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FORMULARIO UNICO DE OPOSICION
2000-12

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OPOSICION A:

- | | |
|---|---|
| ☒ Patente de Invención | ☐ Marca Colectiva |
| ☐ Patente de Modelo de Utilidad | ☐ Marca de Certificación |
| ☐ Diseño Industrial | ☐ Lema Comercial |
| ☐ Esquema de Trazado para Circuitos Integrados | |
| ☐ Marcas de Productos o Servicios | |

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Número del expediente: 09 047.482.

GACETA No. 618 del 19/07/2010 N° de publicación: 618.

Solicitante: IRM LLC.

SOLICITUD Apoderado: CARLOS R. OLARTE.

PUBLICADA Título de la nueva creación o denominación del signo:
ANTICUERPOS AGONISTAS DEL TRKB Y USOS DE LOS MISMOS.

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OPOSICION PRESENTADA POR:

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	Domicilio: Bogotá, D.C.		

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FUNDAMENTOS DE LA OPOSICION:

Causal: Incumplimiento de requisitos de patentabilidad (Título II, Capítulo I, Decisión 486 de 2000). Se violan los artículos 14, 16 (las patentes de invención deben ser nuevas, tener nivel inventivo y ser susceptibles de aplicación industrial) y 18 (una invención tiene nivel inventivo si no es obvia para una persona versada en la materia).

Justificación: VER ANEXO

162

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Comprobante de pago No. 1086041 (Oposición) Fecha Octubre 13 / 2010
(Prórroga) Fecha _____

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Anexos:

- ☒ Comprobante de pago de la tasa
- ☒ Poderes, si fuere el caso
- ☒ Pruebas de los fundamentos de la oposición
- ☒ Certificado de existencia y representación legal de las partes, cuando sean personas jurídicas
- ☐ Documentos que acrediten el legítimo interés, si fuere el caso
- ☒ Copia de la oposición para el traslado al solicitante.

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NOMBRE: JOSÉ LUIS REYES VILLAMIZAR

FIRMA: _____

C.C.No. 79.152.473 T.P. No. 44655

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

E.

S.

D.

REF: Expediente 09 047.482

TÍTULO SOLICITADO: ANTICUERPOS AGONISTAS DEL TRKB Y USOS DE LOS MISMOS.

PUBLICACIÓN: Gaceta 618 de 19/07/2010, N° de publicación 432.

PRIORIDAD: US 60/858.169 de 09/11/2006

SOLICITANTE: IRM LLC.

APODERADO: CARLOS R. OLARTE.

TRÁMITE: PRESENTACIÓN DE OPOSICION

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 **LAFRANCOL S.A.**, sociedad constituida y vigente de conformidad con las leyes colombianas, con domicilio en Cali, Valle, en ejercicio de lo dispuesto por el artículo 42 de la Decisión 486 de 2000, muy respetuosamente me permito formular la siguiente

I. PETICIÓN

Negar el beneficio patentario a la solicitud contenida en el expediente **09 047.482**, por no cumplir con los requisitos de patentabilidad a que se refieren los artículos 14, 16, 18 y 20 literal d) de la Decisión 486 de la Comunidad Andina de Naciones, con base en los fundamentos de hecho y de derecho que me permito exponer a continuación.

II. LEGÍTIMO INTERÉS

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

III. FUNDAMENTO DE LA OPOSICIÓN

La solicitud colombiana en trámite **09 047.482**, que se refiere a un anticuerpo agonista aislado del receptor B tirosinquinasa (TrkB), una composición farmacéutica que lo contiene solo o en combinación con agentes que reducen los niveles de glucosa en sangre y/o el peso corporal de un individuo ; incumple con los requisitos fundamentales para poder otorgársele el privilegio de patente de invención, tal y como se demuestra a continuación:

En primer lugar, 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 reducir los niveles de glucosa en sangre y/o el peso corporal de un individuo, tal como lo señala en sus reivindicaciones 18 a 25, queda claramente expuesta la improcedencia directa para otorgar el privilegio de patente de invención a esta solicitud.

En un segundo aspecto, y esta vez refiriéndonos a la combinación farmacológica que reivindica la solicitud colombiana en cuestión, específicamente en sus reivindicaciones 17 a 23, es relevante mencionar el concepto de la Doctora en Bioquímica Silvia Susana Giarcovich¹ en su opinión escrita acerca de "Asociaciones y Patentes" en donde afirma que las asociaciones farmacológicas están restringidas por ciertos principios y lineamientos preestablecidos por varias

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

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.
- 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.

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

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 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."

Además, se puede considerar que todo efecto sinérgico o terapéutico entre fármacos no se refiere propiamente a una invención, sino que corresponde mejor a la categoría de un descubrimiento, dado que dicho efecto sinérgico o terapéutico conseguido mediante la combinación de fármacos ocurre dentro del organismo y se hace evidente a través de los respectivos ensayos preclínicos y/o clínicos por los que todo fármaco o combinación de fármacos deben ser evaluados. Así mismo es importante enfatizar en que la combinación de fármacos para tratar ciertas patologías, hace parte de las actividades de la profesión médica, y no de las actividades industriales.

Finalmente podemos demostrar que existe suficiente información científica publicada con anterioridad, que afecta de manera directa el nivel inventivo de la solicitud colombiana, veamos:

Novel Agonist Monoclonal Antibodies Activate Trkb Receptors And Demonstrate Potent Neurotrophic Activities³.

Abstract: Tyrosine kinase receptor B (TrkB) mediates neurotrophic effects of brain-derived neurotrophic factor (BDNF) to increase neuronal survival, differentiation, synaptic plasticity, and neurogenesis. The therapeutic potential of TrkB activation using BDNF has been demonstrated well in several preclinical models of CNS diseases, validating TrkB as a promising drug target. Therefore, we aimed to develop TrkB-specific receptor agonists by using a monoclonal antibody approach. After generation of hybridoma clones and assessment of their binding and functional activity, we identified five mouse monoclonal antibodies that show highly selective binding to TrkB and that induce robust activation of TrkB signaling. Epitope mapping studies using competition analysis showed that each of the monoclonal antibodies recognizes a unique binding site on TrkB, some of which are distinct from BDNF docking sites. These antibodies behave as true agonists based on their ability to both activate proximal and secondary signaling molecules downstream of TrkB receptors and promote neuronal survival and neurite outgrowth. The binding affinities and the functional efficacy of these antibodies are comparable to those of BDNF, whereas they do not bind to the p75 low-affinity neurotrophin receptor at all. Therefore, they could represent novel reagents to explore the pathophysiological roles of TrkB and its potential therapeutic utility in treating CNS disorders.

³ Qian MD, Zhang J, Tan XY, Wood A, Gill D, Cho S. J Neurosci. 2006 Sep 13;26(37):9394-403.

Brain-Derived Neurotrophic Factor Modulates Glucagon Secretion From Pancreatic Alpha Cells: Its Contribution To Glucose Metabolism⁴.

Abstract: AIM: Brain-derived neurotrophic factor (BDNF) reduces plasma glucose levels in obese db/db diabetic mice and is speculated to produce its effects via the hypothalamus, the regulatory centre of satiety and the autonomic nervous system. The potential effect of BDNF directly on peripheral endocrine organs, however, remains to be clarified. Here we report the effects of BDNF on hormonal secretion from pancreatic islets of Langerhans using their isolated culture.

METHODS AND RESULTS: In an immunohistochemical study, mouse pancreatic alpha cells were stained specifically with the anti-TrkB (a specific receptor for BDNF) antibody. After 7 days culture of isolated islets of the normal mouse pancreas, 10 ng/ml BDNF decreased the secretion of glucagon per 6 h significantly less than that of the control (p = 0.016). In contrast, there were no significant changes in insulin secretion or glucagon and insulin contents in the islets cultured under the same conditions. In vivo administration of BDNF (10 mg/kg/day) to normal mice for 7 days significantly decreased their food consumption (p < 0.05). The fasting plasma glucose levels were decreased on day 7 compared with day 1 more significantly in BDNF-treated mice (p = 0.043) than in pair-fed control mice (p = 0.14). In newborn BDNF-knockout mice, fasting plasma glucose levels increased in the order of homozygote, heterozygote and wild type (p = 0.033). No apparent immunohistochemical abnormality was observed for pancreatic glucagon in the BDNF-knockout mice.

CONCLUSION: These data suggest that BDNF affects glucose metabolism not only with its anorectic effect but also with modulated glucagon secretion from pancreatic alpha cells.

Brain-Derived Neurotrophic Factor Improves Blood Glucose Control And Alleviates Fasting Hyperglycemia In C57BLKS-Lepr(Db)/Lepr(Db) Mice⁵.

