por

los artículos 14, 16, 18 y 20 literal d) de la Decisión 486 de 2000, y que por ello no merece recibir el beneficio patentario, veamos:

En primer lugar, y ante la reivindicación textual del uso de los anticuerpos que se reivindican para el tratamiento de trastornos relacionados a la amiloidosis, específicamente en sus reivindicaciones 1 a 12, es relevante señalar que dicha pretensión resulta inaceptable a la luz de la Decisión 486, en tanto los “usos” son una categoría excluida del concepto de “Productos o Procedimientos” que establece el artículo 14 de la norma citada. Lo anterior, además, en consonancia con reiteradas interpretaciones de la SIC sobre este punto.

Sin perjuicio de lo anterior me permito hacer notar que, aún si en gracia de discusión los usos o métodos terapéuticos fuesen permitidos en nuestro medio, éstos deberían cumplir necesariamente, y en forma simultánea, con los requisitos materiales de patentabilidad, es decir, novedad, nivel Inventivo y aplicación industrial. Además de anotar que respecto del pretendido “uso”, el tercer requisito resulta de imposible cumplimiento, en tanto la reproducción industrial del uso separado, combinado o secuencial de medicamentos para tratar ciertas patologías sería del resorte de la profesión médica, y no, por supuesto, de las actividades industriales.

De la misma manera la Decisión 486 de la Comunidad Andina de Naciones en su aparte d) del artículo 20 del capítulo I, acerca de los requisitos de patentabilidad, menciona que *“no serán patentables los métodos terapéuticos o quirúrgicos para el tratamiento humano o animal, así como los métodos de diagnóstico aplicados a los seres humanos o a animales”*, y si tenemos en cuenta que la solicitud en cuestión reivindica también un método para el tratamiento de un paciente que padece una recaída de cáncer HER2 positivo, durante o después del tratamiento con un anticuerpo anti-HER2, tal como lo señala en sus reivindicaciones 1 a 16, queda claramente expuesta la improcedencia directa para otorgar el privilegio de patente de invención a esta solicitud.

En otro aspecto, y en vista que la solicitud colombiana en cuestión revela en sus reivindicaciones 1 a 16 una combinación de dicho anticuerpo anti-VEGF (Bevacizumab) junto con un anticuerpo anti-HER2 (Trastuzumab), es relevante

mentonar el concepto de la Doctora en Bioquímica Silvia Susana Giarcovich¹ en su opinión escrita a cerca de "Asociaciones y Patentes" en donde afirma que las asociaciones farmacológicas están restringidas por ciertos principios y lineamientos preestablecidos por varias entidades, puesto que dichas combinaciones deben estar bajo el precepto de eficacia, seguridad y eficiencia terapéutica, veamos:

"3.3.2. Lineamientos FDA:

A continuación se mencionarán lineamientos que la agencia sanitaria de EEUU ha establecido para el caso de los llamados "*Combination Drug Products*":

3.3.2.1. CFR

El CFR establece la política en relación a las combinaciones de drogas a dosis fija para humanos en 21CFR300.50. Allí se establece que dos o más drogas pueden ser combinadas en una única unidad posológica cuando cada componente hace una contribución al efecto propuesto y la dosis de cada componente es tal que la combinación es segura y efectiva (se consideran casos especiales cuando se busca aumentar la seguridad y eficacia del principal componente o minimizar su potencial abuso).

3.3.2.2. Guía "OTC Combination of Drug Products" [Docket No. 78D-0322]²:

Esta guía establece en detalle la política de la agencia para combinar dos o más ingredientes activos OTC, seguros y eficaces y caracteriza diferentes tipos de asociaciones de OTC:

- Ingredientes activos de diferentes categorías terapéuticas para tratar distintos síntomas simultáneamente: Sólo se acepta esta asociación si cada ingrediente está presente dentro de su rango de seguridad y eficacia.

¹ Profesora Regular Adjunta del Departamento de Farmacología de la Facultad de Farmacia y Bioquímica de la UBA y Directora Técnica y Gerente de Estudios Clínicos de DIFFUCAP-EURAND SACIFI

- Ingredientes activos de la misma categoría terapéutica que tienen distintos mecanismos de acción para tratar los mismos síntomas o condición: Sólo se acepta esta asociación si es mejor o igual que cada ingrediente solo en su dosis terapéutica y también pueden usarse a dosis subterapéuticas si corresponde.
- Ingredientes activos de la misma categoría terapéutica que tienen el mismo mecanismo de acción: Estos no deberían ser combinados a menos que haya alguna ventaja con respecto a los ingredientes simples (mayor eficacia, seguridad, aceptación del paciente o calidad de la formulación).
- Ingredientes considerados adyuvantes, que aumenten o alteren el efecto de otro ingrediente activo, serán, considerados ingredientes activos.

3.3.2.3. Guía Draft "Current GMP for Combination Products":

Este documento provee una guía para la industria y para el *staff* de la FDA acerca de la aplicabilidad de la cGMP para las asociaciones (*combination products*) y define a un *combination product*, de acuerdo al CFR (21CFR3.2(e), de forma que no sólo involucra drogas sino *devices*.

Un producto combinado es un producto compuesto de cualquier combinación de una droga y un dispositivo; un producto biológico y un dispositivo; una droga y un producto biológico; o una droga, dispositivo, y un producto biológico. Bajo 21 CFR 3.2 (e), un producto de combinación incluye:

1. *Un producto que comprende dos o más componentes regulados (i.e., droga/dispositivo, biológico/dispositivo, droga/biológico, o*

² Anunciada en el Federal Register (1978), Vol. 43, No. 229.

droga/dispositivo/biológico) que son combinados físicamente, químicamente, o de otra forma, o mezclados y producidos como una entidad única;

2. Dos o más productos separados, empaquetados juntos en un solo paquete, o como una unidad comprendiendo droga y productos del dispositivo, dispositivo y productos biológicos, o biológico y productos de droga;

3. Una droga, dispositivo, o producto biológico empaquetado separadamente que, de acuerdo a su plan de investigación o el etiquetado propuesto, es destinado para utilizarse únicamente con una droga, dispositivo, o producto biológico aprobada especificada individualmente, dónde al mismo tiempo ambos son deben estar aceptados para la indicación o efecto y donde, luego de la aprobación del producto propuesto, el etiquetado del producto aprobado necesitaría ser cambiado (e.g., para reflejar un cambio en la indicación, forma farmacéutica, dosis, ruta de administración, cambio significativo en la posología); ó

4. Cualquier droga en investigación, dispositivo, o producto biológico empaquetado separadamente que, de acuerdo al etiquetado propuesto es para utilizarse únicamente con otra droga en investigación especificada, dispositivo, o producto biológico, dónde a la vez se requiere que ambos sean aceptados para la misma indicación, uso y efecto.

Además, dentro de esta guía es muy interesante el concepto de "parte constitutiva de un *combination product*" porque especifica su identidad regulatoria como droga, *device* o producto biológico.

3.3.2.4. Guía Draft "Combination Products Timeliness of Premarket Reviews":

Esta guía provee información a un postulante a reclamar la resolución de una disputa acerca de la duración de la revisión de una postulación referida a un producto combinado. Es una guía referida simplemente a un problema administrativo que

demuestra cuán regulado se halla este ámbito que tiene una oficina destinada, la Office of Combination Products.

3.3.3. Lineamientos EMEA: Guía "Fixed Combination Medicinal Products":

Esta guía propone evaluar las potenciales ventajas de la asociación en cuanto a mejoramiento de la relación riesgo/beneficio y a simplificación de la terapia. Asimismo, establece criterios a respetar y requisitos a cumplimentar, tanto generales como particulares. Dentro de los particulares hace especial referencia a:

- La indicación.
- Los estudios farmacodinámicos y farmacocinéticos.
- Eficacia y Seguridad: En este caso diferencia dos escenarios concretos:

a) Cuando la combinación fija corresponde exactamente a combinaciones que están ya en uso difundido, un análisis de los datos bibliográficos bien fundamentados debería ser presentado. Con tal que los datos estén bien documentados, este análisis puede ser útil en reducir la cantidad de ensayos clínicos a ser realizada y podría facilitar la selección de dosis para cada sustancia y el rango de dosis propuesto en la combinación fija.

b) Cuando la combinación fija es esencialmente nueva (las sustancias activas no son usualmente combinadas, la composición cuantitativa es inusual entre sustancias usualmente combinadas o una sustancia enteramente nueva), los datos necesarios son similares a los de una nueva entidad química en la situación donde la combinación fija será propuesta (primero línea o segunda de la línea de terapia). La existencia de experiencia con las sustancias debería ser tomada en cuenta.

y especifica Régimen de dosis y Composición, Ensayos Terapéuticos y Aspectos de Seguridad.

- Paquetes combinados de diferentes productos medicinales para administración simultánea o secuencial.

Conclusión:

Las diferentes agencias sanitarias a nivel internacional han regulado fuertemente el registro de asociaciones de forma de garantizar la seguridad y eficacia de las mismas y la buena calidad en su elaboración. Todo estudio, investigación o análisis derivados de estos requisitos representan procedimientos de rutina y no originales ideas llevadas a la práctica."

En otras palabras, se puede afirmar que dicho efecto sinérgico o terapéutico entre sustancias activas no se debe considerar como un aporte inventivo como tal, dado que este puede ser obvio para una persona medianamente versada en la materia y que conozca los mecanismos de acción de dichos principios activos involucrados, y además, dicho efecto sinérgico y/o terapéutico que se puede dar entre dos o mas sustancias corresponde a un simple reporte de un descubrimiento, debido a que dicha sinergia o acción terapéutica de los principios activos combinados ocurre en el organismo y se evidencia a través de los ensayos preclínicos y clínicos rutinarios en el proceso de elaboración de todo medicamento. Además, una combinación farmacológica no pertenece a la categoría de producto o proceso, sino que pertenece al consorte de las actividades médicas.

Finalmente también podemos demostrar que dicha combinación de anticuerpos reivindicada en la solicitud colombiana en tramite carece *per se* de novedad y de nivel inventivo.

Existen publicaciones de carácter científico que exponen con anterioridad la combinación específica entre anticuerpos anti-VEGF y anticuerpos anti-HER2 en el tratamiento de cáncer, veamos:

Combined Biological Therapy Of Breast Cancer Using Monoclonal Antibodies Directed Against HER2/Neu Protein And Vascular Endothelial Growth Factor³.

Abstract: Vascular endothelial growth factor (VEGF) is one of the most important endothelial mitogens involved in the development and differentiation of the vascular system. Vascular endothelial growth factor is a highly conserved, homodimeric glycoprotein with multiple isoforms. The most abundant isoform, VEGF165, binds to vascular endothelial growth factor receptors-1 (Flt-1) and 2 (KDR/Flk-1) with picomolar affinity. Recently, correlation between microvessel density and engineered expression of VEGF in human breast xenografts was observed. A role of VEGF in breast cancer progression is evident from clinical studies showing elevated serum VEGF in invasive breast cancers. Vascular endothelial growth factor in breast tumor cytosols is correlated with microvessel density, and VEGF165 content correlates with disease-free and overall survival in primary breast cancers. Preliminary data indicate a transcriptional upregulation of VEGF in HER2-overexpressing breast cancer cells. We hypothesize that the upregulation of VEGF in HER2-overexpressing breast cancers contributes to the aggressive phenotype observed in HER2-positive cases and that the "angiogenic switch" associated with HER2 can be attenuated by trastuzumab. Although tumor angiogenesis in breast cancer is complex, the VEGF/vascular endothelial growth factor receptor (VEGFR) system provides a useful model for testing new angiogenesis inhibitors that target this pathway. The VEGF/VEGFR system provides a number of opportunities for therapeutic intervention in breast cancer. Understanding the biology of this system is paramount to fully exploiting VEGF as a therapeutic target in breast cancer. We hypothesize that new therapeutic molecules targeting VEGF and/or its receptors, such as recombinant humanized monoclonal anti-VEGF antibody (rhuMAb VEGF), may have unique activity against HER2-overexpressing breast cancers.

Strategies For Delaying Or Treating In Vivo Acquired Resistance To Trastuzumab In Human Breast Cancer Xenografts⁴.

Abstract: PURPOSE: Acquired resistance to trastuzumab (Herceptin) is common in patients whose breast cancers show an initial response to the drug. The basis of this acquired resistance is unknown, hampering strategies to delay or treat such acquired resistance, due in part to the relative lack of appropriate in vivo tumorigenic models.

EXPERIMENTAL DESIGN: We derived an erbB-2-positive variant called 231-H2N, obtained by gene transfection from the highly tumorigenic erbB-2/HER2-negative human breast cancer cell line, MDA-MB-231. Unlike MDA-MB-231, the 231-H2N variants was sensitive to trastuzumab both in vitro and especially in vivo, thus allowing selection of variant resistant to drug treatment in the latter situation after showing an initial response.

³ Pegram MD, Reese DM. Semin Oncol. 2002 Jun;29(3 Suppl 11):29-37.

⁴ du Manoir JM, Francia G, Man S, Mossoba M, Medin JA, Viloria-Petit A, Hicklin DJ, Emmenegger U, Kerbel RS. Clin Cancer Res. 2006 Feb 1;12(3 Pt 1):904-16.

RESULTS: The growth of established orthotopic tumors in severe combined immunodeficient mice was blocked for 1 month by trastuzumab, after which rapid growth resumed. These relapsing tumors were found to maintain resistance to trastuzumab, both in vitro and in vivo. We evaluated various therapeutic strategies for two purposes: (a) to delay such tumor relapses or (b) to treat acquired trastuzumab resistance once it has occurred. With respect to the former, a daily oral low-dose metronomic cyclophosphamide regimen was found to be particularly effective. With respect to the latter, an anti-epidermal growth factor receptor antibody (cetuximab) was effective as was the anti-vascular endothelial growth factor (anti-VEGF) antibody bevacizumab, which was likely related to elevated levels of VEGF detected in trastuzumab-resistant tumors.