Abstract: Systemic administration of brain-derived neurotrophic factor (BDNF) decreases nonfasted blood glucose in obese, non-insulin-dependent diabetic C57BLKS-Lepr(db)/lepr(db) (db/db) mice, with a concomitant decrease in body weight. By measuring percent HbA1c in BDNF-treated and pair-fed animals, we show that the effects of BDNF on nonfasted blood glucose levels are not caused by decreased food intake but reflect a significant improvement in blood glucose control. Furthermore, once established, this effect can persist for weeks after cessation of BDNF treatment. Oral glucose tolerance tests were performed to examine the effects of BDNF on blood glucose control in the fasted state and after an oral glucose challenge. BDNF treatment normalized fasting blood glucose from initially hyperglycemic levels and also showed evidence for beneficial, although less marked, effects on the ability to remove exogenous glucose from blood. One means to lower fasting blood glucose is to reduce the glucose output of peripheral tissues that normally play a part in the maintenance of fasting hyperglycemia.

⁴ Hanyu O, Yamatani K, Ikarashi T, Soda S, Maruyama S, Kamimura T, Kaneko S, Hirayama S, Suzuki K, Nakagawa O, Nawa H, Aizawa Y. Diabetes Obes Metab. 2003 Jan;5(1):27-37.
⁵ Tonra JR, Ono M, Liu X, Garcia K, Jackson C, Yancopoulos GD, Wiegand SJ, Wong V. Diabetes. 1999 Mar;48(3):588-94.

Because the liver is the major endogenous source of glucose in blood during fasting, and because hepatic weight and glucose output are increased in type 2 diabetes, we evaluated the effects of BDNF on liver tissue. BDNF reduced the hepatomegaly present in db/db mice, in association with reduced liver glycogen and reduced liver enzyme activity in serum, supporting the possible involvement of liver tissue in the mechanism of action for BDNF.

Brain-Derived Neurotrophic Factor Reduces Blood Glucose Level In Obese Diabetic Mice But Not In Normal Mice⁶.

Abstract: *Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family. However, it is not yet known if BDNF works on the endocrine system itself. Here we report that BDNF improves hyperglycemia in obese diabetic animals. BDNF reduced the blood glucose level in obese db/db diabetic mice in which the effect of BDNF was age-dependent and high under the condition of hyperinsulinemia, while BDNF showed no effect on non-diabetic db/m mice. These results suggest that BDNF ameliorates insulin resistance by enhancing insulin action in peripheral tissues. Furthermore, BDNF was found to reduce the plasma insulin level in db/db mice. Among the neurotrophin family, NT-3 also reduced the blood glucose level in db/db mice. These results provide a novel insight that neurotrophin functions on the endocrine system as well as the nervous system.*

Como se puede apreciar con claridad en estas publicaciones científicas, la relación entre el receptor B de tirosinquinasa (TrkB), el factor neurotrópico derivado del cerebro (BDNF) y la modulación de la concentración de glucosa en sangre. Al mismo tiempo se hace mención del uso de anticuerpos anti-TrkB en inmunoensayos que lograron demostrar que dichos anticuerpos aumentan la actividad neurotrópica y también que dichos anticuerpos pueden intervenir en la modulación del BDNF sobre la secreción de glucagón de las células alfa del páncreas.

De la misma manera, existen publicaciones en donde no solamente se revela el uso de anticuerpos anti-TrkB en ensayos relacionados a la modulación de la glicemia, sino que además dan información detallada acerca del proceso a través del cual se pueden obtener estos anticuerpos, veamos:

La publicación internacional WO/1996/001837 publicada el 25 de enero de 1996 y titulada "COMPOSITIONS AND METHODS OF MAKING AND USING HUMAN FULL LENGTH TRK-B" revela:

⁶ Ono M, Ichihara J, Nonomura T, Itakura Y, Taiji M, Nakayama C, Noguchi H. Biochem Biophys Res Commun. 1997 Sep 18;238(2):633-7.

The present invention relates to full length version of the human neurotrophic factor receptor TRK-B, (full length human TRK-B), nucleic acid molecules that comprise a nucleotide sequence which encodes a full length version of human TRK-B and fragments thereof and to methods of making and using full length human TRK-B including mutated forms and antibodies that bind to full length human TRK-B.

(...)

The present invention relates to essentially pure and isolated full length human TRK-B protein.

The present invention relates to monoclonal antibodies which specifically binds to an epitope on the intracellular domain of full length human TRK-B protein.

The present invention relates to pharmaceutical compositions that comprises monoclonal antibodies which specifically binds to an epitope on the intracellular domain of full length human TRK-B protein.

(...)

The present invention relates to a kit for determining whether a human neuroblastoma cell expresses full length human TRK-B which comprises a container that includes monoclonal antibodies that specifically binds to an epitope on the intracellular domain of full length human TRK-B and a container that includes a protein to which the antibodies bind.

(...)

Those having ordinary skill in the art can produce monoclonal antibodies which specifically bind to the intracellular domain of the full length human TRK-B protein and are useful as therapeutics using standard techniques and readily available starting materials. The techniques for producing monoclonal antibodies are outlined in Harlow, E. and D. Lane, (1988) *ANTIBODIES: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor NY, which is incorporated herein by reference, provide detailed guidance for the production of hybridomas and monoclonal antibodies which specifically bind to target proteins. Briefly for example,

full length human TRK-B protein, or an immunogenic fragment thereof including the carboxy terminal portion, is injected into mice. The spleen of the mouse is removed, the spleen cells are isolated and fused with immortalized mouse cells. The hybrid cells, or hybridomas, are cultured and those cells which secrete antibodies are selected. The antibodies are analyzed and, if found to specifically bind to the intracellular domain of the full length human TRK-B, the hybridoma which produces them is cultured to produce a continuous supply of antibodies.

(...)

15. Essentially pure and isolated protein having an amino acid sequence encoding by nucleotides 352-2817 of SEQ ID NO:1.

16. A monoclonal antibody which specifically binds to a protein according to claim 15 at an epitope within the amino acid sequence encoded by nucleotides 1750-2817 of SEQ ID NO:1.

Como se puede apreciar, en este documento se revela el proceso de obtención de anticuerpos anti-TrkB a partir de la proteína aislada Trkb, así como la inclusión de los mismos en formulaciones farmacéuticas.

En conclusión, la solicitud colombiana en trámite carece de todos los requisitos fundamentales para poder otorgársele el privilegio de patente de invención, en primer lugar porque incurre en una improcedencia directa de patentabilidad al reivindicar un método de tratamiento, en segundo lugar porque la combinación farmacológica que pretende carece de nivel inventivo y finalmente porque en el mismo estado de la técnica se tiene suficiente información que es claramente una guía para la obtención y aplicación en terapéutica de anticuerpos anti-TrkB como los que pretende reivindicar la solicitud colombiana en cuestión.

Así las cosas, y con fundamento en los argumentos expuestos a lo largo del presente escrito, respetuosamente reitero la solicitud para que su despacho se abstenga de conceder el privilegio de patente solicitado, y ordene en consecuencia el archivo del expediente respectivo.

IV. FUNDAMENTOS DE DERECHO

Son fundamentos de derecho el Capítulo I, artículos 14, 16, 18 y 20 literal d) de (Requisitos de Patentabilidad) y el artículo 42 (Oposiciones) de la Decisión 486 de 2000.

V. PRUEBAS

Ruego que se tengan como tales, sin perjuicio de las que puedan presentarse al vencimiento de la prórroga solicitada, y de aquellas que el Despacho considere oficiosamente, las siguientes:

- ❖ Copia de todas las publicaciones científicas mencionadas en el acápite de fundamentos de la oposición.
- ❖ La publicación internacional WO/1996/001837 publicada el 25 de enero de 1996 y titulada "COMPOSITIONS AND METHODS OF MAKING AND USING HUMAN FULL LENGTH TRK-B"

VI. ANEXOS

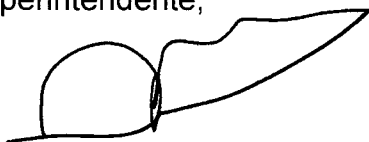
Para efectos del trámite correspondiente, me permito anexar al presente escrito los siguientes anexos

1. Certificado de existencia y representación legal de Lafrancol S.A.
2. Poder para actuar.
3. Constancia de pago de las tasas de tramitación de oposición.
4. Copia para correr traslado al solicitante.
5. El mencionado en el acápite de pruebas.

VIII. NOTIFICACIONES

El suscrito recibirá notificaciones en la secretaría de su Despacho o en mi oficina localizada en la Carrera 17 No. 88-23, Oficina 205, Fax (1) 621.25.42 de esta ciudad. Lafrancol S.A., las recibirá en sus oficinas localizadas en la Carrera 1 N° 46-84 de Cali.

Señor Superintendente,

A handwritten signature in black ink, consisting of a large, rounded initial 'J' followed by a series of loops and a long, sweeping horizontal stroke that extends to the right.

JOSÉ LUIS REYES VILLAMIZAR

C.C. 79'152.473 de Usaquén

T.P.A. 44.655 del C.S.J.

Novel Agonist Monoclonal Antibodies Activate TrkB Receptors and Demonstrate Potent Neurotrophic Activities

Ming D. Qian,¹ Jie Zhang,² Xiang-Yang Tan,¹ Andrew Wood,² Davinder Gill,¹ and Seongeun Cho²

¹Antibody Technologies, Wyeth Research, Cambridge, Massachusetts 02140, and ²Neuroscience Discovery Research, Wyeth Research, Princeton, New Jersey 08543

Tyrosine kinase receptor B (TrkB) mediates neurotrophic effects of brain-derived neurotrophic factor (BDNF) to increase neuronal survival, differentiation, synaptic plasticity, and neurogenesis. The therapeutic potential of TrkB activation using BDNF has been demonstrated well in several preclinical models of CNS diseases, validating TrkB as a promising drug target. Therefore, we aimed to develop TrkB-specific receptor agonists by using a monoclonal antibody approach. After generation of hybridoma clones and assessment of their binding and functional activity, we identified five mouse monoclonal antibodies that show highly selective binding to TrkB and that induce robust activation of TrkB signaling. Epitope mapping studies using competition analysis showed that each of the monoclonal antibodies recognizes a unique binding site on TrkB, some of which are distinct from BDNF docking sites. These antibodies behave as true agonists based on their ability to both activate proximal and secondary signaling molecules downstream of TrkB receptors and promote neuronal survival and neurite outgrowth. The binding affinities and the functional efficacy of these antibodies are comparable to those of BDNF, whereas they do not bind to the p75 low-affinity neurotrophin receptor at all. Therefore, they could represent novel reagents to explore the pathophysiological roles of TrkB and its potential therapeutic utility in treating CNS disorders.

Key words: neuroprotection; neurite outgrowth; binding; agonists; monoclonal antibodies; phosphorylation

Introduction

Tyrosine receptor kinases are a family of single transmembrane glycoproteins that mediate various trophic effects of the neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. A large body of evidence demonstrates crucial roles of neurotrophins in a wide spectrum of neuronal responses such as neurogenesis, differentiation, and neurite growth (Connor and Dragunow, 1998). In particular, BDNF, a cognate ligand to tyrosine kinase receptor B (TrkB), has been shown to exert strong survival and neuroprotective effects on the neurons of the CNS. For example, BDNF protects hippocampal neurons from glutamate toxicity (Lindholm et al., 1993) and rescues cerebellar neurons from programmed cell death (Leeds et al., 2005). In animal studies, BDNF has been shown to reduce ischemic injury (Schabitz et al., 2000; Kurozumi et al., 2004) and to improve functional recovery and postinjury regeneration (Koda et al., 2004).

These preclinical data strongly support the rationale for clinical evaluation of BDNF as a therapeutic agent for various neurological disorders. However, the outcome of several clinical trials using recombinant BDNF has been disappointingly negative (Ochs et al., 2000; Beck et al., 2005), and it is potentially because of the short *in vivo* half-life of BDNF. In addition, BDNF also

binds to the p75 neurotrophin receptor (p75NTR), a promiscuous NTR that is known to activate cell death pathways via recruitment of multiple adaptor proteins (Chao, 1994; Friedman and Greene, 1999). In fact, BDNF has been shown to exacerbate cytotoxicity when TrkB expression is not present (Friedman and Greene, 1999), which might have also contributed to the lack of efficacy in the clinic. To date, no examples of exogenous agents exist that act as potent and selective *in vivo* agonists of TrkB.

TrkB is one of the most widely distributed NTRs in the brain, whose expression is high in such areas as the neocortex, hippocampus, striatum, and brainstem (Muragaki et al., 1995; Shelton et al., 1995). It is a multidomain transmembrane protein that consists of an extracellular ligand binding domain, a transmembrane region, and an intracellular tyrosine kinase domain (McDonald and Chao, 1995). BDNF binding to TrkB induces autophosphorylation of TrkB and, subsequently, phosphorylation of several mediator kinases, including extracellular signal-regulated kinase [mitogen-activated protein kinase (MAPK)], phosphatidylinositol 3-kinase/Akt, phospholipase C- γ , and their downstream targets (Middlemas et al., 1994; Encinas et al., 1999). The binding of neurotrophins to Trk receptors has been shown to induce receptor dimerization that leads to conformational changes and activation of intracellular signal transduction pathways (Jing et al., 1992). In the case of TrkA, a receptor for NGF, it was shown that receptor cross-linking of TrkA by use of polyclonal or monoclonal antibodies (mAbs) mimicked the biological effects of NGF (Clary et al., 1994; LeSauter et al., 1996). These Abs appear to share binding sites with NGF as evidenced by competition with NGF binding and the inhibition of NGF-mediated neurite outgrowth. The role of other extracellular

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regions of TrkA in receptor dimerization and signaling activation remains elusive.

In this study, we present novel TrkB-selective mAbs with diverse binding epitopes that fully recapitulate the trophic activities of BDNF. They effectively activate TrkB signaling and promote neuronal survival and neurite outgrowth, thus representing potent agonists of TrkB.

Materials and Methods

Immunization schedules. Five 8-week-old female BALB/c mice were immunized with 10 μ g of recombinant human TrkB-extracellular domain (rhTrkB-ECD) (R & D Systems, Minneapolis, MN) and 1 μ g of recombinant mouse TrkB-ECD (rmTrkB) (R & D Systems) that were premixed with complete Freund's adjuvant. The animals were immunized four times, biweekly. Mice were bled 1 week after the last immunization, and the Ab responses in the antisera were evaluated. Three mice were chosen for hybridoma preparation. They received one additional boost of 10 μ g of rhTrkB and 1 μ g of rmTrkB 3 d before the cell fusion.

Generation of mouse anti-TrkB mAbs. Splenocytes from three BALB/c mice immunized with rhTrkB and rmTrkB were fused with mouse myeloma cells (P3X63Ag8.653; American Type Culture Collection, Manassas, VA) at a 4:1 ratio using 50% polyethylene glycol (MW 1500; Roche Diagnostic Systems, Somerville, NJ). After fusion, cells were seeded and cultured in 96-well plates at 1×10^5 cells/well in the RPMI 1640 selection medium containing 20% fetal bovine serum (FBS), 5% Origen (IGEN International, Gaithersburg, MD), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES, and $1 \times$ hypoxanthine-aminopterin-thymidine (Sigma, St. Louis, MO). Hybridoma supernatants were screened for binding to human TrkB (hTrkB) by ELISA using rhTrkB-Fc (R & D systems) and by fluorescence-activated cell sorting (FACS) analysis on human embryonic kidney 293 (HEK293) cells stably expressing hTrkB (Zhang et al., 2006). Hybridoma supernatants with a positive binding signal were also tested for functional agonist activity using a luciferase reporter assay (see below, Luciferase reporter assay). Selected hybridomas were subcloned four times by serial dilution and once by FACS. Conditioned media were harvested from the stable hybridoma cultures, and Igs were purified using Prosep-A Montage Ab purification spin columns (Millipore, Bedford, MA). The Ig class of mAb was determined with a mouse mAb isotyping kit (IsoStrip; Boehringer Mannheim, Indianapolis, IN).

Cell culture. HEK293 cells stably expressing full-length hTrkB and 3xCRE_{Luc} (3x cAMP-responsive element-Luc vector; Mercury Signal System; Clontech, Palo Alto, CA), referred to as HEK-TrkB, were described previously (Zhang et al., 2006). These cells were grown in standard DMEM containing 10% fetal calf serum supplemented with 1% penicillin/streptomycin, 250 μ g/ml geneticin/G418, 2 μ g/ml puromycin, and 350 μ g/ml hygromycin. HEK-TrkA and HEK-TrkC cells were cultured in the same standard media as for HEK-TrkB. Chinese hamster ovary (CHO)-p75NTR stable cells were grown in α -medium (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal calf serum and 20 nM utx-methotrexate. Human neuroblastoma SH-SY5Y cells were grown in DMEM:F-12 (1:1) supplemented with 2 mM L-glutamine, 15% fetal calf serum, and 1% penicillin/streptomycin. Primary neuronal cultures were prepared from rat and mouse cerebella and rat cortex. After dissection of the cerebellum from postnatal day 5–7 animals, tissues were cut into small pieces and enzymatically digested for 30 min using a papain dissociation kit (Worthington, Freehold, NJ). After dispersion by gentle trituration and centrifugation at $300 \times g$ for 5 min, dissociated cerebellar granule neurons (CGNs) were reconstituted in a standard Neurobasal growth medium containing B27 supplement, 0.5% FBS, 0.5 mM L-glutamine, and 25 mM potassium chloride and plated in multiwell Bio-coat plates precoated with poly-D-lysine (BD Biosciences, Franklin Lakes, NJ). Rat cortical neurons were isolated from postnatal day 1–2 rats using the same protocol described above for CGNs and plated in a B27-supplemented Neurobasal medium. All cultures were maintained at 37°C in 5% CO₂ and 95% air.