CONCLUSIONS: Our results provide a possible additional rationale for combined biological therapy using drugs that target both erbB-2/HER2 and VEGF and also suggest the potential value of combining less toxic metronomic chemotherapy regimens not only with targeted antiangiogenic agents but also with other types of drug such as trastuzumab.

Como se puede apreciar claramente en estas publicaciones científicas, es evidente que se reconoce con anterioridad la utilidad terapéutica de la combinación entre anticuerpos anti-HER2 junto con anticuerpos anti-VEGF, cuando se presentan cuadros de retroceso en los resultados del tratamiento de pacientes con cáncer que inicialmente se venían tratando con un protocolo anti-HER2. Específicamente se hace mención de Trastuzumab y de Bevacizumab.

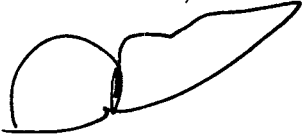
En conclusión, la solicitud colombiana en trámite carece de absolutamente todos los requisitos para podersele otorgar el privilegio de patente de invención, en primer lugar porque hace referencia directa a un uso de una combinación de anticuerpos como un método de tratamiento, lo que es claramente una improcedencia en la patentabilidad directa según lo dispuesto en la Decisión 486 de 2000, y finalmente porque dicha combinación farmacológica entre los anticuerpos que se mencionan allí ya se reconocía con anterioridad en el estado de la técnica

Por todo lo anterior, ruego se analicen los argumentos expuestos con el rigor y detenimiento habituales, y que en consecuencia el Despacho decrete, además de la negación de la solicitud en comento, el archivo del expediente que lo contiene.

II. ANEXOS

- ❖ Copia de todas las publicaciones mencionadas en el acápite de Complemento de Información.

Señor Superintendente,



JOSE LUIS REYES VILLAMIZAR

C.C. 79.152.473 de Usaquén
T.P. 44655 del C. S de la J.

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Semin Oncol. 2002 Jun;29(3 Suppl 11):29-37.

Combined biological therapy of breast cancer using monoclonal antibodies directed against HER2/neu protein and vascular endothelial growth factor.

Pegram MD, Reese DM.

Division of Hematology-Oncology and Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA 90095, USA.

Abstract

Vascular endothelial growth factor (VEGF) is one of the most important endothelial mitogens involved in the development and differentiation of the vascular system. Vascular endothelial growth factor is a highly conserved, homodimeric glycoprotein with multiple isoforms. The most abundant isoform, VEGF165, binds to vascular endothelial growth factor receptors-1 (Flt-1) and 2 (KDR/Flk-1) with picomolar affinity. Recently, correlation between microvessel density and engineered expression of VEGF in human breast xenografts was observed. A role of VEGF in breast cancer progression is evident from clinical studies showing elevated serum VEGF in invasive breast cancers. Vascular endothelial growth factor in breast tumor cytosols is correlated with microvessel density, and VEGF165 content correlates with disease-free and overall survival in primary breast cancers. Preliminary data indicate a transcriptional upregulation of VEGF in HER2-overexpressing breast cancer cells. We hypothesize that the upregulation of VEGF in HER2-overexpressing breast cancers contributes to the aggressive phenotype observed in HER2-positive cases and that the "angiogenic switch" associated with HER2 can be attenuated by trastuzumab. Although tumor angiogenesis in breast cancer is complex, the VEGF/vascular endothelial growth factor receptor (VEGFR) system provides a useful model for testing new angiogenesis inhibitors that target this pathway. The VEGF/VEGFR system provides a number of opportunities for therapeutic intervention in breast cancer. Understanding the biology of this system is paramount to fully exploiting VEGF as a therapeutic target in breast cancer. We hypothesize that new therapeutic molecules targeting VEGF and/or its receptors, such as recombinant humanized monoclonal anti-VEGF antibody (rhUMAb VEGF), may have unique activity against HER2-overexpressing breast cancers.

PMID: 12138395 [PubMed - indexed for MEDLINE]

Publication Types, MeSH Terms, Substances

LinkOut - more resources

Strategies for Delaying or Treating *In vivo* Acquired Resistance to Trastuzumab in Human Breast Cancer Xenografts

Jeanne M. du Manoir,¹ Giulio Francia,¹ Shan Man,¹ Miriam Mossoba,² Jeffrey A. Medin,² Alicia Vilorio-Petit,³ Daniel J. Hicklin,⁴ Urban Emmenegger,¹ and Robert S. Kerbel¹

Abstract **Purpose:** Acquired resistance to trastuzumab (Herceptin) is common in patients whose breast cancers show an initial response to the drug. The basis of this acquired resistance is unknown, hampering strategies to delay or treat such acquired resistance, due in part to the relative lack of appropriate *in vivo* tumorigenic models.

Experimental Design: We derived an erbB-2-positive variant called 231-H2N, obtained by gene transfection from the highly tumorigenic erbB-2/HER2-negative human breast cancer cell line, MDA-MB-231. Unlike MDA-MB-231, the 231-H2N variant was sensitive to trastuzumab both *in vitro* and especially *in vivo*, thus allowing selection of variant resistant to drug treatment in the latter situation after showing an initial response.

Results: The growth of established orthotopic tumors in severe combined immunodeficient mice was blocked for 1 month by trastuzumab, after which rapid growth resumed. These relapsing tumors were found to maintain resistance to trastuzumab, both *in vitro* and *in vivo*. We evaluated various therapeutic strategies for two purposes: (a) to delay such tumor relapses or (b) to treat acquired trastuzumab resistance once it has occurred. With respect to the former, a daily oral low-dose metronomic cyclophosphamide regimen was found to be particularly effective. With respect to the latter, an anti-epidermal growth factor receptor antibody (cetuximab) was effective as was the anti-vascular endothelial growth factor (anti-VEGF) antibody bevacizumab, which was likely related to elevated levels of VEGF detected in trastuzumab-resistant tumors.

Conclusions: Our results provide a possible additional rationale for combined biological therapy using drugs that target both erbB-2/HER2 and VEGF and also suggest the potential value of combining less toxic metronomic chemotherapy regimens not only with targeted antiangiogenic agents but also with other types of drug such as trastuzumab.

One of the major significant clinical advances in cancer therapeutics over the last decade has been the discovery, development, and approval of a number of drugs, both antibodies and small molecules, which target the products of oncogenes. These drugs include trastuzumab (Herceptin), the humanized antibody to the erbB-2/HER2 receptor tyrosine

kinase (1); imatinib (Gleevec), a small molecule drug that targets the bcr-abl, c-kit, and platelet-derived growth factor receptor tyrosine kinases (2); and cetuximab (C225/Erbitux), the chimeric antibody to the epidermal growth factor receptor (EGFR; erbB-1) and small molecule antagonists of the EGFR (3). Unfortunately, the success of these drugs in responding patients is compromised by the same problem, which plagues other systemic therapies, such as chemotherapy, hormone receptor antagonizing drugs, or hormonal ablation therapeutics i.e., the development of acquired resistance (4). In particular, the striking responses frequently induced by imatinib in indolent chronic myelogenous leukemia or gastrointestinal stromal tumor patients followed by relapse and the discovery of mutations in the respective target genes that lead to loss of tumor response (5) have highlighted the profound effect that acquired drug resistance mechanisms can have on limiting the durability of tumor responses to such promising drugs (4).

One of the surprising aspects of this area of research is the lack of any published detailed studies devoted to defining the mechanisms of acquired resistance to trastuzumab. Despite the fact that this drug has been in development for >15 years and has by now been used in the treatment of many thousands of breast cancer patients, there are as yet no published preclinical studies on isolating and characterizing trastuzumab-resistant variants/mutants from initially erbB-2-positive

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Note: R.S. Kerbel is Canada Research Chair in Molecular Medicine.

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and responsive human tumors *in vivo*, and only a few reports using cell lines selected *in vitro* for acquired resistance to trastuzumab (6–8). The study of acquired resistance to trastuzumab, especially variants derived during *in vivo* therapy, can be very useful for devising possible therapeutic strategies to delay acquired trastuzumab resistance, or to treat tumors that have actively acquired resistance, based on the discovery of new targets expressed in resistant variant cells that might be exploitable using second-line therapies. This is an important clinical problem because about 40% of metastatic patients with erbB-2 overexpressing breast cancer respond to first-line trastuzumab therapy as a single agent (9), but the median duration of response is only about 8 months. With respect to *intrinsic* resistance to trastuzumab, there have been a few recent studies in this area. For example, an important clinical study by Nagata et al. reported that activation of the PTEN tumor suppressor protein seems to contribute to trastuzumab tumor responsiveness in human breast cancer patients, such that loss of PTEN predicts trastuzumab resistance (10). However, whether this type of genetic alteration contributes to some forms of *acquired* trastuzumab resistance is unknown. In addition, Tanner et al. generated a breast cell line (called JIMT-1) from a patient treated with trastuzumab, but who did not respond (i.e., showed intrinsic resistance to the drug; ref. 11). The cell line showed no identifiable mutation in erbB-2. However, the accessibility of erbB-2 seems to be reduced by MUC4 overexpression (12).

There are several practical problems associated with studying acquired resistance to trastuzumab using erbB-2 breast cancer xenografts, which may help explain the lack of relevant studies in this preclinical area of research. First, there are very few human breast cancer cell lines, which overexpress erbB-2, and those few that do, such as SKBR3 and BT-474, tend to be poorly or even nontumorigenic, making it difficult to have the proper selective pressure conditions for isolating authentic variants having an acquired drug-resistant phenotype. Second, long-term therapy experiments are rare, in part because of the relatively weak responses to the drug (which are usually manifested in the form of tumor growth delays, but not frank tumor regressions), which, once again, make it difficult to isolate authentic mutants or variants that have acquired resistance to trastuzumab in a preclinical setting. To overcome these problems, we studied a variant of a highly tumorigenic human tumor cell line, MDA-MB-231 (which expresses barely detectable levels of erbB-2), from which a stable erbB-2 overexpressing variant cell population was obtained by gene transfection. The variant, called 231-H2N, was grown orthotopically in severe combined immunodeficient (SCID) mice and subjected to trastuzumab therapy alone, or combined with chemotherapy. We used chemotherapy in combination with trastuzumab for some of the experiments because trastuzumab is normally administered with chemotherapy (1, 13), and this could obviously affect the kinetics and outcome of acquired resistance to trastuzumab. We used cyclophosphamide as the chemotherapeutic for our studies, which was administered either in a daily low-dose (metronomic) fashion through drinking water (14) or alternatively, in a more conventional maximum tolerated dose (MTD) regimen with 2-week-long drug-free breaks between weekly cycles of MTD therapy (15). Part of the original rationale for the metronomic chemotherapy experiments was based on the prospect of undertaking long-

term therapy studies in mice when using such relatively nontoxic chemotherapy regimens, either alone, or when these are combined with targeted therapies, such as antiangiogenic agents (16). We reasoned this paradigm might be extended to trastuzumab, especially because this drug may have antiangiogenic effects, which contribute to its antitumor efficacy (17, 18). Thus, we hypothesized that combination of this drug with a metronomic chemotherapy regimen might also result in better disease control (and overall less toxicity) compared with trastuzumab combined with MTD regimens of cyclophosphamide. We also speculated that such long-term therapy experiments in mice (assuming the combination therapy would be highly effective) could be helpful in the eventual experimental selection and overgrowth of variants having acquired resistance to trastuzumab.

Our results show that it is indeed possible to select trastuzumab-resistant variants *in vivo* using the aforementioned experimental approaches, whether using trastuzumab alone or in combination with chemotherapy. The resistant variants we selected were partially characterized, and the results indicated they had a significantly elevated vascular endothelial growth factor (VEGF) expression, a finding that is similar to the properties of variants of other human tumor cell lines selected for acquired resistance *in vivo* to erbB-1/EGFR targeting antibodies, such as C225/cetuximab (19). Consequently, addition of bevacizumab (the humanized anti-VEGF antibody) to trastuzumab, transiently restored responsiveness to trastuzumab-resistant variants, suggesting this type of approach might be of value to delay or to treat variants with acquired trastuzumab resistance, as a second-line or follow-up therapy. The resistant variants also responded to trastuzumab when combined with an EGFR targeting antibody, which may be related to an observed up-regulation of transforming growth factor- α (TGF- α) in the resistant variants. Finally, we also obtained preliminary evidence for the superiority of combining trastuzumab with metronomic versus standard MTD chemotherapy, both in terms of reduced toxicity and prolonged survival.

Materials and Methods

Cells and culture conditions. Human breast tumor cell lines, MDA-MB-361, BT-474, and SK-BR3 were purchased from the American Type Culture Collection (Rockville, MA) and maintained as monolayers in DMEM supplemented with 10% fetal bovine serum. The human breast tumor cell line, MDA-MB-231, was kindly provided by Dr. Janet E. Price (M.D. Anderson Cancer Centre, University of Texas). MDA-MB-231 cells were transfected to stably overexpress erbB-2. Briefly, amphotropic GP+envAM12 packaging cells, cotransfected with pUMFG/her2/neu/IRES/enYFP containing the full-length human c-erbB-2 cDNA and the plasmid pGT-N28 (New England Biolabs, Beverly, MA), were used to transduce MDA-MB-231 cells. The cell line 231-H2N was derived by fluorescence-activated cell sorting to select the top 10% of erbB-2 expressors. Both MDA-MB-231 and 231-H2N were maintained as monolayers in DMEM with 5% fetal bovine serum. All cell cultures were maintained in a humidified incubator at 37°C and 5% CO₂.