ELISA. Binding of hybridoma supernatants and purified Abs to hTrkB was evaluated using ELISA. Ninety-six-well plates (Maxisorp; Nunc,

Naperville, IL) were coated with either 1 μ g/ml rhTrkB (R & D systems) or rmTrkB (R & D systems) and incubated overnight at 4°C. After washing and blocking with PBS containing 1% BSA and 0.05% Tween 20, 100 μ l of sample (diluted immune serum, hybridoma supernatants, or purified Abs, as indicated in the figures) was added and incubated for 1 h at room temperature. The plates were washed, and the bound anti-TrkB Abs were detected using peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL), followed by incubation with the substrate TMB (BioFX Laboratories, Owings Mills, MD) for color development. Absorbance values were determined at 450 nm in a spectrophotometer. The concentrations of mAbs were determined using peroxidase-labeled goat anti-mouse IgG (Fc γ) (Pierce) and a standard curve generated by a purified isotype-matching mouse IgG.

FACS analysis. Cells were suspended in PBS containing 1% BSA at a density of 4×10^5 cells/ml. Cells were incubated with 100 μ l of suspended sample (diluted immune sera, hybridoma supernatants, or purified Abs) for 30 min at 4°C. After washing, cells were incubated with phycoerythrin (PE)-labeled goat anti-mouse IgG, F(ab')₂ (Dako, Carpinteria, CA) for 30 min at 4°C in the dark. Cell-associated fluorescence signals were measured by a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) using 5000 cells per treatment. Propidium iodide was used for identification of dead cells, which were excluded from the analysis.

Determination of relative Ab-binding epitopes on hTrkB. To determine whether TrkB mAbs affect BDNF binding to TrkB, we conducted competition ELISA binding assays. Ninety-six-well plates were coated with 0.3 μ g/ml BDNF (R & D Systems) overnight at 4°C. After washing and blocking the wells with PBS containing 1% BSA and 0.05% Tween 20, 100 μ l of preincubated mixtures containing Ab samples (diluted immune sera, hybridoma supernatants, or purified Abs) and rhTrkB-Fc (150 ng/ml) was added to each well and incubated for 1 h at room temperature. Plates were washed, and BDNF-bound rhTrkB-Fc was detected using Fc γ (Pierce), followed by incubation with the substrate TMB (BioFX Laboratories) for color development. Binding of rhTrkB-Fc to BDNF without any competing Abs or with diluted preimmune serum was used as a control and defined as 100% binding.

We used similar competition approaches to determine the relative binding epitopes between each pair of selected mAbs. First, 1 μ g/ml purified mAb was added to each well of a 96-well plate and defined as a "competing" Ab. Subsequently, 100 μ l of one of the mAbs (20 μ g/ml) premixed with rhTrkB-Fc (150 ng/ml), referred to as "prebound" Ab, was added to the wells and incubated for 1 h at room temperature. After washing, the amount of rhTrkB-Fc remaining on the plate that was bound to the competing Abs was determined using Fc γ , as described above.

Luciferase reporter assay. Induction of luciferase was measured to assess the agonistic activities of TrkB mAbs, as described previously (Zhang et al., 2006). In brief, HEK-TrkB cells were plated at a density of 1.5×10^5 cells/ml in 96-well plates in standard DMEM. The next day, cells were stimulated with BDNF or Abs, as indicated in the figures, and the luciferase activity was measured after 16 h of incubation using the Steady-Glo assay kit (Promega, Madison, WI). After replacement of culture media with 100 μ l of PBS, an equal volume of Steady-Glo assay reagent was added to induce cell lysis and the plates were shaken on a Titer Plate Shaker (Lab Line Instruments, Melrose Park, IL) for 5 min. The luminescence signal was measured using TopCount NXT (Packard, Meriden, CT), and the results were presented as fold increases over basal activity.

TrkB autophosphorylation assay/Western blot. HEK-TrkB cells were plated in 24-well plates at 2×10^5 cells/well in standard DMEM. The next day, cells were incubated in serum-free DMEM for 90 min before stimulation with BDNF or Abs at indicated concentrations for 30 min at 37°C. Stimulation was terminated by removal of the medium followed by one wash with PBS. Cells were lysed in 100 μ l of Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and heated to 95°C. Cellular lysates were filtered through a QiaShredder column (Qiagen, Valencia, CA), and 20 μ l of samples was resolved on 4–12% Bis-Tris gels (Invitrogen) and processed for Western blot analysis. For testing of primary neurons, rat CGNs were plated in six-well plates at a density of 1×10^6 cells/well. Cells were serum deprived for 2 h, treated with BDNF or Abs for 30 min, and lysed in 100

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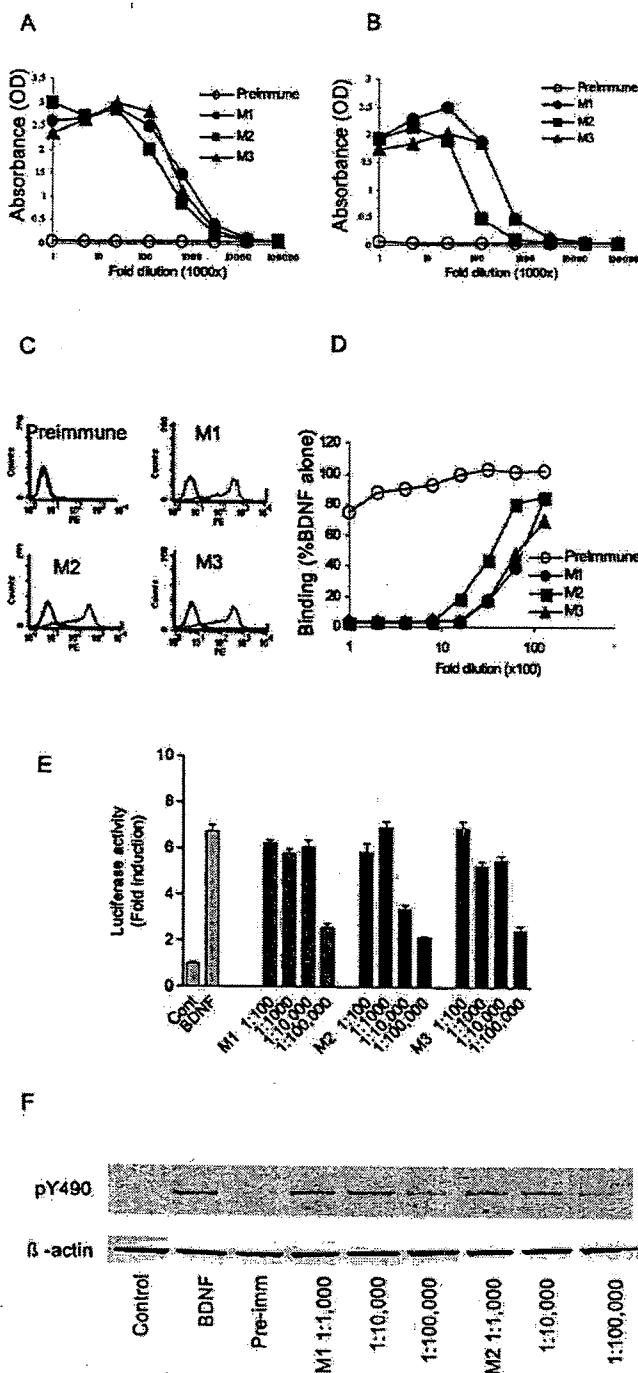


Figure 1. Immunization with hTrkB and mTrkB elicited cross-reactive Ab responses with agonistic activities. Immune sera from three BALB/c mice (M1, M2, and M3) were obtained 7 d after the fourth immunization. **A**, Immune sera were diluted in PBS containing 1% BSA and subjected to ELISA to examine their binding activities to hTrkB (**A**) or mTrkB (**B**), as described in Materials and Methods. Preimmune sera were used as a negative control. **C**, Immune or preimmune sera at a 1:1000 dilution were incubated with HEK-TrkB cells (gray traces) or parental HEK cells (black traces), followed by secondary incubations with PE-labeled goat anti-mouse Abs, and the staining intensities were analyzed using FACS. **D**, Various dilutions of immune or preimmune sera were incubated with rhTrkB-Fc (150 ng/ml) for 30 min and added to ELISA plates precoated with BDNF. After wash, the amount of rhTrkB-Fc bound to BDNF was measured using peroxidase-conjugated goat anti-human IgG. The binding response of rhTrkB-Fc and BDNF without immune sera was defined as a total binding and was expressed as 100%. The addition of preimmune serum had no significant effect on rhTrkB-Fc binding to BDNF, whereas immune sera competed with this interaction in a dose-dependent manner. **E**, HEK-TrkB cells in 96-well plates were stimulated with BDNF (50 ng/ml) or immune sera at various dilutions, and the luciferase activities were measured 16 h later. All three immune sera (M1, M2, and M3) induced