Protein isolation and Western blots. Cells were grown to ~80% confluency in normal culture conditions. Plates were placed on ice, washed with ice-cold PBS, and cold lysis buffer [20 mmol/L Tris (pH 7.5) containing 137 mmol/L NaCl, 100 mmol/L NaF, 10% glycerol, 1% NP40, 2 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, and 10 μ g/mL leupeptin] was added directly to plates. Cells were scraped from plates, incubated on ice for

40 minutes, and centrifuged at 10,000 rpm for 15 minutes. Protein concentrations of the supernatant was determined using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA); 50 µg/lane of total proteins were resolved by 8% SDS-PAGE, transferred onto Immobilon-P membrane (Millipore Canada Ltd., Etobicoke, Ontario, Canada), blocked with 10% milk in TBST, and probed with primary antibody. Anti-neu, anti-EGFR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-β-actin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) were incubated for 1 hour at room temperature, whereas Akt, p44/42 mitogen-activated protein kinase, phospho-erbB-2 Tyr¹²⁴⁸, phospho-AKT, phospho-EGFR, phospho-mitogen-activated protein kinase (all from Cell Signaling Technologies, Beverly, MA), PTEN (MMAC1 Ab-2, Mediacorp, Montreal, Canada), PTEN (Santa Cruz Biotechnology), and extracellular erbB-2 Ab-20 (L87 + 2ERB19 from Mediacorp) were incubated overnight at 4°C. All primary antibodies were diluted 1:1,000 in 5% milk in TBST, detected with anti-rabbit IgG^{HRP} or anti-mouse IgG^{HRP} (Promega, Madison, WI) diluted 1:5,000, and detected with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

Northern blot of total RNA. Tumor tissues were stored at -70°C in RNAlater. Trizol extraction (Life Technologies, Inc., Gaithersburg, MD) was carried out according to manufacturer's instruction. VEGF Northern blots were done as previously described (20). The VEGF probe spanning the 200-bp sequence common for all VEGF isoforms was a generous gift of Drs. Brygida Berse and Harold Dvorak (Beth Israel Hospital, Boston, MA). Glyceraldehyde-3-phosphate dehydrogenase was probed as a loading control.

In vitro determination of drug sensitivity. Two thousand cells were plated in 96-well flat-bottomed tissue culture plates in a volume of 100 µL media per well and incubated overnight at 37°C and 5% CO₂. Drug treatments were added to wells in a volume of 100 µL media and were carried out for 72 hours. Cells were then pulsed with 2 µCi/well of methyl-[³H]thymidine (Amersham Biosciences) for 6 hours. DNA was harvested onto Unifilter filtermat plates (Perkin-Elmer Life Sciences, Woodbridge, Ontario, Canada) using the Filtermate Harvester (Perkin-Elmer Life Sciences). Incorporated radioactivity was measured by scintillation counting using a Packard Topcount NXT microplate counter (Perkin-Elmer Life Sciences).

In vivo tumor growth assessment. Four-week-old female C.B-17 (SCID) mice were purchased from Charles River Canada (Saint-Constant, Quebec, Canada). Mice were allowed to acclimatize and mature for 2 weeks before implantation of tumor cells. To prepare cells for injection, subconfluent plates were harvested with 1% trypsin-EDTA (Life Technologies Bethesda Research Laboratories, Gaithersburg, MD), and cells were then washed and resuspended in DMEM at 4×10^7 /mL. Two million cells were injected into the inguinal mammary fat pad of anaesthetized mice. Wounds were secured with surgical staples.

Mice were monitored twice weekly for tumor growth and fluctuations in body weight. Treatments were initiated when the average tumor volume reached 250 mm³ (unless otherwise indicated) as measured by Vernier calipers and calculated by $(\text{length} \times \text{width}^2) / 2$. Institutional guidelines were followed for end point of experiments. Plasma was collected by cardiac puncture into Microtainer Plasma Separator tubes (Becton Dickinson, Franklin Lakes, NJ) and stored at -70°C until analysis. Tumors were removed from mice, divided into three sections, and placed into RNAlater (Ambion, Inc., Austin, TX), Prefer fixative (Anatech, Battle Creek, MI), or frozen section medium (Stephens Scientific, Riverdale, NJ).

Drug treatments. Cyclophosphamide was purchased from the institutional pharmacy and prepared for oral or i.p. administration as previously described (14). Oral cyclophosphamide was administered at an estimated dose of 20 mg/kg/d. Mice administered the MTD schedule were injected i.p. with 100 mg/kg, on days 1, 3, and 5 of each 21-day cycle.

Trastuzumab (Herceptin) was generously provided by Genentech (San Francisco, CA). A 20 mg/mL stock solution was prepared by adding sterile PBS to the lyophilized antibody. Immediately before i.p. injection, the trastuzumab stock solution was diluted 1:5 in normal saline and was administered at 20 mg/kg twice weekly. Additional

in vivo dosing studies were carried out with the erbB-2 heterodimerization inhibitor antibody pertuzumab (Omnitarg/2C4) and the humanized anti-VEGF antibody bevacizumab (Avastin; both provided by Genentech), as well as the anti-EGFR antibody cetuximab (Erbix/C225), provided by Imclone Systems, Inc. (New York, NY). All antibody reagents were diluted in sterile normal saline and administered i.p., twice weekly, together with trastuzumab. Drugs were administered at the following doses: 400 µg pertuzumab, 200 µg bevacizumab (21), 500 µg cetuximab (22, 23) per mouse, twice weekly.

Derivation of trastuzumab-resistant cell lines. 231-H2N orthotopic mammary tumors were grown to ~250 mm³ before treatment with trastuzumab. Trastuzumab was administered i.p. at 20 mg/kg twice weekly. Tumor growth was inhibited for ~30 days of treatment, at which time tumors rapidly progressed. Once tumors reached a volume of 1,700 mm³, mice were sacrificed, and tumor tissue was removed under sterile conditions, minced into small ~2 mm³ pieces, and

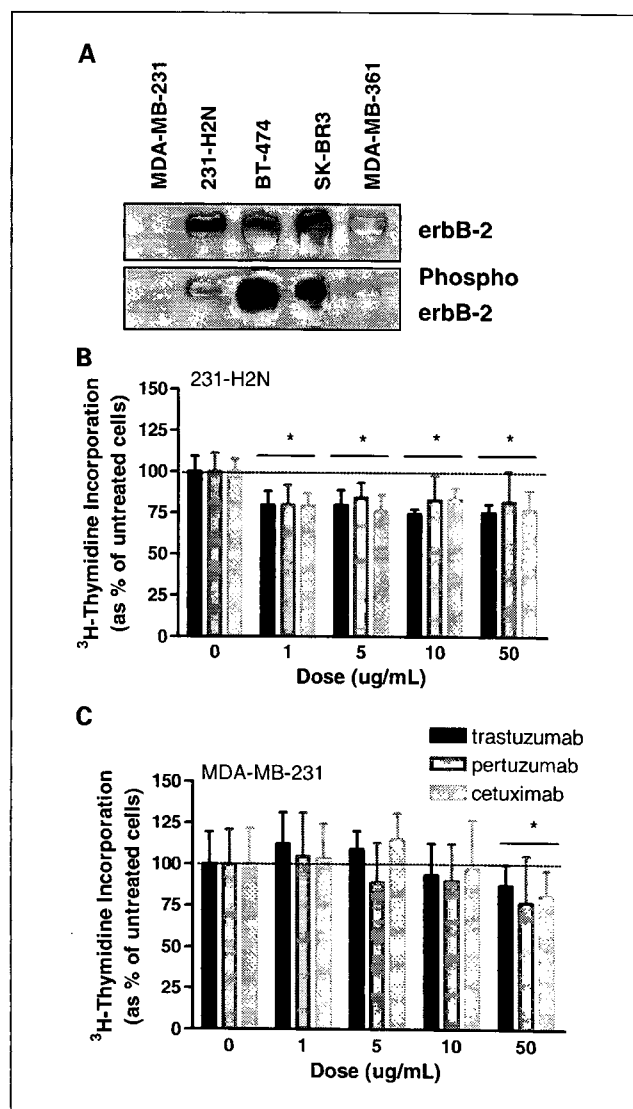


Fig. 1. A, Western blot to determine levels of erbB-2 and phospho-erbB-2 in whole-cell lysates of the 231-H2N-transfected cells compared with parental MDA-MB-231 and endogenous erbB-2 overexpressing cell lines BT-474, SK-BR3, and MDA-MB-361. *In vitro* inhibition of cell proliferation of (B) 231-H2N cells and (C) of MDA-MB-231 cells, after 72 hours of treatment with the erbB-targeted antibodies trastuzumab, pertuzumab, or cetuximab as measured by [³H]thymidine incorporation and reported as % cell proliferation relative to untreated cells. Columns, mean ($n = 8$); bars, SD. *, $P < 0.05$.

digested with an enzyme cocktail of serum-free DMEM containing 2 mg/mL Collagenase 3 (Worthington Biochemical Corp., Lakewood, NJ), 1 mg/mL hyaluronidase (Sigma-Aldrich), and 1 mg/mL Collagenase IV (Sigma-Aldrich). After incubation on ice for 30 minutes, followed by a 30-minute incubation at 37°C with shaking, the cells were washed and plated in DMEM supplemented with 5% FCS, Fungizone (Invitrogen Life Technologies, Burlington, Ontario, Canada) and penicillin/streptomycin (Invitrogen, San Diego, CA). Cells were grown and passaged thrice to allow for tumor cell enrichment and also elimination of host cells before further study of the cell lines. Cells were maintained in DMEM with 5% FCS.

In vivo angiogenesis assessment by Matrigel plug and tumor perfusion assays. The perfusion assays with FITC-dextran were carried out as previously described, with minor modifications (16). Briefly, two Matrigel preparations were injected s.c. into the flanks of 8-week-old female CB-17 SCID mice, the first containing 500 ng/mL of the angiogenic growth factor basic fibroblast growth factor, and the second containing 3×10^6 231-H2N tumor cells. Treatment commenced 4 days after implantation and was carried out for 7 days. For tumor perfusion assays, 231-H2N tumors were established as indicated above, and treatment commenced when tumors reached a volume of 400 mm³. Mice were treated for 21 days.

Measurement of TGF- α and VEGF by ELISA. Cells (1×10^5) from parental and resistant variants were plated in 24-well plates and grown for 24 hours. Medium was replaced with DMEM containing 1% fetal bovine serum, and the cells were incubated for an additional 48 hours. Conditioned medium was then collected and assayed on the human TGF- α ELISA and human VEGF kit, respectively, following the manufacturer's protocol (R&D Systems, Minneapolis, MN). The detected growth factor levels in the conditioned medium were corrected for final cell number per well.

Statistical analysis. Results are reported as mean $\pm$ SD or SE. Statistical significance of differences was assessed by PRISM software (version 4.00; GraphPad, San Diego, CA). The level of significance was set at $P < 0.05$.

Results

Establishment of a trastuzumab-responsive orthotopic breast cancer mouse model. To establish a highly tumorigenic erbB-2-positive breast tumor model in mice, the tumorigenic human breast tumor cell line MDA-MB-231, which does not normally overexpress erbB-2, was stably transfected to derive an erbB-2 overexpressing variant termed 231-H2N. MDA-MB-231 was selected as an ideal breast tumor model for these studies, due in part to the reported involvement of VEGF-dependent angiogenesis in the progression of these tumors (24). In addition, *in vivo* tumor growth of MDA-MB-231 mammary tumors has been shown to be effectively delayed by treatment of mice with metronomic cyclophosphamide administered through the drinking water (14), and we wanted to evaluate and compare the effects of trastuzumab alone, or combined with a chemotherapy drug (i.e., cyclophosphamide), administered either in a frequent low-dose (metronomic) fashion or in a conventional MTD manner to determine which of the two would be superior, and how each might affect kinetics of acquired resistance to trastuzumab. We wish to emphasize that some naturally erbB-2-positive breast cancer cell lines were also tested but not selected for our studies due to poor or complete lack of *in vivo* growth (e.g., BT-474 and SK-BR3), even when the cells were injected into the mammary fat pads of SCID mice (data not shown).

Expression levels of erbB-2 were determined by Western blot for 231-H2N cells relative to the parental MDA-MB-231 cell

line and several endogenous erbB-2 overexpressing cell lines. Although the parental cell line expresses minimal levels of erbB-2, 231-H2N cells were found to express erbB-2 and, importantly, do so in the range of endogenous erbB-2 overexpressing cell lines (Fig. 1A). In addition, when the blot was probed for phospho-erbB-2, the receptor was found to be constitutively phosphorylated in 231-H2N cells (Fig. 1A), suggesting that the receptor is biologically activated. To confirm that erbB-2 was functional and involved in the activation of downstream intracellular signaling pathways within the transfected cell line, *in vitro* assessment of the cytostatic effects of erbB-2-targeted therapies was carried out on the 231-H2N and MDA-MB-231 cell lines. The erbB-2-targeted antibody, trastuzumab, as well as the EGFR-erbB-2 heterodimerization inhibitory antibody, pertuzumab, and the EGFR-targeted antibody cetuximab (C225) were shown to inhibit cell proliferation of 231-H2N cells by 25% at antibody concentrations as low as 1 μ g/mL (Fig. 1B and C). Such modest levels of growth inhibition mediated by trastuzumab *in vitro* are consistent with the results of others and our own previous results using other cell lines (17). In contrast, significant inhibition of the parental cell line was observed only at very high concentrations of 50 μ g/mL.

We then undertook preliminary *in vivo* studies, where tumor "take" in the mammary fat pad was determined. 231-H2N tumors were palpable in all mice within 7 days of injection, and tumors reached 150 to 250 mm³ within 5 weeks with acceptable variation in tumor size (data not shown). Untreated tumors reached a volume of 1,700 mm³ within 10 weeks and remained encapsulated within the mammary fat pad. No evidence of macroscopic metastases was observed in the lungs of these mice.

Effect of single or combined trastuzumab therapies on tumor growth. One of the main purposes of our studies was to assess the antitumor effects (and development of resistance) of trastuzumab administered alone, compared with the drug used in combination with low-dose, chronically administered cyclophosphamide, as a first step to evaluate the potential value of this type of chemotherapy treatment regimen for erbB-2 overexpressing breast cancer tumors. Two independent *in vivo* experiments designed to evaluate the effects of metronomic cyclophosphamide, trastuzumab, and combination therapy, compared with a standard MTD dosing schedule commonly used in mice produced similar results. In the experiment shown in Fig. 2, orthotopic 231-H2N tumors were established in the abdominal mammary fat pad of female CB-17 SCID mice and grown to ~ 250 mm³ before initiation of treatment (day 0). Treatments were continued until tumors reached a volume of 1,700 mm³ or mice experienced a loss of 15% of their initial body weight, according to institutional animal care guidelines.

Although untreated control tumors progressed rapidly, reaching a volume of 1,700 mm³ in <40 days, daily oral metronomic cyclophosphamide effectively delayed tumor growth to this volume for 60 days (Fig. 2A), as previously reported using parental MDA-MB-231 tumors (14). A complete block of tumor growth was observed for trastuzumab-treated 231-H2N tumors for almost a month, after which time the tumors rapidly progressed, clearly suggestive of the emergence of variants with acquired resistance to the drug. The initial response of the primary tumors to trastuzumab was much greater than expected, as *in vitro* studies of 231-H2N monolayer cultured cells indicated only a 25% inhibition of proliferation at increasing concentrations of trastuzumab. This impressive

in vivo effect of trastuzumab is likely due to one or more host dependent effects, including, potentially, antibody-dependent cell-mediated cytotoxicity (25) and the postulated antiangiogenic properties of this drug (17, 18), which, acting alone or together in concert with a direct inhibition of tumor cell proliferation, might be responsible for this surprising degree of initially potent tumor growth suppression. With respect to "relapse," both MTD cyclophosphamide and metronomic cyclophosphamide, when combined with trastuzumab, delayed this apparent rapid onset of resistance, in the case of metronomic cyclophosphamide by an additional 75 to 90 days, illustrating the greatly improved efficacy of the combination of trastuzumab with chemotherapeutic regimens, as reported by Pegram et al. (26) and others. However, mice in the MTD cyclophosphamide + trastuzumab group had to be sacrificed due to severe toxicity after three treatment cycles (Fig. 2B). To emphasize the advantage of the metronomic cyclophosphamide and trastuzumab combination therapy, compared with the combination with standard MTD, an event-free survival curve was plotted, whereby event-free survival time was defined as a time to progression of the tumor to $>1,500 \text{ mm}^3$ or loss of $>15\%$ body weight. As seen in Fig. 2C, the combination of MTD cyclophosphamide and trastuzumab showed little benefit with respect to event-free survival compared with trastuzumab therapy alone due to the toxicity of the chemotherapy (as indicated by substantial weight loss), whereas combination

therapy of metronomic cyclophosphamide and trastuzumab provided a significant survival advantage compared with trastuzumab therapy alone, due to both a prevention of tumor progression for 120 days and an absence of obvious toxic side effects. As illustrated in Fig. 2B, mice receiving trastuzumab, metronomic cyclophosphamide, or a combination of these drugs showed no significant treatment-related fluctuations in weight over the duration of the experiment. In contrast, mice administered MTD cyclophosphamide alone or in combination experienced weight loss following the administration of cyclophosphamide. The third MTD dosing cycle was followed by an irreversible weight loss of $>25\%$, an indication of severe, cumulative toxicity. Although not carried out in these experiments, a detailed analysis of the toxic effects of low-dose metronomic versus MTD cyclophosphamide in mice was reported recently by our group (27); with the exception of lymphopenia, toxic side effects, such as myelosuppression, were not observed in low-dose metronomic cyclophosphamide regimens, despite the ultrasensitivity of SCID mice to DNA damaging agents such as cyclophosphamide.

To determine whether the effects of metronomic chemotherapy and trastuzumab could be extended to situations involving larger, more established tumors, an experiment was carried out where treatment was commenced when tumors were $\sim 800 \text{ mm}^3$ in volume and continued for 3 weeks. Although single-agent therapy with either trastuzumab or metronomic cyclophosphamide

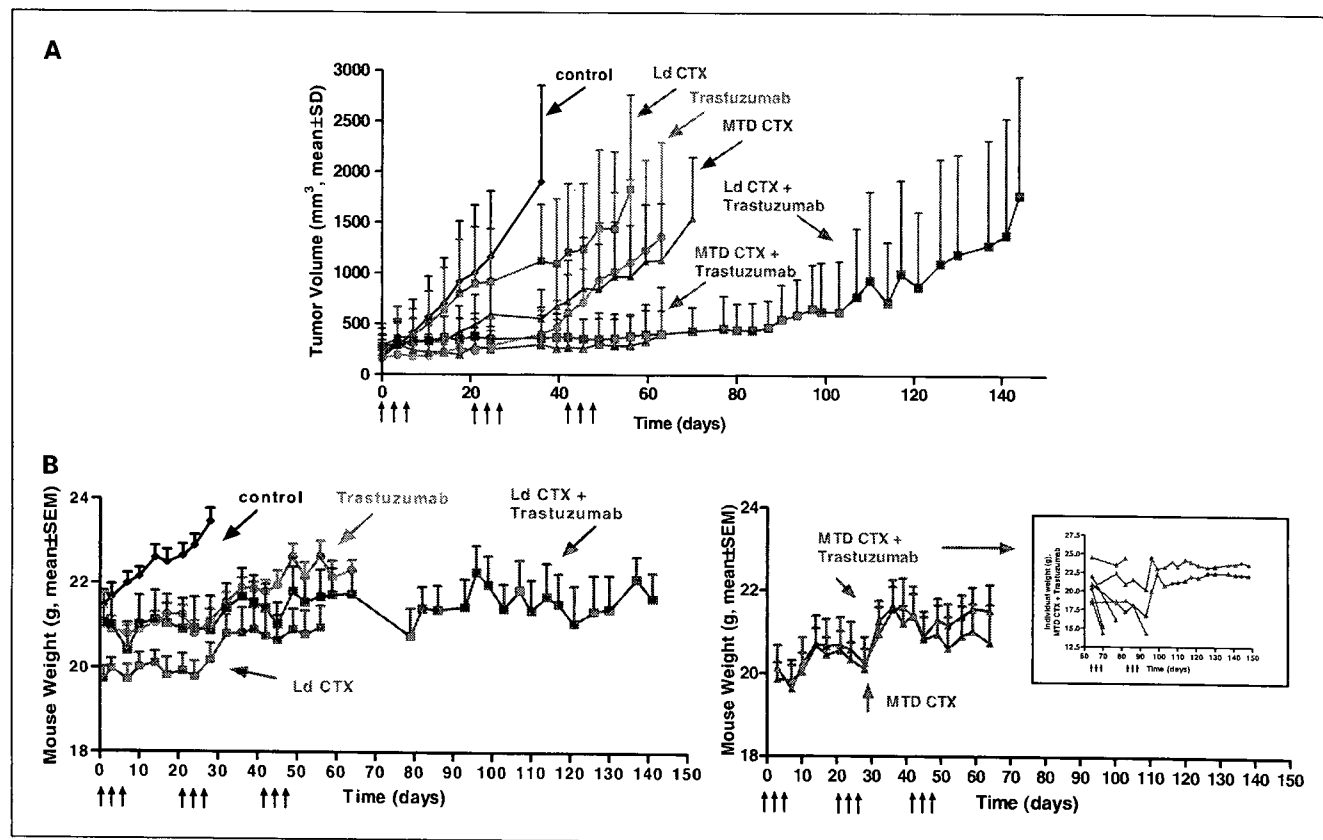


Fig. 2. *In vivo* treatment of 231-H2N mammary tumors growing orthotopically in SCID mice. **A**, antitumor effects of low-dose metronomic cyclophosphamide (Ld CTX), MTD dosing of cyclophosphamide (MTD CTX), or trastuzumab alone, and combination regimens using low-dose metronomic cyclophosphamide plus trastuzumab, or MTD cyclophosphamide plus trastuzumab. $\uparrow$, time of MTD dosing. Symbols, mean ($n = 7-8$); bars, SD. **B**, corresponding mouse weight as a measurement of relative toxicity of the different therapeutic regimens.

showed some delay in tumor growth, combination therapy completely blocked tumor progression during this treatment period (Fig. 2D). In addition, MDA-MB-231 tumors were treated with trastuzumab to confirm the lack of response of the parental cell line to trastuzumab, as found above and *in vitro*.

Analysis of erbB-2 levels in cell lines derived from trastuzumab-resistant tumors. Because the mechanisms of resistance to trastuzumab have been rarely studied in an *in vivo* context, we decided to further explore the underlying causes of the development of resistance to trastuzumab following relatively long-term trastuzumab administration.

Initially, *in vivo* clonal selection for cells expressing lower levels of the erbB-2 target was suspected as the cause for loss of response to trastuzumab, as the cell line 231-H2N was derived from a polyclonal population of erbB-2-transfected cells. Cell lines were therefore derived from untreated primary tumors and tumors that had become unresponsive to trastuzumab therapy alone, or in combination with metronomic cyclophosphamide, and were analyzed for retention or loss of erbB-2

expression. Analysis of whole-cell lysates for total erbB-2 and EGFR protein by Western blot indicated that all cell lines maintained expression of EGFR, and five of eight of these established resistant tumor cell lines still expressed high levels of the erbB-2 protein (Fig. 3A), as has also been shown by others (6, 28). Although the remaining three cell lines were found to express lower levels of the full-length erbB-2 protein, it remains to be determined if these cell lines express a truncated or mutant form of the erbB-2 receptor that is not recognized by the antibody used to probe the membranes. In addition, blots were probed for total protein levels of Akt and p44/42 (mitogen-activated protein kinase); no significant changes in expression levels of these two proteins were observed.

Increased expression of VEGF as a potential mechanism of resistance. Previously, we reported that VEGF is up-regulated in A431 human tumor xenografts selected *in vivo* for acquired resistance to anti-EGFR antibody therapy and, moreover, that this alteration seems to contribute to the resistant phenotype (19). Given the similar nature of the therapy, we decided to

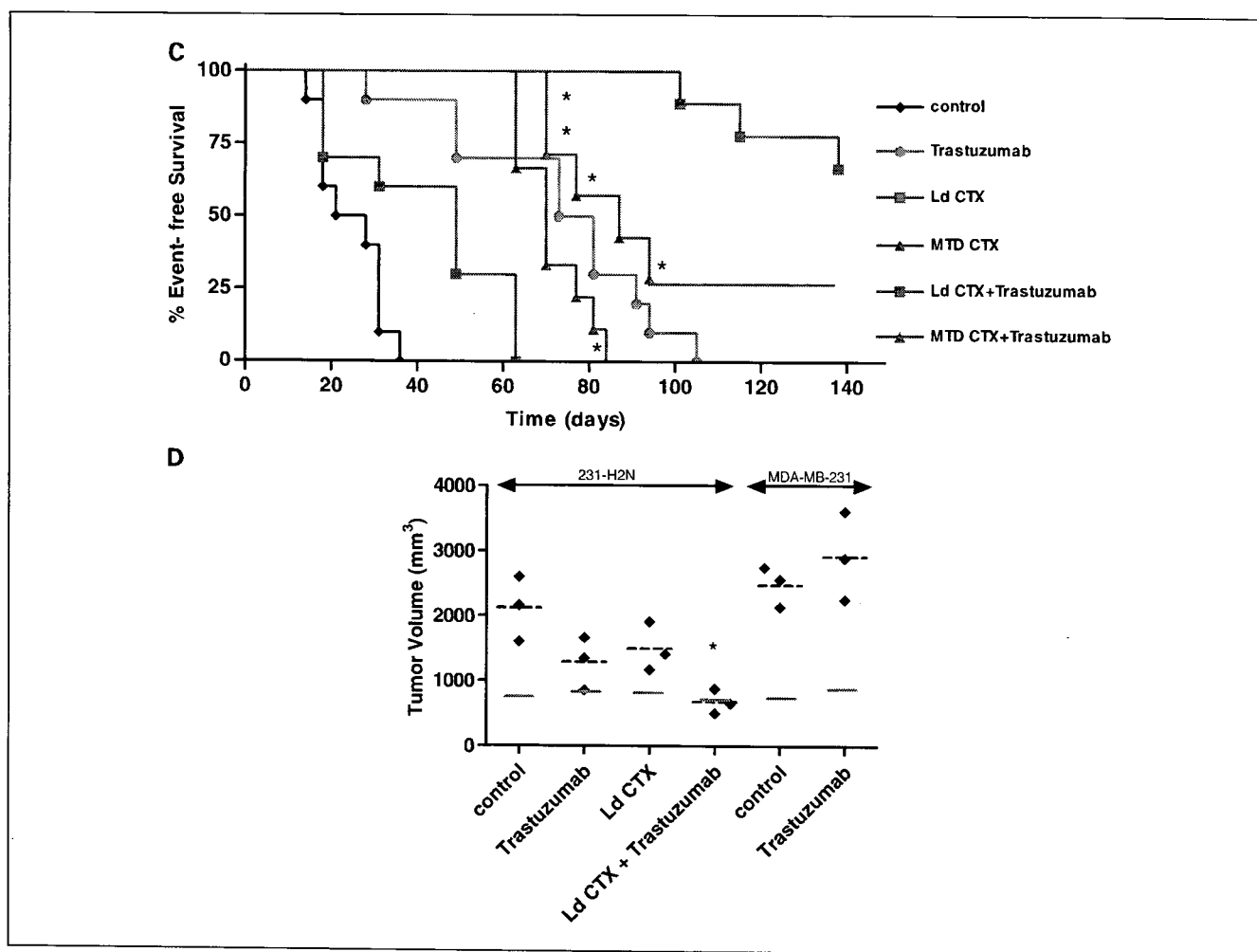


Fig. 2. Continued. C, effects of therapeutic regimens on event-free survival (Kaplan-Meier analysis), where duration of event-free survival is defined as time to primary tumor progression to 1,500 mm³ or >15% weight loss. †, indicating time of MTD dosing. *, weight loss of >15%. Significant event-free survival was observed with low-dose metronomic cyclophosphamide plus trastuzumab (*Ld CTX + Trastuzumab*) compared with the other treatment regimens. D, *in vivo* antitumor effects of cyclophosphamide and trastuzumab therapies on well-established (>800 mm³) 231-H2N and MDA-MB-231 mammary tumors growing orthotopically in SCID mice. 231-H2N tumors treated with the metronomic cyclophosphamide + trastuzumab regimen remained at ~800 mm³ throughout the 3-week period of this experiment. Gray bars, mean tumor volume at start of treatment; black bars, mean tumor volume after 3 weeks of treatment (symbols represent individual tumor volumes). *, *P* < 0.05.