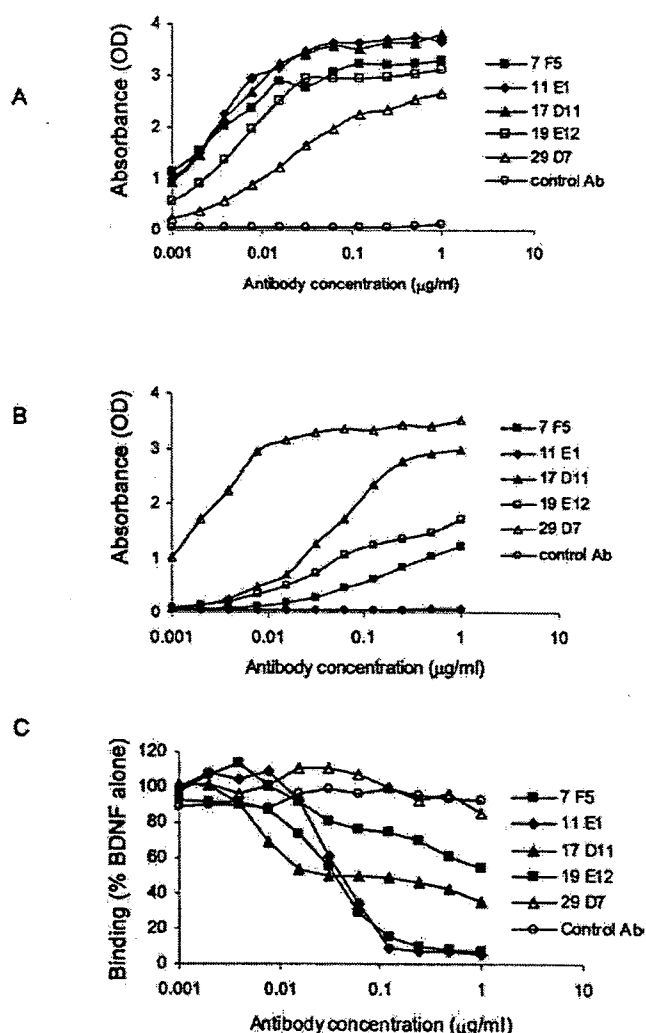


Figure 2. Binding properties of TrkB mAbs. **A**, **B**, Dose–response curves demonstrating the binding activities of five mAbs to hTrkB (**A**) and mTrkB (**B**), as determined by ELISA. **C**, Various concentrations of TrkB mAbs were incubated with rhTrkB-Fc (150 ng/ml) for 30 min, and the mixture was added to ELISA plates precoated with BDNF. After wash, the amount of rhTrkB-Fc bound to BDNF was measured using peroxidase-conjugated goat anti-human IgG. The binding of rhTrkB-Fc to BDNF in the absence of any mAbs was determined as a total binding and was expressed as 100%. In all assays, isotype-matched mouse Abs (mIgG1, mIgG2a) were used as a negative control.

μl of RIPA buffer. Protein concentrations were determined, and 20 μg of total proteins was loaded on the 4–12% Bis-Tris gel (Invitrogen). After transfer to the nitrocellulose membranes, immunoblotting was performed using PY490 phospho-Trk-specific Ab (9141, 1:100; Cell Signaling, Beverly, MA), anti-phospho-MAPK Ab (1:2000; Cell Signaling), anti-MAPK (1:5000; Cell Signaling), anti-phospho-Akt (1:1000; Cell Signaling), or anti-β-actin (1:3000; Sigma), followed by incubation with HRP-conjugated secondary Abs (Invitrogen). The signal was developed using the ECL plus kit (Amersham Biosciences, Piscataway, NJ).

Neurite outgrowth assay. For the neurite outgrowth assay, SH-SY5Y

dose-dependent increases of the luciferase activities. Data are presented as mean fold induction over the basal level ± SEM from triplicate samples. Cont, Control. **F**, HEK-TrkB cells were treated with BDNF (50 ng/ml), a preimmune serum (pre-imm; 1:1000), or immune sera at indicated dilutions for 30 min. Equal amounts of cell lysates were resolved in the SDS-PAGE gel, and phosphorylation levels of TrkB were detected using anti-pY490 as described in Materials and Methods. β-Actin was used to verify equal loading of proteins in each lane. The blot shown here represents one of the two independent experiments with similar results.

neuroblastoma cells were plated in 96-well tissue culture plates at a density of 4×10^3 cells/well and incubated with $10 \mu\text{M}$ all-trans-retinoic acid (RA) to induce neuronal differentiation. On the third day, the medium was replaced with fresh growth medium with or without BDNF and/or testing Abs, as indicated in the results. After an additional 3 d in culture, cells were fixed by IC-Fix (Biosource, Camarillo, CA) for 30 min at room temperature and also processed for immunostaining of β -tubulin III. First, cells were permeabilized by brief incubation in 0.2% Triton in PBS (TPBS). Subsequently, samples were incubated in 1.5% normal goat serum (NGS) in TPBS for 30 min to block nonspecific binding, followed by incubation with anti- β -tubulin III mAb (TuJ1, 1:1000; Covance, Berkeley, CA) in 1.5% NGS/TPBS. TuJ1 signal was detected using Alexa 488 mouse anti-goat Abs (1:500; Invitrogen), and neurite outgrowth was analyzed using Cellomics (Pittsburgh, PA) ArrayScan. For quantification, ~800 cells were analyzed from each well, and typically each condition was run in quadruplicate.

For the evaluation of neurite-promoting effects of Abs in primary neurons, rat and mouse CGN cultures were seeded at a density of 1.2×10^4 cells/well in 96-well plates precoated with poly-D-lysine (BD Biosciences). Cells were treated with BDNF or testing Abs for 24 h, fixed with IC-Fix for 30 min at room temperature, and processed for TuJ1 immunostaining as described above.

Cell survival assay. SH-SY5Y cells were plated in 96-well plates at 1×10^4 cells/well and incubated with RA ($10 \mu\text{M}$) to induce neuronal differentiation. After 3 d, the culture medium was switched to a standard growth medium without serum (serum-free DMEM) supplemented with BDNF, testing Abs, or a vehicle. After an additional 2 d in culture, cell viability was measured by MTT conversion using the CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol.

Neuroprotection assay. Rat or mouse CGNs were plated in 96-well Biocoat plates precoated with poly-D-lysine (BD Biosciences) at a density of 7.3×10^4 cells/well. Twenty-four hours after plating, cells were subjected to potassium serum deprivation (KSD) injury, which is known to result in significant death of CGNs (Skaper et al., 1998). Some sister cultures were treated with BDNF or testing Abs under KSD conditions to monitor their neuroprotective efficacies. After a 24–96 h incubation, cell viability was measured using the CellTiter 96 Cell Proliferation Assay kit (Promega). Rat cortical neurons were grown in poly-D-lysine-coated 96-well plates (BD Biosciences) in a Neurobasal medium supplemented with B27 at a density of 6.5×10^4 cells/well. Cells were treated with indicated concentrations of a TrkB Ab or a vehicle for 48 h, and cell viability was measured using the CellTiter 96 Cell Proliferation Assay kit (Promega).

Results

Evaluation of Ab responses from TrkB-immunized mice

We wanted to raise Abs that cross-react with hTrkB and mouse TrkB (mTrkB), which would then allow us to assess their activities in both experimental models and human systems. Therefore, we used a mixture of hTrkB and mTrkB proteins as immunogens. After a series of immunizations, immune bleeds were drawn for initial evaluation of Ab response to TrkB. All immunized mice generated high titers against rhTrkB and rmTrkB, as determined by ELISA (Fig. 1A,B). These immune bleeds also bound to membrane-anchored full-length hTrkB expressed on the surface of HEK-TrkB cells, as determined by FACS analysis (Fig. 1C). We then questioned whether the binding sites of these Abs overlapped with the BDNF docking site on TrkB. We immobilized

Table 1. Characterization of anti-TrkB mAbs

mAb	ELISA binding		BDNF competition
	hTrkB [ED_{50} (nM)]	mTrkB [ED_{50} (nM)]	
7F5	0.016	Weak binding	0.3
11E1	0.02	ND	0.3
17D11	0.02	0.3	Partial blocking
19E12	0.031	Weak binding	Partial blocking
29D7	0.12	0.013	ND

For determination of binding avidities of mAbs to TrkB, a series of concentrations of purified Abs was incubated with rhTrkB or rmTrkB coated onto 96-well plates. After a 1 h incubation period, plates were washed and the amounts of Abs bound to TrkB were measured using peroxidase-conjugated secondary Abs. For binding competition with BDNF, preincubated mixtures of mAbs with rhTrkB-Fc were added to ELISA plates coated with BDNF. After a 1 h incubation period, BDNF-bound rhTrkB-Fc was detected using peroxidase-conjugated goat anti-human IgG. ND, Not detectable.

Table 2. Mapping of relative Ab-binding epitopes

Prebound mAb	Competing mAb					Control mAb ^a
	7F5	11E1	17D11	19E12	29D7	
7F5	+	+/-	—	—	—	—
11E1	+	+	—	—	+/-	—
17D11	—	—	+	+/-	—	—
19E12	—	—	+	+	—	—
29D7	—	—	—	—	+	—
Control mAb ^a	—	—	—	—	—	N/A

A preformed complex of each mAb ($20 \mu\text{g/ml}$) with rhTrkB-Fc (150 ng/ml) was added to each well of 96-well plates coated with competing Abs. After a 1 h incubation period, plates were washed with PBS and the amounts of rhTrkB-Fc remaining in the wells were quantified using peroxidase-conjugated goat anti-human IgG. +, Complete competition; +/-, partial competition; —, no competition; N/A, not applicable.

^aIsotype-matched mouse mAb.

BDNF on the ELISA plate and performed competitive binding using rhTrkB-Fc pre-equilibrated with immune sera. Immune sera at high concentrations completely blocked the binding of BDNF to rhTrkB-Fc (Fig. 1D). However, with increasing dilution of immune sera, we detected displacement of immune sera with BDNF, allowing the binding of BDNF to rhTrkB (Fig. 1D). Although we observed dose-dependent binding responses of immune sera from all three mice, immune serum from mouse 2 in particular showed a binding curve clearly left-shifted from those of mice 1 and 3 (Fig. 1D), suggesting it might contain fewer Abs against the BDNF docking site on TrkB or the epitopes important for TrkB–BDNF interaction. Interestingly, immune sera from mice 1 and 3, which displayed higher binding potencies to TrkB as shown in Figure 1, A and B, also appeared to interfere more with BDNF binding to rhTrkB than mouse 2 did. None of the preimmune sera showed evidence of TrkB binding in either ELISA or FACS assay (Fig. 1A–D).

We then tested whether our TrkB binding immune sera were functionally agonistic. The luciferase reporter assay using HEK-TrkB has been shown to reflect activation of TrkB in a receptor- and ligand-selective manner (Zhang et al., 2006). Thus, TrkB-selective ligands, BDNF and NT-4, induced robust increases in the luciferase signal, whereas other growth factors including NGF and basic FGF had no effects (data not shown). Using this assay, we observed that treatment of cells with immune sera significantly increased the luciferase signal in a dose-dependent manner (Fig. 1E). Again, immune sera from mice 1 and 3 enhanced luciferase activity with higher potency than mouse 2, consistent with their higher binding to TrkB as shown in Figure 1A. To confirm that the immune sera indeed activated TrkB, we examined the autophosphorylation of TrkB receptors using Western blot analysis. Treatment of HEK-TrkB cells with BDNF induced a marked increase in the protein band intensity detected by an Ab specifically recognizing phosphorylated Trk (PY490) (Fig. 1F and our unpublished data). Also, incubation with immune sera resulted in dose-dependent increases of TrkB phosphorylation (Fig. 1F). Cell lysates harvested after treatment with preimmune

bleeds were devoid of any phosphorylation signals similar to the control.

Production and characterization of mAbs

Splenocytes from three mice were fused with mouse myeloma cells, and the supernatants from the resultant hybridomas were screened for TrkB binding using ELISA and FACS. We identified clones showing robust binding to rhTrkB, some of which cross-reacted with rmTrkB (data not shown). The agonist activities of all of the binding clones were tested in a luciferase assay, and the agonist clones were subjected to additional subcloning by serial dilution and FACS to achieve monoclonality. These steps led us to isolate a final set of five unique mAbs, as described below.

Illustrated in Figure 2, A and B, are the dose-dependent binding activities of mAbs to hTrkB and mTrkB. All five Abs showed high ELISA binding avidities to rhTrkB with ED_{50} values of 10^{-10} – 10^{-11} M, as summarized in Table 1. We also confirmed their binding activities to membrane-associated hTrkB expressed on the cell surface using FACS analysis (see Fig. 4C). We then evaluated whether our Abs could compete with BDNF binding to TrkB. Results from ELISA analysis revealed varying degrees of competition by each Ab with BDNF binding to rhTrkB (Fig. 2C). Clones 7F5 and 11E1 effectively blocked TrkB–BDNF interaction with an IC_{50} of 3×10^{-10} M, whereas clones 17D11 and 19E12 were able to only partially interfere with BDNF binding, even at the highest concentrations tested (1 μ g/ml). Notably, clone 29D7 did not affect BDNF docking to TrkB at all. Furthermore, our Abs also varied in their ELISA binding avidities to mTrkB. Clones 17D11 and 29D7 bound rmTrkB with high avidities ($ED_{50} = 10^{-10}$ – 10^{-11} M), whereas the rest of our Abs showed very weak or no binding to rmTrkB (Fig. 2B). The variable binding characteristics of these Abs suggest that they each might recognize a unique epitope on TrkB.

Next, we evaluated the relative binding epitopes of our Abs by examining the capability of each Ab to block the binding of other Abs to rhTrkB. Our analysis showed that clones 7F5 and 11E1 might share binding epitopes, as do clones 17D11 and 19E12. The binding site of clone 29D7 appeared unique, not overlapping with the other Abs in our panel (Table 2).

The agonist activities of purified Abs were examined using the luciferase assay. Purified Abs resulted in dose-dependent increases in luciferase signal with EC_{50} values of ~ 0.03 μ g/ml ($\sim 10^{-10}$ M) (Fig. 3A). The maximum signal induced by Abs was sixfold to eightfold higher than the background level. Sister cultures treated with BDNF as a positive control showed increases in activity by 5- and 6.2-fold over background with 100 and 200 ng/ml BDNF, respectively, demonstrating comparable signal windows with Ab treatments. Induction of luciferase activity by TrkB Abs was completely prevented by coincubation with hrTrkB-Fc protein (Fig. 3B), also confirming that Ab-mediated signal activation requires functional TrkB.

To provide direct evidence that the luciferase signaling was indeed mediated through TrkB, we took a proximal measure of TrkB activation by assessing autophosphorylation of TrkB. HEK-TrkB cells treated with agonist mAbs were evaluated in a Western blot analysis using an anti-phospho-Trk Ab, PY490. The immunoblots in Figure 3C clearly show that treatment with agonist Ab led to robust TrkB phosphorylation. The Ab responses were effectively blocked by cotreatment with K252a, a receptor tyrosine kinase inhibitor, also confirming the selective involvement of TrkB rather than a general induction of kinase pathways (Fig. 3C). Treatment with our Abs also induced robust phosphorylation of MAPK and Akt kinases, downstream signaling mediators