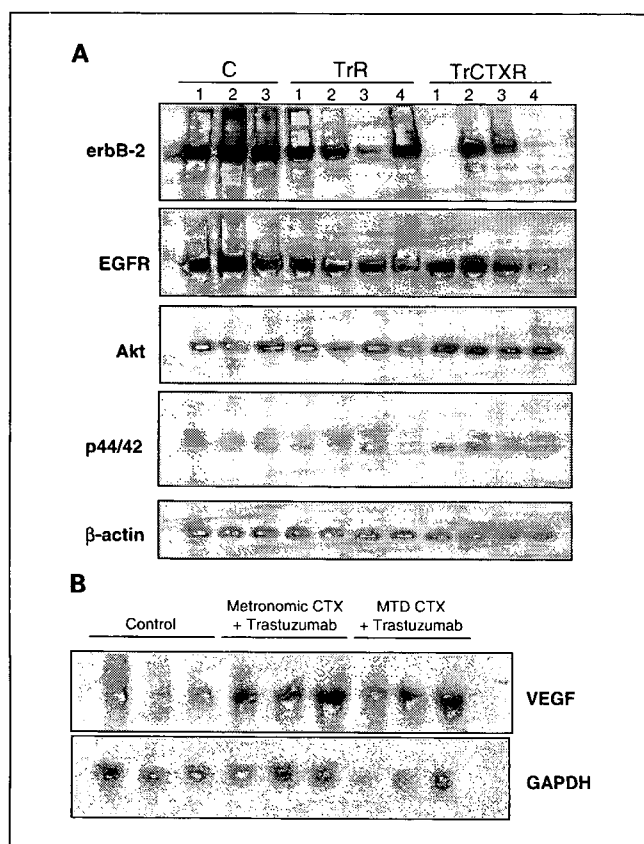


Fig. 3. A, Western blot to determine expression of erbB-2 (anti-neu), EGFR, Akt, and p44/42 MAPK in cells isolated from untreated 231-H2N tumors (C), compared with cells isolated from tumors that developed resistance to either trastuzumab therapy alone (TrR), or resistance to trastuzumab and metronomic cyclophosphamide (CTX) combination therapy (TrCTXR). β -Actin (bottom) was used as a loading control. B, Northern blot showing upregulation of VEGF mRNA expression in tumors progressing after long-term therapy using combinations of cyclophosphamide and trastuzumab, compared with untreated control tumors. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

determine if a similar mechanism might be associated with the development of acquired resistance of 231-H2N tumors to trastuzumab. Total RNA was extracted from tumors following progression and probed for VEGF by Northern blot. Indeed, we observed an increase in the expression of VEGF mRNA in tumors resistant to combination therapy regimens (Fig. 3B) compared with control tumors.

Acquired resistance to trastuzumab is observed *in vitro* and *in vivo*. To determine if the acquired drug resistance was due to host-related mechanisms (i.e., increased drug clearance, poor drug penetration, or loss of antibody-dependent cell-mediated cytotoxicity function) versus genetic or epigenetic alterations in the tumor cell population, cell lines isolated from trastuzumab-resistant tumors were assessed for *in vitro* and *in vivo* sensitivity to trastuzumab. Two cell lines (TrR2 and TrR3) from trastuzumab-resistant tumors were selected for further investigation and were compared with a cell line derived from an untreated 231-H2N tumor (C1). The *in vitro* antiproliferative effects of trastuzumab initially observed for the 231-H2N cell line (Fig. 1B) and in the C1 cell line were abolished in the two resistant cell lines (Fig. 4A), suggesting that the mechanism of resistance is not a transient or

entirely host-mediated effect. To further strengthen this conclusion, tumor cells established from untreated tumors and from tumors resistant to trastuzumab were injected into the mammary fat pads of female SCID mice, and tumor growth was monitored. Although trastuzumab was able to block growth of tumors established from previously untreated 231-H2N primary tumors (C1; Fig. 4B), the two trastuzumab-resistant tumor derived cell lines tested *in vivo* showed only a slight, statistically insignificant, response to drug treatment (TrR3) or a complete lack of response (TrR2), respectively, similar to that observed for the parental untransfected cell line MDA-MB-231 (Fig. 4B).

Analysis of cell lines derived from trastuzumab-resistant tumors. To get an insight as to the mechanisms responsible for the evolution of trastuzumab resistance, we analyzed cell lines derived from trastuzumab-resistant tumors in more detail. Analysis of VEGF secretion into the conditioned media (measured by ELISA) showed that three of four cell lines expressed significantly higher levels compared with the controls (4,580 pg/10⁶ cells/48 hours; Fig. 5A). This result suggested that perhaps one contributing factor to trastuzumab resistance could be the increased expression of this proangiogenic factor.

Overexpression of EGFR has been proposed as a mechanism of resistance to trastuzumab (29, 30). Human MDA-MB-231 breast cancer cells have been shown to have autocrine activation of the EGFR/TGF- α signaling cascade, which in turn has been reported to have a role in the regulation of VEGF expression (31). Therefore, levels of TGF- α secreted into the conditioned media of cell lines derived from tumors resistant to trastuzumab were measured by ELISA (Fig. 5B). Cell lines derived from untreated 231-H2N xenografts (C1, C2, C3) expressed on average 6.66 pg TGF- α /10⁶ cells/48 hours. Three cell lines derived from trastuzumab-resistant tumors (TrR1, TrR2, TrR3) expressed significantly elevated levels of TGF- α compared with the mean of the control group.

We next sought to determine if there was a genetic change that could explain the development of trastuzumab resistance and the concurrent increase in VEGF expression. To do so, we compared the parental cell lines with the variants TrR1 and TrR2, as these showed the highest increase in VEGF protein expression. To our surprise, we found no significant difference in VEGF mRNA expression (Fig. 5C), suggesting that the increase in VEGF protein secretion (shown in Fig. 5A) is due to post-transcriptional changes.

We next tested to see if changes in the erbB-2 protein, or components of its signaling pathway, could explain the observed acquired resistance. Western blotting analysis showed that although both variants expressed the intracellular domain of erbB-2 (Fig. 5D), the expression was reduced in one variant (TrR2). This variant also showed low or barely detectable levels of phospho-HER-2 or phospho-EGFR (Fig. 5E). In contrast, no significant changes were noted between parental and trastuzumab-resistant variants in terms of phospho-AKT or phospho-mitogen-activated protein kinase levels. Therefore, although one variant (TrR2) showed changes in the target pathways of trastuzumab as a possible mechanism for resistance to this drug, no unifying event was identified to explain the observed resistance. Rather, we believe a number of mechanisms can independently contribute to trastuzumab resistance, any number of which may be observed in a trastuzumab-resistant tumor population.

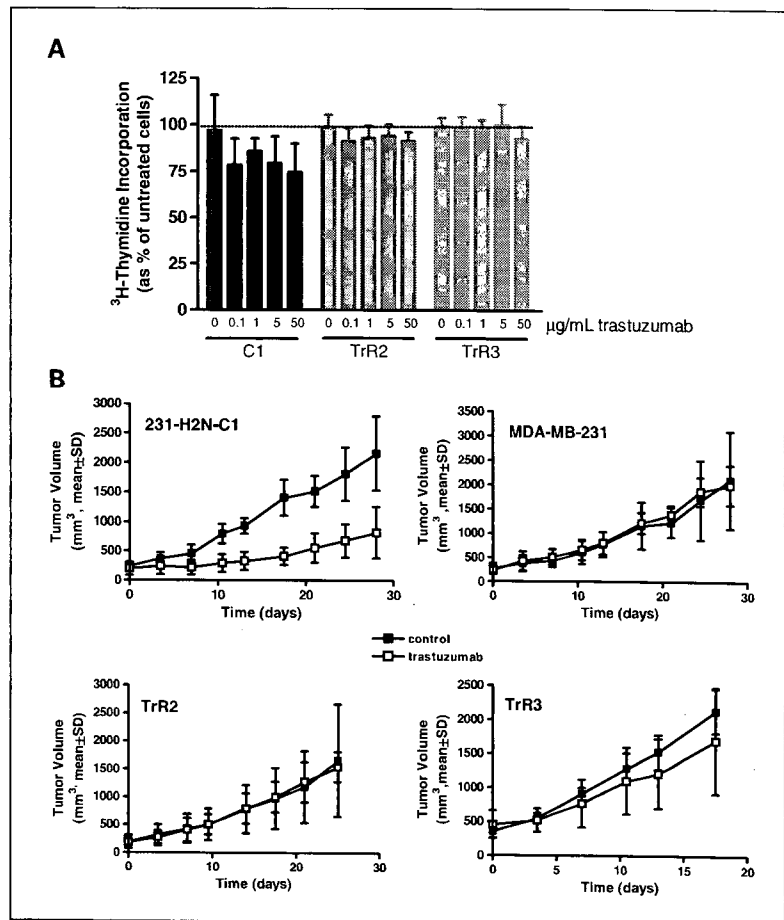
Finally, because loss of PTEN has been reported to predict *intrinsic* clinical resistance to trastuzumab (10), we examined various cell lines for PTEN status. However, we did not find any consistent or obvious changes in PTEN expression in the variants selected for acquired resistance (data not shown).

Antiangiogenic activity of trastuzumab plus cyclophosphamide treatment regimens. Because our results suggested that increased angiogenic activity might account at least in part for the acquired resistance to trastuzumab, we undertook a series of FITC-dextran perfusion studies. Our aim was to test the possible antiangiogenic effects of the various treatment regimens as used in Fig. 2A. In established orthotopic 231-H2N xenografts, all single-agent therapies produced a reduction in tumor perfusion relative to controls after one 21-day treatment cycle, ranging from 10% (trastuzumab) to 38% and 41% (metronomic and MTD cyclophosphamide groups, respectively; Fig. 6A). Combination therapies with trastuzumab resulted in additive antiangiogenic activity, reducing tumor perfusion by 50% and 55% for metronomic and MTD cyclophosphamide combination therapies, respectively. Because trastuzumab exerts its antiangiogenic effects indirectly, by modifying the angiogenic profile of tumors (18), it was not altogether unexpected that trastuzumab alone had no effect in the basic fibroblast growth factor Matrigel plug assay due to the lack of the target (i.e., erbB-2–positive tumor cells; Fig. 6B and D). Accordingly, we noted no additive activity of the combination regimens. However, trastuzumab, metronomic cyclophosphamide, MTD cyclophosphamide, and the corresponding combination regi-

mens noticeably reduced angiogenesis within tumor cell containing Matrigel plugs (Fig. 6C and E).

Second-line therapies of trastuzumab-resistant tumors. Although addition of chemotherapy, especially metronomic cyclophosphamide, to trastuzumab significantly prolonged responses, we also wanted to evaluate therapy options that might be effective in treating tumors that have already acquired overt resistance to the drug. In this regard, the aforementioned results stimulated us to undertake studies testing the anti-human VEGF antibody, bevacizumab, as one such potential second-line therapy. Another potential mechanism of resistance to trastuzumab is the overexpression of EGFR ligands (30, 32). We therefore tested the EGFR targeting antibody cetuximab (C225) and the erbB-2 heterodimerization inhibitor pertuzumab as additional means to overcome trastuzumab resistance. Mice bearing 231-H2N tumors were treated with trastuzumab and metronomic cyclophosphamide until tumors began to progress around 100 days after treatment initiation as shown in Fig. 2A. Mice were then continued on this therapy, and treatment with bevacizumab, cetuximab or pertuzumab was added. Monitoring of tumor growth illustrated that both bevacizumab and cetuximab were effective at delaying tumor growth for ~25 days (i.e., around day 125), before further progression (Fig. 7). In contrast, the addition of pertuzumab provided no benefit compared with those mice maintained on trastuzumab and metronomic cyclophosphamide only, although it shows antiproliferative activity in 231-H2N *in vitro*. These results suggest that in our breast carcinoma model, VEGF

Fig. 4. Resistance to trastuzumab is sustained both *in vitro* and *in vivo* in cells derived from 231-H2N trastuzumab-resistant tumors. Cells were isolated from 231-H2N tumors that became resistant to trastuzumab (TrR2 and TrR3) and from untreated control 231-H2N tumors (231-H2N-C1). **A**, *in vitro* inhibition of cell proliferation was observed in 231-H2N-C1 cells but not in TrR2 and TrR3 cells after 72 hours of treatment with trastuzumab. [³H]thymidine incorporation measurements, reported as % cell proliferation relative to untreated cells. Columns, mean ($n = 8$); bars, SD. **B**, *in vivo* growing orthotopic mammary tumors in SCID mice treated with and without trastuzumab. 231-H2N C1 tumors showed significant growth inhibition when treated with trastuzumab, whereas MDA-MB-231 tumors showed no response. TrR2 and TrR3 tumors showed limited or no response to trastuzumab therapy. Symbols, mean ($n = 4$); bars, SD.



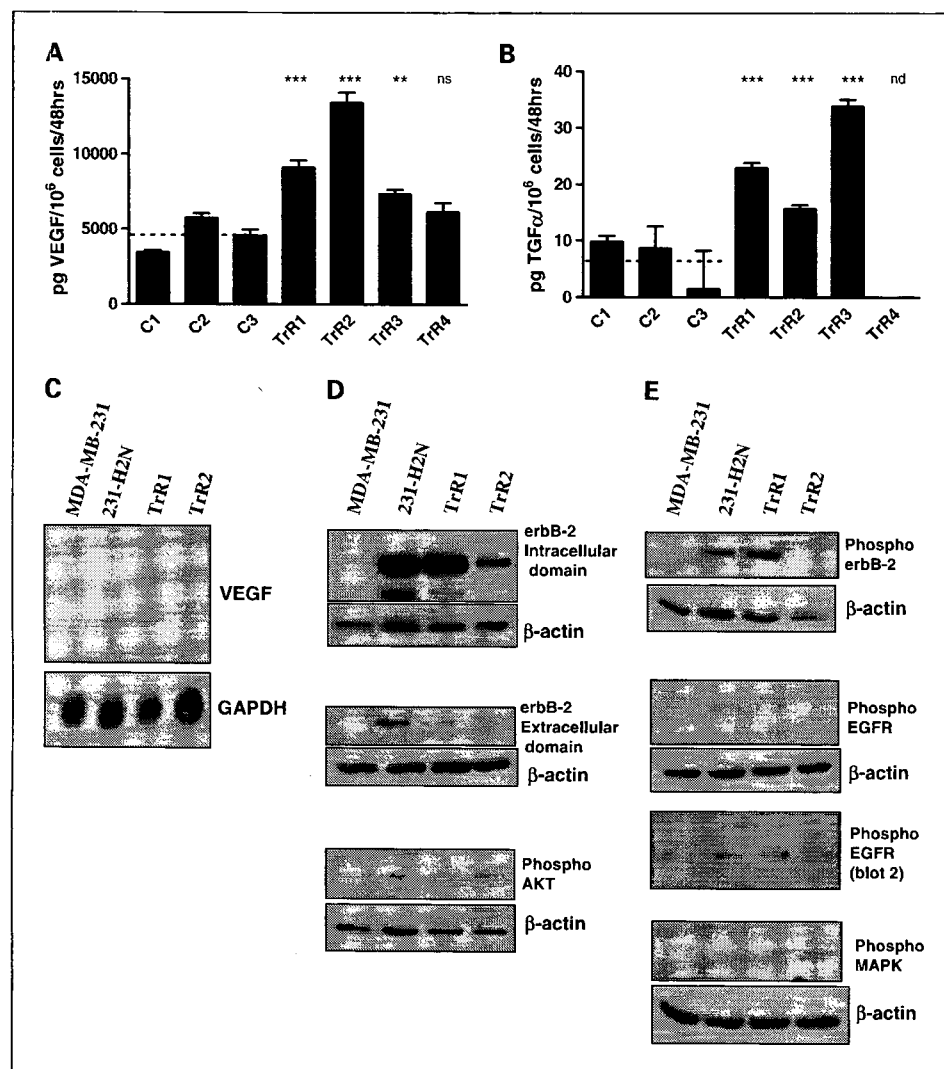


Fig. 5. VEGF (A) and TGF- α (B) secretion by cell lines derived from 231-H2N tumors resistant to trastuzumab (TrR) and control tumors (C), cultured for 48 hours in serum-reduced media, as measured by ELISA. Columns, mean ($n = 4$ for VEGF and $n = 3$ for TGF- α); bars, SD. **, $P < 0.01$; ***, $P < 0.001$. ns, $P > 0.05$; nd, not detected. C, Northern blot of VEGF expression, showing no gross changes in VEGF mRNA levels in trastuzumab-resistant variants (TrR1 and TrR2) compared with the parental 231-H2N cell line. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. D-E, Western blot analysis for expression of erbB-2 intracellular and extracellular domains, as well as for levels of phospho erbB-2, phospho-EGFR, phospho-AKT, and phospho-mitogen-activated protein kinase (phospho-MAPK) levels in the parental cell line MDA-MB-231, the erbB-2 transfectant 231-H2N, and trastuzumab-resistant variants. β -actin was used as a loading control. Phospho-erbB-2 levels were undetectable in the trastuzumab-resistant cell line TrR2 that lacks the extracellular erbB-2 domain. TrR2 shows also relatively low/undetectable levels of phospho-EGFR.

might indeed be involved in the progression of tumors resistant to trastuzumab, suggesting that bevacizumab treatment may be a therapeutic option to consider as a second-line additional agent for the treatment of breast cancer patients who are no longer responding to trastuzumab. Whether the improved response observed by addition of bevacizumab to trastuzumab requires continuation of trastuzumab therapy is unknown and is an issue that should be explored in detail in future investigations. Implicit as well is the possibility that upfront combination of these two drugs may be more effective than either alone, in some cases, by delaying resistance/prolonging responses. In addition, EGFR-targeted drugs, such as cetuximab, may be effective agents in breast cancer patients with both erbB-2 amplification and expression of EGFR, as suggested by the observed delay in tumor progression in our studies when cetuximab was administered to trastuzumab-resistant tumors, which express both erbB-2 and EGFR.

Discussion

The prolonged delay in human breast cancer xenograft growth followed by rapid progression in those treatment

groups receiving trastuzumab therapy with or without chemotherapy (cyclophosphamide) was not unexpected due to the frequent response, and then relapse, of responding patients treated with trastuzumab for metastatic erbB-2-positive breast cancer. Nevertheless, to our knowledge, our study is the first to report such a preclinical relapse *in vivo* following a reasonably prolonged treatment for mice (1 month), as a review of the literature revealed that there are few examples of longer-term studies using trastuzumab therapy. Considering the clinical importance of acquired resistance to trastuzumab, it is surprising that this area is only starting to be explored preclinically in more detail. Our results suggest that the acquired resistance to trastuzumab detected in our system is likely the consequence of heritable genetic alteration(s) in the cells and not due to host-mediated effects, because the resistant phenotype was maintained both *in vitro* and *in vivo* when reinjected into mice. Also interesting is that the majority of cell lines obtained from resistant tumors in this study maintained overexpression of the *erbB-2* gene, suggesting that other tumor cell alterations are required to lead to the resistant phenotype.

Although an up-regulation of VEGF (and TGF- α) in tumors resistant to trastuzumab therapy was observed in our studies,

further work is required to confirm this observation (especially at the clinical level) and identify the mechanism(s) by which this occurs. In this regard, it is perhaps noteworthy that Konecny et al. have reported an association between *erbB-2*/HER2 and VEGF expression, which predicts clinical outcome in the treatment of breast cancer (33). There are a number of mechanisms that could potentially be involved in an up-regulation of VEGF in the trastuzumab-resistant variants (e.g. up-regulation of insulin-like growth factor-IR signaling; refs. 34–36) or other tyrosine kinase receptors, acquisition of PTEN mutations leading to loss of function (37), constitutive activation of phosphatidylinositol-3 kinase (38), or nuclear factor- κ B (39), the development of hypoxia-inducible factor- α -independent VEGF expression (40) or changes in expression or degradation of hypoxia-inducible factor- α (41), among others. Because the resistant phenotype of the variants was expressed not only *in vivo* but *in vitro*, the contribution of elevated VEGF expression to the observed resistance can be called into question. This assumes that VEGF can *only* function as a paracrine factor and affect host dependent processes, such as tumor angiogenesis. However, it is possible that VEGF may act in an *autocrine* fashion due to expression of VEGF binding receptors on breast cancer cells (e.g., VEGFR-1, VEGFR-2, or neuropilin-1; refs. 42–44). We are currently evaluating whether the trastuzumab-resistant variants also (over)express such receptors, and if so, whether the elevated

VEGF levels in the variants might directly contribute to the resistant phenotype *in vitro*.

Regardless of the mechanism by which tumors become resistant to trastuzumab, addition of an anti-VEGF-directed regimen, such as the antibody bevacizumab, to trastuzumab therapy can be rationalized by both the up-regulation of VEGF through the *erbB-2* oncogene (41) and our present finding that VEGF is up-regulated within the tumor microenvironment upon acquisition of a resistant phenotype. The preliminary studies presented here, using our orthotopic tumor model, indicate that resumed tumor growth due to resistance to trastuzumab can be halted for a time by the addition of bevacizumab, the anti-VEGF monoclonal antibody, to the drug cocktail. It would be interesting to determine whether or not the administration of these two drugs before the development of resistance to trastuzumab therapy, as suggested by Konecny et al. (33) and Pegram and Reese (45), could delay tumor regrowth due to drug resistance. Indeed, a clinical trial has been initiated testing the combination of bevacizumab and trastuzumab for treatment of metastatic breast cancer, and early (interim) results look extremely promising (46).

The administration of the anti-EGFR antibody cetuximab to trastuzumab-resistant tumors was also effective at delaying tumor progression compared with those mice maintained only on metronomic cyclophosphamide with trastuzumab.

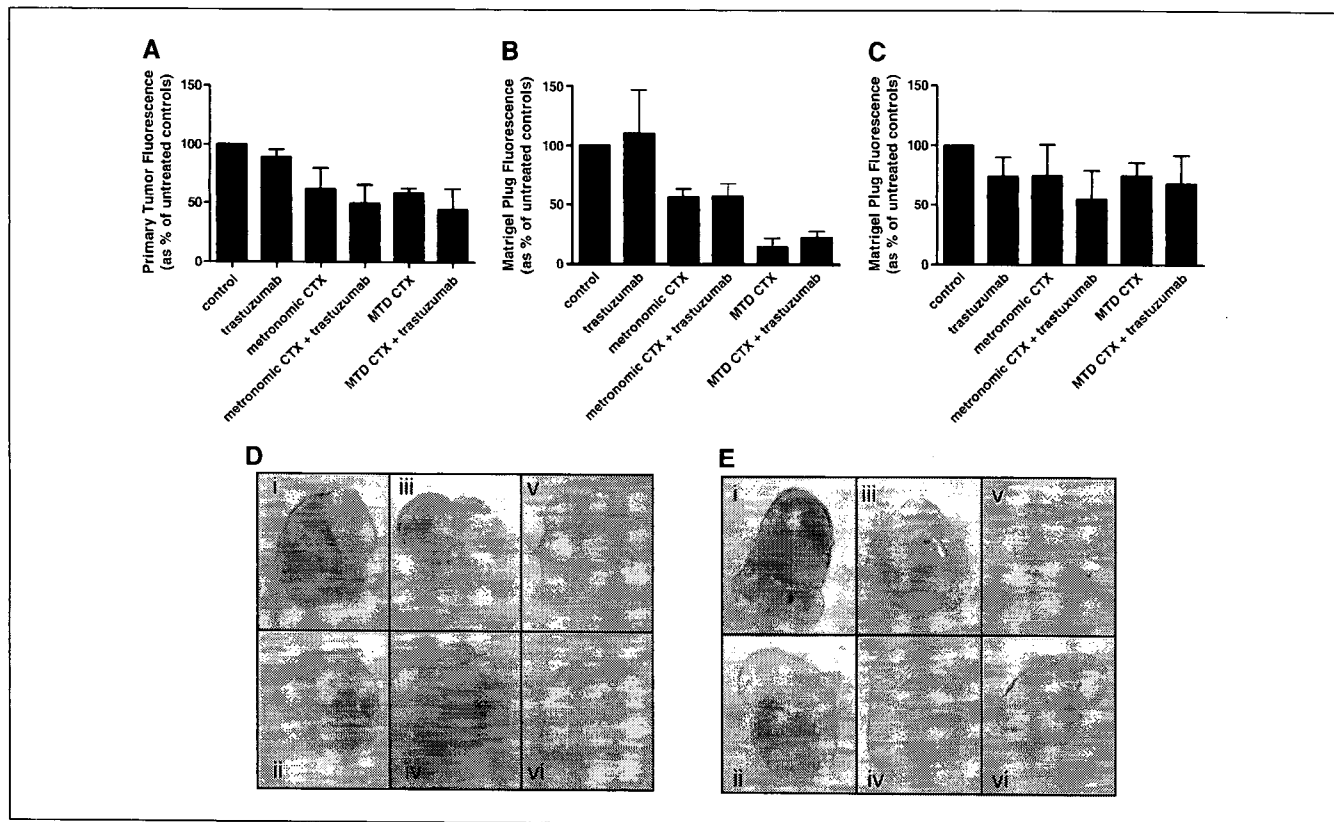


Fig. 6. *In vivo* assessment of antiangiogenic effects of trastuzumab and cyclophosphamide (CTX) therapies. **A**, tumor perfusion assay. The decrease in intravascular FITC-dextran fluorescence reflects the changes in tumor perfusion in established 231-H2N orthotopic tumors treated with various cyclophosphamide and trastuzumab therapies for a 3-week cycle. **B**, basic fibroblast growth factor Matrigel assay. **C**, tumor cell Matrigel assay. The decrease in FITC-dextran fluorescence in the Matrigel plugs indicates inhibition of angiogenic processes by therapeutic regimens administered for 7 days. All data reported as fluorescence relative to untreated controls. Columns, mean ($n = 5$); bars, SD. Appearance of (**D**) basic fibroblast growth factor and (**E**) tumor cell Matrigel plugs excised from mice after 7 days of treatment. i, control; ii, trastuzumab; iii, metronomic cyclophosphamide; iv, metronomic cyclophosphamide + trastuzumab; v, MTD cyclophosphamide; vi, MTD cyclophosphamide + trastuzumab.