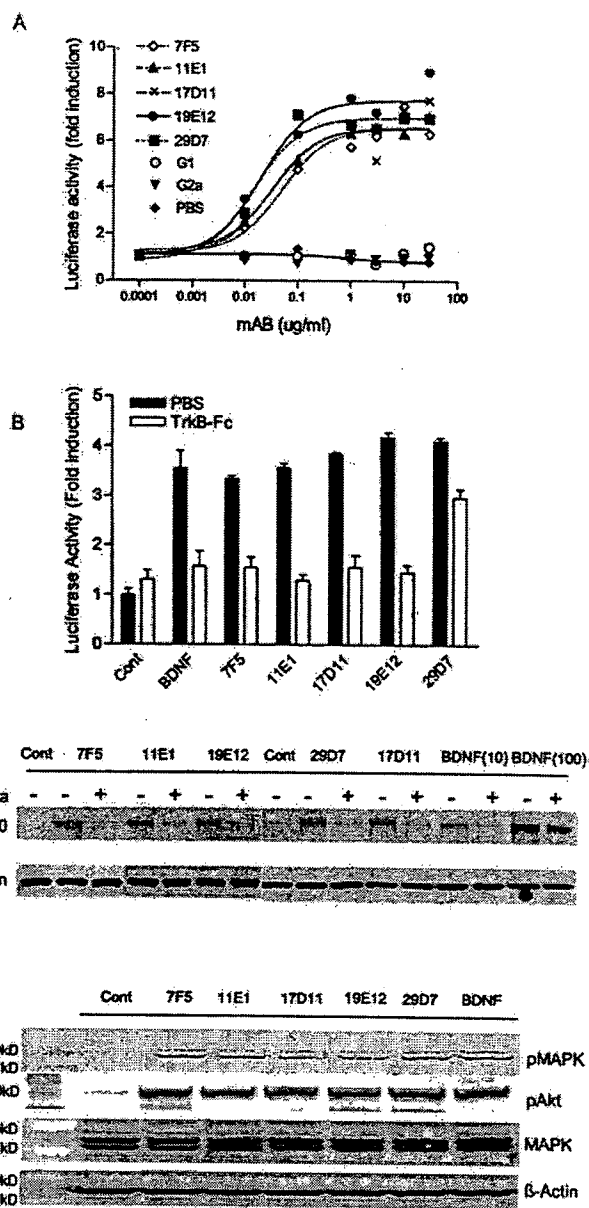


Figure 3. Activation of TrkB-dependent luciferase signaling and phosphorylation cascades by TrkB mAbs. *A*, HEK-TrkB cells in 96-well plates were treated with purified TrkB mAbs, isotype-matching control Abs (G1, mlgG1; G2a, mlgG2a), or a vehicle (PBS), and the accumulated luciferase activities were measured after 16 h. Data are presented as mean fold induction over the basal level $\pm$ SEM from triplicate samples. *B*, HEK-TrkB cells were treated with TrkB mAb (0.1 μ g/ml) or BDNF (25 ng/ml) in the presence or absence of hrTrkB-Fc (2 μ g/ml), and the induction of luciferase activities was assessed as in *A*. *C*, HEK-TrkB cells were stimulated with each mAb (1 μ g/ml) or with BDNF (10 or 100 ng/ml) in the presence (+) or absence (–) of K252a. Cell lysates were resolved in the SDS-PAGE gel, and phosphorylation levels of TrkB were detected using anti-pY490 as described in Materials and Methods. β -Actin was used to verify equal loading of proteins in each lane. All mAbs as well as BDNF led to robust phosphorylation of TrkB, which was effectively blocked by K252a. *D*, HEK-TrkB cells were stimulated with indicated mAbs (1 μ g/ml) or BDNF (100 ng/ml), and the cell lysates were analyzed for phosphorylation of MAPK and Akt kinases using phospho-specific Abs. The same samples were also probed for a total MAPK protein and β -actin to verify equal loading. Cont, Control.

of TrkB cascades, which presumably would have led to cAMP response element-binding protein (CREB)-mediated transcriptional activation and luciferase induction (Fig. 3D).

To demonstrate that the activities of these mAbs are selective for TrkB, we used HEK cells stably expressing either TrkA or TrkC and followed luciferase activation. Treatment of HEK-

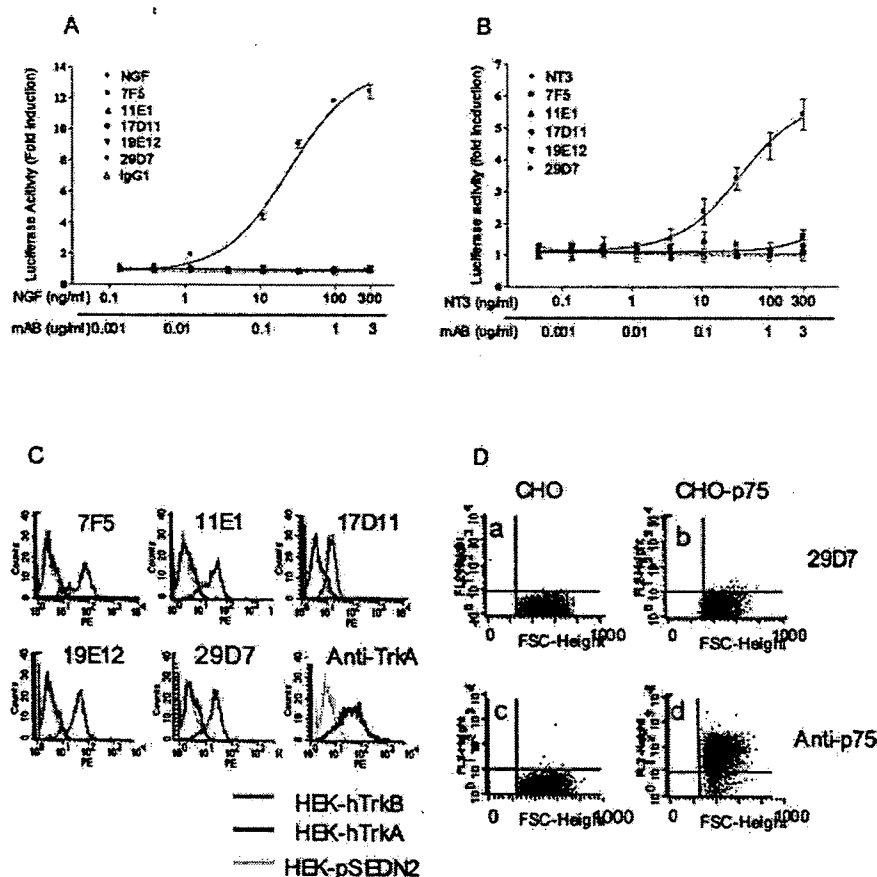


Figure 4. TrkB mAbs are not cross-reactive with TrkA, TrkC, or p75NTR. *A, B*, HEK-TrkA (*A*) or HEK-TrkC (*B*) cells were seeded in 96-well plates and stimulated with NGF, NT-3, TrkB mAbs, or a control Ab as indicated in the figures. Sixteen hours later, the accumulated luciferase activities were detected as a measure of receptor activation. Data are presented as the mean fold induction over the basal level $\pm$ SEM from triplicate samples, and the experiment was repeated at least twice. *C*, Purified TrkB mAbs diluted at 1:1000 were added to HEK-TrkB (gray traces), HEK-TrkA (black traces), or pSEDN2 vector-transfected (HEK-pSEDN2; light gray traces) cells. Cellular binding of mAbs was detected using PE-labeled goat anti-mouse Abs, and the staining intensities were analyzed using FACS. TrkB mAbs specifically bound to TrkB-expressing cells but not to TrkA- or vector-expressing cells. An anti-TrkA Ab was used as a positive control to demonstrate its binding to HEK-TrkA cells. *D*, A 29D7 TrkB mAb or an anti-p75NTR Ab (0.5 μ g/ml) was incubated with parental (CHO; *a, c*) or p75NTR-expressing (CHO-p75; *b, d*) cells. After a 30 min incubation, binding signals were detected using PE-labeled goat anti-mouse Abs, and cell-associated fluorescence was detected by FACS analysis.

TrkA or HEK-TrkC cells with BDNF had no effect on the luciferase activities (data not shown). In contrast, treatment of HEK-TrkA cells with NGF and HEK-TrkC cells with NT-3 resulted in dose-dependent increases in their luciferase activities, suggesting the intactness of the cellular signaling system (Fig. 4*A, B*). Remarkably, none of our TrkB-specific Abs showed any activity in these assays, indicating that they did not cross-react with TrkA or TrkC. FACS analysis using TrkA cells also confirmed lack of binding of TrkB mAbs to TrkA (Fig. 4*C*). In addition, we tested binding activities of TrkB Abs on p75NTR using a recombinantly prepared CHO-p75NTR cell line. As shown in Figure 4*D*, there was no detectable binding of 29D7 to either parental or p75NTR-expressing cells. Similar results were observed with other TrkB Abs (data not shown). In contrast, a known p75NTR-binding Ab (R & D Systems) increased the fluorescence signal when added to CHO-p75NTR cells, as expected (Fig. 4*D*).

Functional activities of mAbs

It is known that BDNF promotes neurite outgrowth and survival of TrkB-expressing neuronal cells. We therefore tested whether TrkB mAbs could mimic the biological effects of BDNF via acti-

vation of endogenous TrkB receptors. Because our Abs bound both hTrkB and mTrkB, we first used human neuroblastoma SH-SY5Y cells, which are known to express TrkB receptors after RA-induced neuronal differentiation (Kaplan et al., 1993; Encinas et al., 1999). Consistent with previous reports (Encinas et al., 1999), we observed that the addition of BDNF promoted neurite outgrowth as demonstrated by increases in neurite length and the number of branch points (Fig. 5). All of our TrkB Abs also significantly increased neurite outgrowth of differentiated SH-SY5Y cells in a dose-dependent manner, with maximum responses comparable or even superior to BDNF (Fig. 5*A, B*), as shown in representative pictures (Fig. 5*C*). Subsequent full dose-response studies determined the neurite outgrowth EC_{50} of Abs to be between 0.05 and 0.5 nM (Table 3).

In another set of experiments, SH-SY5Y cells were treated with RA to induce neuronal differentiation and were then subsequently challenged with serum deprivation. This resulted in the death of differentiated SH-SY5Y neurons (Encinas et al., 1999; Iglesias et al., 2003), which could be partially rescued by BDNF treatment (Fig. 6). Treatment with TrkB Abs also protected these cells from serum deprivation-induced injury in a dose-dependent manner. Clone 11E1 was even more potent than BDNF in this assay (Fig. 6).