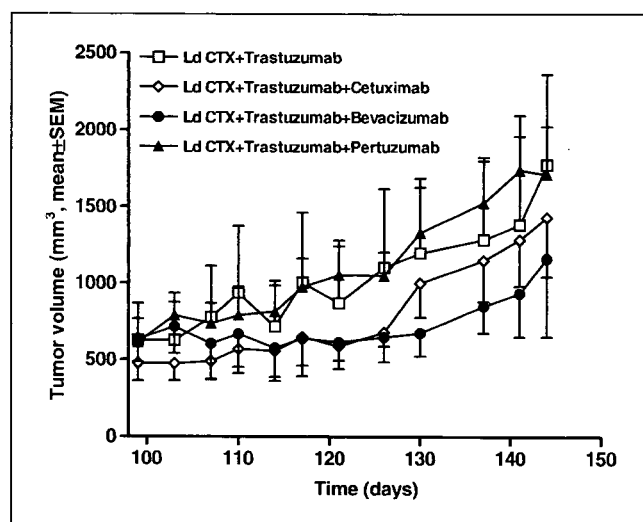


Fig. 7. Addition of second-line therapies to block rapid tumor growth observed after long-term treatment of 231-H2N tumors with trastuzumab and metronomic cyclophosphamide (CTX). 231-H2N cells were injected orthotopically into SCID mice, and tumors were then treated with low-dose (Ld) metronomic cyclophosphamide plus trastuzumab. After 98 days, 231-H2N tumors that began progressing to trastuzumab + metronomic cyclophosphamide therapy were maintained on the same regimen with and without additional treatment of bevacizumab, or cetuximab, or pertuzumab as described in Materials and Methods. Both the additional bevacizumab and the additional cetuximab treatments effectively delayed further tumor growth for 25 to 30 days. Symbols, mean ($n = 4$); bars, SE. $P < 0.001$ for low-dose cyclophosphamide + trastuzumab + bevacizumab and low-dose cyclophosphamide + trastuzumab + cetuximab versus low-dose cyclophosphamide + trastuzumab. $P > 0.05$ for low-dose cyclophosphamide + trastuzumab + pertuzumab versus low-dose cyclophosphamide + trastuzumab.

Although both EGFR and erbB-2 homodimers induce the expression of VEGF, two heterodimers (erbB-2/erbB3 and EGFR/erbB-2) induce even more potently VEGF expression and enhance tumor angiogenesis (47). Therefore, by simultaneously blocking EGFR and erbB-2 signaling with cetuximab and trastuzumab, respectively, the formation of the most potent heterodimer to regulate VEGF is prevented, in addition to more efficiently inhibiting tumor cell proliferation/survival mediated by EGFR signaling. Whereas 25% to 30% of breast cancer patients overexpress erbB-2, only 5.4% overexpress both EGFR and erbB-2 (48). It might be speculated that unless EGFR is up-regulated through the development of resistance to trastuzumab, anti-EGFR treatment strategies may not be as appropriate a second-line drug therapy as bevacizumab or other direct antiangiogenic inhibitors, because so few breast cancer patients would seemingly fit the criteria for cetuximab therapy. However, overexpression of EGFR may not be necessary as a determinant of potential responsiveness, especially if one or more of the ligands for EGFR is up-regulated. In any case, it will be of considerable interest to determine if resistance to trastuzumab in the clinic is accompanied by elevated expression or function of normal (wild type) or mutated EGFRs (and/or relevant ligands). In this regard, it is interesting to note that acquired resistance to the estrogen receptor antagonist tamoxifen, and loss of progesterone expression, can be accompanied by an increase in EGFR and erbB-2 expression and activity (49, 50).

Overexpression of erbB-2/HER2 is detected in the tumor cells of 25% to 30% of breast cancer patients (51), who are therefore candidates for therapy with trastuzumab, or other

erbB-2-targeting agents. Although trastuzumab has been approved as a single-agent therapy for advanced metastatic disease (52), this drug is more commonly combined with paclitaxel- or platinum-based chemotherapy regimens, thereby eliminating or minimizing the potential nontoxic benefits of trastuzumab. Furthermore, as the use of trastuzumab is extended into the adjuvant setting (53, 54), there may be a need to assess whether this drug can be combined with an alternative to highly toxic, MTDs of chemotherapy, especially after relapses to such a combination therapy occur. This brings us to the other major purpose of our studies: to determine whether responses to trastuzumab can be significantly prolonged by metronomic chemotherapy-based regimens.

Our results indicate that low-dose, metronomic cyclophosphamide in combination with trastuzumab was as effective as MTD cyclophosphamide combination therapy at blocking tumor growth for extended periods of time as well as delaying the development of resistance to trastuzumab. Yet, unlike MTD cyclophosphamide combination therapy, which showed evidence of toxicity through weight fluctuations and severe weight loss, the chronic administration of oral metronomic cyclophosphamide on a daily basis had no indications of serious long-term toxicity, even for treatment periods extending for up to 140 days, using SCID mice known to be particularly sensitive to this type of drug. We suggest that the prolonged antitumor efficacy of metronomic combination therapy may in part be due to the additive antiangiogenic side effects of these two drugs. In this regard, antiangiogenic properties have been attributed to trastuzumab by several groups (17, 18). Moreover, it has been reported by others (52) that trastuzumab, when combined with weekly low-dose paclitaxel, more effectively suppresses tumor growth, metastasis, and erbB-2-mediated angiogenesis, than either therapy alone, in an orthotopic breast tumor model. Our results confirm and extend this observation with an additional low-dose metronomic chemotherapy regimen, cyclophosphamide through the drinking water, which mimics the long-term treatment of metastatic breast cancer patients with daily low-dose cyclophosphamide with no breaks (55) and supports the additive antiangiogenic effects of combination therapy.

The decision to use cyclophosphamide for our studies may be questioned in view of the fact that trastuzumab is often combined with taxane- or platinum-based drugs in the clinic. There are several reasons for our choice of chronic administration of cyclophosphamide. First, we have accumulated considerable information about metronomic cyclophosphamide regimens, including the use of a regimen of delivering the drug orally on a daily basis, in our studies through the drinking water (14). This type of regimen is convenient for the types of prolonged therapy studies that we (16) and others (56) have undertaken. Second, we have determined the optimal daily dose for this metronomic regimen, which is 20 to 25 mg/kg/d. Third, our results could lay the groundwork for future clinical trials involving daily low-dose cyclophosphamide and trastuzumab, in the metastatic setting, and perhaps in the adjuvant therapy setting. For example, based on preclinical results and phase II clinical trial studies of daily low-dose oral cyclophosphamide (50 mg) in breast cancer (55), trials have been initiated in advanced breast or ovarian cancer using daily low-dose cyclophosphamide and bevacizumab, administered every 2 weeks (refs. 57, 58; <http://www.ClinicalTrials.gov>; identifier NCT00083031), and an adjuvant metronomic cyclophosphamide plus bevacizumab trial has been

initiated in early-stage operable breast cancer (<http://www.ClinicalTrials.gov>; identifier NCT00121134). Another possible methodologic concern regarding the model and approach we studied is the use of an erbB-2 transfectant. Breast cancer cell lines, which express endogenous erbB-2, may obviously behave differently with respect to how they acquire resistance to trastuzumab. However, the poor tumorigenic properties of the few such lines that are available led us to adopt the transfection approach as an experimental alternative.

In conclusion, we have outlined an experimental approach to study acquired resistance *in vivo* to the erbB-2-specific anticancer drug, trastuzumab. Using this model, we have shown that eventual relapses during trastuzumab therapy can be significantly delayed by prolonged combination therapy with metronomic low-dose cyclophosphamide chemotherapy, which seems as efficacious but much less toxic compared with

combination with MTD cyclophosphamide therapy. In addition to delaying acquired resistance to trastuzumab, we have outlined some potential therapeutic approaches to treating tumor variants, which have actually acquired resistance (i.e., treatment with either anti-VEGF and anti-EGFR antibodies). In this regard, our results would seem to provide further support for the rationale of treating breast cancer using combinations of the targeted therapeutics, such as trastuzumab and bevacizumab (46). Other possible drug combination may be implicated by future studies designed to uncover the biochemical/molecular basis of the resistant phenotype.

Acknowledgments

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References

- Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;344:783–92.
- Capdeville R, Buchdunger E, Zimmermann J, Matter A. Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. *Nat Rev Drug Discov* 2002;1:493–502.
- Castillo L, Etienne-Grimaldi MC, Fischel JL, Formento P, Magne N, Milano G. Pharmacological background of EGFR targeting. *Ann Oncol* 2004;15:1007–12.
- McCormick F. New-age drug meets resistance. *Nature* 2001;19:281–2.
- Gorre ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 2001;293:876–80.
- Kute T, Lack CM, Willingham M, et al. Development of Herceptin resistance in breast cancer cells. *Cytometry* 2004;57A:86–93.
- Nahta R, Esteva FJ. *In vitro* effects of trastuzumab and vinorelbine in trastuzumab-resistant breast cancer cells. *Cancer Chemother Pharmacol* 2004;53:186–90.
- Nahta R, Takahashi T, Ueno NT, Hung MC, Esteva FJ. P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. *Cancer Res* 2004;64:3981–6.
- Cardoso F, Piccart MJ, Durbecq V, Di Leo A. Resistance to trastuzumab: a necessary evil or a temporary challenge? *Clin Breast Cancer* 2002;3:247–57.
- Nagata Y, Lan KH, Zhou X, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* 2004;6:117–27.
- Tanner M, Kapanen AI, Junttila T, et al. Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer. *Mol Cancer Ther* 2004;3:1585–92.
- Nagy P, Friedlander E, Tanner M, et al. Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a Herceptin-resistant, MUC4-expressing breast cancer cell line. *Cancer Res* 2005;65:473–82.
- Konecny GE, Thomssen C, Luck HJ, et al. Her-2/*neu* gene amplification and response to paclitaxel in patients with metastatic breast cancer. *J Natl Cancer Inst* 2004;96:1141–51.
- Man S, Bocci G, Francia G, et al. Antitumor and antiangiogenic effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. *Cancer Res* 2002;62:2731–5.
- Browder T, Butterfield CE, Kraling BM, Marshall B, O'Reilly MS, Folkman J. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* 2000;60:1878–86.
- Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* 2000;105:R15–24.
- Viloria-Petit AM, Rak J, Hung M-C, Rockwell P, Goldstein N, Kerbel RS. Neutralizing antibodies against EGF and ErbB-2/*neu* receptor tyrosine kinases down-regulate VEGF production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997;151:1523–30.
- Izumi Y, Xu L, di Tomaso E, Fukumura D, Jain RK. Herceptin acts as an anti-angiogenic cocktail. *Nature* 2002;416:279–80.
- Viloria-Petit A, Crombet T, Jothy S, et al. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies *in vivo*: a role for altered tumor angiogenesis. *Cancer Res* 2001;61:5090–101.
- Francia G, Mitchell SD, Moss SE, Hanby AM, Marshall JF, Hart IR. Identification by differential display of Annexin-VI, a gene differentially expressed during melanoma progression. *Cancer Res* 1996;56:3855–8.
- Gerber HP, Kowalski J, Sherman D, Eberhard DA, Ferrara N. Complete inhibition of rhabdomyosarcoma xenograft growth and neovascularization requires blockade of both tumor and host vascular endothelial growth factor. *Cancer Res* 2000;60:6253–8.
- Ciardiello F, Bianco R, Damiano V, et al. Antiangiogenic and antitumor activity of anti-epidermal growth factor receptor C225 monoclonal antibody in combination with vascular endothelial growth factor antisense oligonucleotide in human GEO colon cancer cells. *Clin Cancer Res* 2000;6:3739–47.
- Prewett MC, Hooper AT, Bassi R, Ellis LM, Waksal HW, Hicklin DJ. Enhanced antitumor activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumor xenografts. *Clin Cancer Res* 2002;8:994–1003.
- Zhang W, Ran S, Sambade M, Huang X, Thorpe PE. A monoclonal antibody that blocks VEGF binding to VEGFR2 (KDR/Flk-1) inhibits vascular expression of Flk-1 and tumor growth in an orthotopic human breast cancer model. *Angiogenesis* 2002;5:35–44.
- Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. *Nat Med* 2000;6:443–6.
- Pegram M, Hsu S, Lewis G, et al. Inhibitory effects of combinations of HER-2/*neu* antibody and chemotherapeutic agents used for treatment of human breast cancers. *Oncogene* 1999;18:2241–51.
- Emmenegger U, Man S, Shaked Y, et al. A comparative analysis of low dose metronomic cyclophosphamide reveals absent or low grade toxicity on tissues highly sensitive to the toxic effects of maximum tolerated dose regimens. *Cancer Res* 2004;64:3994–4000.
- Spiridon CI, Ghetie MA, Uhr J, et al. Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antigrowth activity of a human breast cancer cell line *in vitro* and *in vivo*. *Clin Cancer Res* 2002;8:1720–30.
- Ritter CA, Bianco R, Dugger T, et al. HER2-overexpressing breast cells selected for Herceptin (trastuzumab) resistance *in vivo* retain HER2 gene amplification and overexpress HER1/ERG receptor ligands. *Proc Am Assoc Cancer Res* 2003;44:969–70.
- Valabrega G, Montemurro F, Sarotto I, et al. Mechanisms responsible for resistance to trastuzumab in human breast cancers. AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics. 2003. Ref Type: Abstract.
- Masuda M, Suzui M, Lim JT, Deguchi A, Soh JW, Weinstein IB. Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction. *J Exp Ther Oncol* 2002;2:350–9.
- Ritter CA, Bianco R, Dugger T, et al. Mechanisms of resistance development against trastuzumab (Herceptin) in an *in vivo* breast cancer model. *Int J Clin Pharmacol Ther* 2004;42:642–3.
- Konecny GE, Meng YG, Untch M, et al. Association between HER-2/*neu* and vascular endothelial growth factor expression predicts clinical outcome in primary breast cancer patients. *Clin Cancer Res* 2004;10:1706–16.
- Oh JS, Kucab JE, Bushel PR, et al. Insulin-like growth factor-1 inscribes a gene expression profile for angiogenic factors and cancer progression in breast epithelial cells. *Neoplasia* 2002;4:204–17.
- Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. *J Biol Chem* 2002;277:38205–11.
- Stoeltzing O, Liu W, Reinmuth N, et al. Regulation of hypoxia-inducible factor-1 α , vascular endothelial growth factor, and angiogenesis by an insulin-like growth factor-I receptor autocrine loop

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SUPERINTENDENCIA

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Trámite :	11 PATENTEIYII	
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Actuación :	432 COMUNICACTOADM	
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Actuación : 432 COMUNICACION ACTO ADMINISTRATIVO

Comendidamente le solicito presentarse a este Despacho ubicado en la Carrera 13 No. 27 - 00 Piso Tercero (3), de la ciudad de Bogotá D.C., dentro del término de cinco (5) días hábiles contados a partir del envío de la presente citación, en horario de 8:00 a.m. a 4:30 p.m. jornada continua, con el fin de notificarle personalmente el contenido de la resolución de la referencia proferida dentro del expediente tramitado con el número de radicación citado en el asunto.

De no hacerse presente en el término indicado anteriormente, la providencia en mención se notificará por edicto, el cual se fijará por el término de diez (10) días en el Centro de Documentación e Información.

Para su comodidad el texto completo de la resolución se encontrará disponible en la página web de la Entidad desde el día hábil siguiente al de su notificación, la cual podrá ser consultada desde un computador con acceso a Internet, en la página www.sic.gov.co, con el número de resolución a través de la opción trámites y servicios - de carácter general transversal - consulta actos administrativos y providencias.

En caso de que se haya notificado, haga caso omiso de este aviso.

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RESOLUCION **31105**

Por la cual se declara abandonada una solicitud

Rad. PCT/ 09-18558

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

en ejercicio de sus facultades legales, en especial de las conferidas en el numeral 38 del artículo 3° del Decreto 1687 de 2010, y

CONSIDERANDO:

PRIMERO: Que mediante escrito radicado en esta Superintendencia el día 24 de febrero de 2009 con el N° 09-018558-00000-0000, la doctora Alicia Lloreda Ricaurte, en su calidad de apoderada de F. Hoffmann - La Roche AG, presentó la solicitud de patente de invención titulada "TERAPIA TUMORAL CON UN ANTICUERPO ANTI-VEGF", correspondiente a la solicitud internacional PCT/EP 2007/007276 de fecha 17 de agosto de 2007, para que se iniciara la fase nacional en virtud del Tratado de Cooperación en Materia de Patentes (PCT).

SEGUNDO: Que efectuado el examen de forma en cuanto a los requisitos que debe llenar la solicitud y cumplidas las exigencias legales, se ordenó publicar el extracto en la Gaceta de la Propiedad Industrial N° 615 del 20 de abril de 2010.

TERCERO: Que mediante escritos radicados en esta Entidad los días 16 y 19 de julio de 2010 con los números 09-018558-00002-0000 y 09-018558-00003-0000, encontrándose dentro del término establecido para el efecto, los doctores Cielo Ángela Peña Rodríguez, en su calidad de apoderada de Procaps S.A., y José Luis Reyes Villamizar, en su calidad de apoderado del Laboratorio Franco Colombiano Lafrancol S.A., respectivamente, presentaron oposiciones fundamentadas, las cuales cumplieron los requisitos formales previstos por la normativa andina. (Fls. 90 a 152 y 161 a 177).

CUARTO: Que el artículo 44 de la Decisión 486 de la Comisión de la Comunidad Andina, establece que: *"Dentro del plazo de seis meses contados desde la publicación de la solicitud, independientemente que se hubieren presentado oposiciones, el solicitante deberá pedir que se examine si la invención es patentable. Los Países Miembros podrán cobrar una tasa para la realización de este examen. Si transcurriera dicho plazo sin que el solicitante hubiera pedido que se realice el examen, la solicitud caerá en abandono."*

QUINTO: Que contados seis meses desde la publicación en la Gaceta de la Propiedad Industrial, no se presentó petición de parte de la interesada para efectuar el examen de fondo, por lo que este Despacho considera abandonada la solicitud en los términos del mencionado artículo 44 de la Decisión 486 de la Comisión de la Comunidad Andina y, en consecuencia,

RESUELVE:

31105



Industria y Comercio

SUPERINTENDENCIA

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCION

Por la cual se declara abandonada una solicitud **#31105**

Rad. PCT/ 09-18558

ARTÍCULO PRIMERO: Declarar abandonada la solicitud de patente de invención titulada "TERAPIA TUMORAL CON UN ANTICUERPO ANTI-VEGF", que entró en fase nacional en virtud del Tratado de Cooperación en Materia de Patentes (PCT).

ARTÍCULO SEGUNDO: Notificar personalmente el contenido de la presente resolución a la doctora Alicia Lloreda Ricaurte, en su calidad de apoderada de F. Hoffmann - La Roche AG, y a los doctores Cielo Ángela Peña Rodríguez, en su calidad de apoderada de Procaps S.A., y José Luis Reyes Villamizar, en su calidad de apoderado del Laboratorio Franco Colombiano Lafranco S.A., sociedades opositoras, o a quienes hagan sus veces, entregándoles copia de la misma y advirtiéndoles que contra ella procede el recurso de reposición, ante el Superintendente de Industria y Comercio, el cual podrá ser interpuesto en el momento de la notificación o dentro de los cinco 5 días hábiles siguientes a ella.

NOTIFÍQUESE Y CÚMPLASE

Dada en Bogotá, D.C., a los

31 MAYO 2011

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO,

JOSÉ MIGUEL DE LA CALLE RESTREPO

Doctora

ALICIA LLOREDA RICAURTE

Calle 72 N° 5 – 83, Piso 5.

Bogotá D.C.

Doctora

CIELO ÁNGELA PEÑA RODRÍGUEZ

Carrera 13 N° 119 – 95, Of. 104.

Bogotá D.C.

Doctor

JOSÉ LUIS REYES VILLAMIZAR

Carrera 17 N° 88 – 23, Of. 205.

Bogotá D.C.

202021

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
EL SECRETARIO GENERAL

Certifica que la Resolución 31105 de fecha 31/05/2011
fue notificado mediante listado número 90
fijado el 10/06/2011 y desfijado el 23/06/2011

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Compareció Bladim Camacho
Identificado con 30863097

y T.P. No. _____
Con el fin de notificarse de la presente resolución
Recibió copia auténtica de la misma y se advirtió que
Contra ella proceden los recursos de reposición ante
La División de Signos Distintivos y el de apelación
Para ante el delegado para la Propiedad Industrial
Bentín de los cinco (5) días hábiles siguientes a esta
Notificación.
Fecha 08 JUN. 2011

Anexo Poder-

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
NOTIFICADOR

AVISO DE NOTIFICACION
CORREO CERTIFICADO**Industria y Comercio**
SUPERINTENDENCIA

Radicación :	9-18558- -7-0	Fecha: 2011-06-01 08:45:38
Trámite :	11 PATENTEIYII	
Evento :	378 FASENACIONALI	
Actuación :	432 COMUNICACTOADM	
Destinatario :	CIELO ANGELA PEÑA RODRIGUEZ Folios 1	

Señor (a) (es)
CIELO ANGELA PEÑA RODRIGUEZ
CARRERA 13 # 119-95 OFC. 104
BOGOTA D.C.

Referencia RESOLUCION : 31105
Fecha : 31/05/2011
Expediente : 9-18558
Trámite : 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II
Evento : 378 FASE NACIONAL INCLUIDO CAPITULO I
Actuación : 432 COMUNICACION ACTO ADMINISTRATIVO

Comendidamente le solicito presentarse a este Despacho ubicado en la Carrera 13 No. 27 - 00 Piso Tercero (3), de la ciudad de Bogotá D.C., dentro del término de cinco (5) días hábiles contados a partir del envío de la presente citación, en horario de 8:00 a.m. a 4:30 p.m. jornada continua, con el fin de notificarle personalmente el contenido de la resolución de la referencia proferida dentro del expediente tramitado con el número de radicación citado en el asunto.

De no hacerse presente en el término indicado anteriormente, la providencia en mención se notificará por edicto, el cual se fijará por el término de diez (10) días en el Centro de Documentación e Información.

Para su comodidad el texto completo de la resolución se encontrará disponible en la página web de la Entidad desde el día hábil siguiente al de su notificación, la cual podrá ser consultada desde un computador con acceso a Internet, en la página www.sic.gov.co, con el número de resolución a través de la opción trámites y servicios - de carácter general transversal - consulta actos administrativos y providencias.

En caso de que se haya notificado, haga caso omiso de este aviso.

Secretaría General
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Al contestar favor indique el número de radicación consignado en el sticker

Sede Centro: Carrera 13 No. 27-00 pisos 3, 5, 7 y 10 PBX: 5870000
Sede CAN: Av. Carrera 50 No. 26- 55 int. 2 Tel 5880070
Call Center 6513240 . Línea 018000-910165
Web: www.sic.gov.co e-mail: info@sic.gov.co
Bogotá D.C. Colombia

AVISO DE NOTIFICACION
CORREO CERTIFICADO**Industria y Comercio**
SUPERINTENDENCIA

Radicación :	9-18558- -8-0	Fecha: 2011-06-01 08:45:38
Trámite :	11 PATENTEIYII	
Evento :	378 FASENACIONALI	
Actuación :	432 COMUNICACTOADM	
Destinatario :	JOSE LUIS REYES VILLAMIZAR Folios 1	

Señor (a) (es)
JOSE LUIS REYES VILLAMIZAR
CARRERA 17 NO 88 - 23 OF 205
BOGOTA D.C.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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Bogotá D.C. Colombia

REYES & REYES
A B O G A D O S

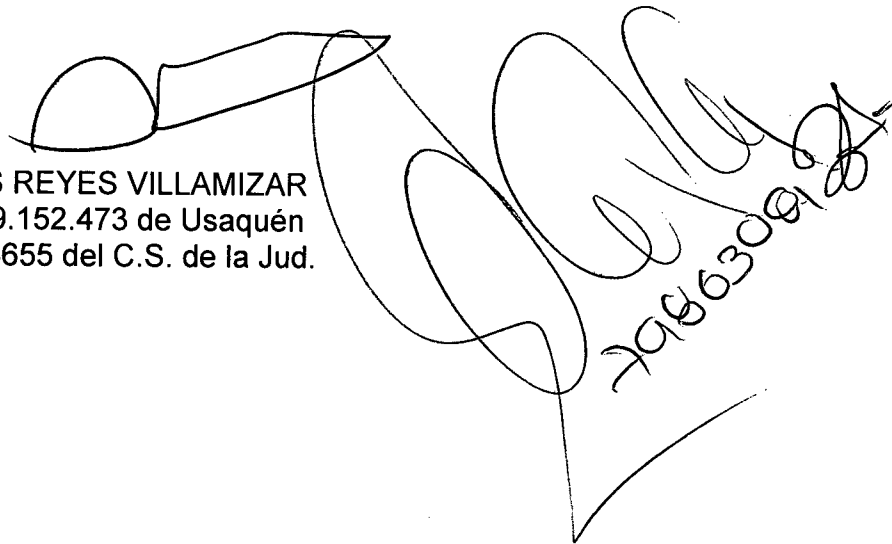
Señor (a)
Director (a) de la División de Nuevas Creaciones
Superintendencia de Industria y Comercio
E. S. D.

Ref.: Resolución : 31105
Fecha : 31/05/2011
Expediente No. : 09-18558

Yo JOSE LUIS REYES VILLAMIZAR, identificado como aparece al pie de mi firma, autorizo al señor BLADIMIR GOMEZ identificado con C.C. No.79.863.081 de Bogotá, para que en mi nombre se notifique de la resolución enunciada en la referencia.

Atentamente,

JOSE LUIS REYES VILLAMIZAR
C.C. No. 79.152.473 de Usaquén
T.P. No. 44655 del C.S. de la Jud.

A large, stylized handwritten signature in black ink, followed by the handwritten identification number 79.863.081.