Among the five Abs, we selected two clones, 29D7 and 17D11, which also bind to mTrkB, as demonstrated above (Fig. 2). Therefore, we wanted to test their functional activities in rodent primary neuronal cultures. We isolated CGN from rats

and mice and first confirmed that BDNF increased neurite outgrowth of cultured neurons as reported in the literature (Fig. 7*A, B*). We then tested the neurite-promoting effects of TrkB Abs. Clone 29D7 increased the total neurite length and neurite branching of both cerebellar neurons in a dose-dependent manner (Fig. 7*A, C, D*). Consistent with this efficacy in neurite outgrowth, treatment with 29D7 also afforded significant protection of CGN against apoptotic death induced by potassium and serum deprivation. Thus, 29D7 increased neuronal survival by 70% at 24 h after injury (88% viability by 29D7 treatment vs 60% in control) (Fig. 7*E*). Subsequent longer-term studies demonstrated the effectiveness of these treatments in promoting neuronal viability up to 96 h, resulting in 50, 22, and 12% increases in survival at 48, 72, and 96 h, respectively, compared with control (Fig. 7*F*). In contrast, clone 17D11 did not show any functional efficacy in neuronal cultures from either rats or mice, despite our ELISA data that showed robust binding to mTrkB (ED_{50} = 0.3 nM) (Table 1). To also confirm that 29D7 is capable of activating endogenous TrkB, we measured phosphorylation of TrkB as well as activation of intracellular signaling molecules. After a 30 min treatment of CGN with 29D7, there were clear increases in the

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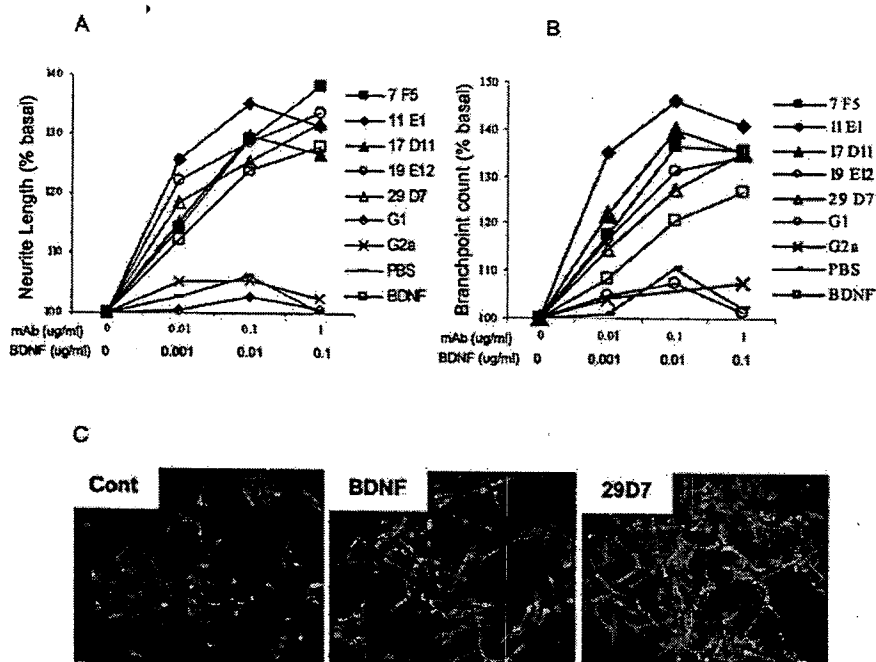


Figure 5. TrkB mAbs promote neurite outgrowth of differentiated SH-SY5Y cells. SH-SY5Y cells were plated in 96-well tissue culture plates and incubated with $10 \mu\text{M}$ all-trans-RA to induce neuronal differentiation. Three days later, the cells were replenished with fresh growth medium containing BDNF or testing Abs. After an additional 3 d in culture, cells were fixed and stained with anti- β -tubulin III (TuJ1) to trace neurite outgrowth using Cellomics ArrayScan. *A*, Neurite length, indicating the average of total neurite length for all TuJ1-positive cells, was analyzed. *B*, Branchpoint counts indicating the average number of branch points for all TuJ1-positive cells analyzed. *C*, Representative images of TuJ1 staining of SH-SY5Y cells after 3 d treatments with a vehicle control, BDNF (10 ng/ml), or a TrkB Ab 29D7 (0.1 $\mu\text{g/ml}$). Cont, Control.

Table 3. TrkB Ab-induced neurite outgrowth in differentiated SH-SY5Y cells

mAb	EC ₅₀ (nM)
7F5	0.26
11E1	0.05
17D11	0.07
19E12	0.05
29D7	0.47

SH-SY5Y cells were plated in 96-well plates at a density of 4×10^3 cells/well and incubated with $10 \mu\text{M}$ m RA for 3 d to induce neuronal differentiation. Subsequently, cells were treated with varying concentrations of indicated mAbs, and the neurite outgrowth was measured using the Cellomics ArrayScan.

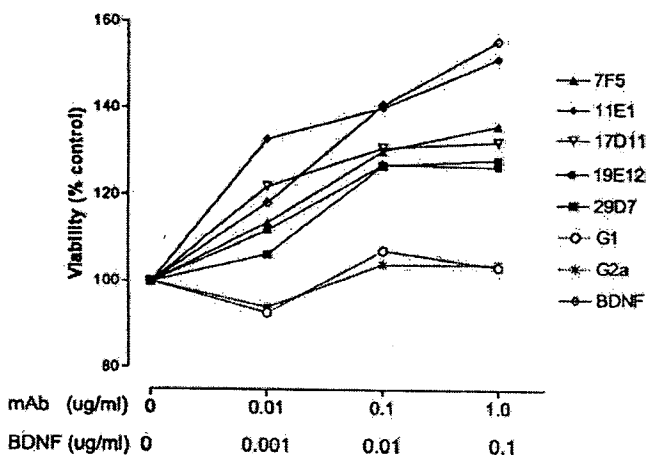


Figure 6. TrkB mAbs enhance the survival of differentiated SH-SY5Y cells. SH-SY5Y cells in 96-well plates were incubated with $10 \mu\text{M}$ all-trans-RA to induce neuronal differentiation. After 3 d, cells were incubated with serum-free media containing BDNF, TrkB mAbs, or isotype-matching control Abs (G1, mIgG1; G2a, mIgG2a), and cell viability was measured 2 d later using Cell Titer Cell Proliferation Assay. Data are presented as the percentage of increase viability over the vehicle-treated control.

levels of phospho-TrkB, phospho-MAPK, and phospho-Akt, as shown by immunoblot analyses (Fig. 7G), demonstrating the effectiveness of 29D7 toward activating neuronal TrkB receptors. We also tested the survival-promoting effects of 29D7 for cortical neurons. The survival of postnatal primary cortical neurons maintained in a B27-supplemented growth medium decreased in culture, resulting in a $\sim 75\%$ viability at 48 h compared with cultures grown in 5% serum-containing media. When varying concentrations of 29D7 were added to culture media, however, we observed dose-dependent rescue of cortical neurons, resulting in a significant increase in viability by 1 and 3 $\mu\text{g/ml}$ 29D7 (124 ± 5 and $126 \pm 1\%$ viability vs vehicle control, respectively; $p < 0.05$; representative images of neurons shown in Fig. 7H).

Discussion

In this study, we describe selective and potent novel TrkB mAbs capable of activating TrkB receptors and thereby exerting neuroprotective and neurotrophic effects. These Abs bind functional TrkB receptors and are completely negative against TrkA or TrkC, confirming their specificity as TrkB receptor ligands. Analyses of signal transduction demonstrating TrkB phosphorylation, activation of MAPK and Akt, and CREB-mediated transcriptional activation provide evidence of bona fide TrkB-mediated responses after Ab treatment. Furthermore, unlike the endogenous agonists BDNF and NT-4, these TrkB Abs have no affinity toward p75NTR, as demonstrated by the lack of binding to p75NTR-overexpressing cells. Activation of this promiscuous low-affinity NTR negates the trophic effects of BDNF and even initiates cell death pathways and myelin inhibition (Bamji et al., 1998; Wong et al., 2002). Therefore, the Abs described here appear to possess a superior pharmacological profile that is not achieved by native ligands.

Most kinase growth factor receptors, including TrkB, are believed to be activated by agonist-induced dimerization, as evidenced by cross-linking studies (Jing et al., 1992). Although the details of this process remain to be elucidated, it has been postulated that agonist-induced receptor dimerization initiates structural transformation and stabilizes the tyrosine kinase activities associated with the intracellular domain of the receptors. The obligatory requirement of bivalency of ligands in receptor activation has been shown in a study by Clary et al. (1994), in which they demonstrated that monovalent anti-TrkA Fab fragments inhibited the biological effects of NGF, whereas bivalent TrkA IgGs were effective as NGF-mimetics in stimulating receptor activation, cell survival, and neurite outgrowth. More recently, Williams et al. (2005) reported that a cyclic peptide containing tandem repeats of a NT-4/TrkB-interacting motif could activate TrkB presumably by receptor dimerization, whereas a cyclic peptide with a single binding motif acted as a TrkB antagonist. Results from these studies suggest that monovalent agents behaved as competitive antagonists by simply occupying a ligand docking site and that receptor dimerization/oligomerization may be required to mimic the functional effects of ligands. Indeed, all

neurotrophins have been shown to form homodimers or heterodimers (Arakawa et al., 1994; Butte et al., 1998; Robinson et al., 1999), thus capable of bringing two Trk receptors together after binding. However, in one report, Saragovi and colleagues (Le-Sauteur et al., 1996) observed a functional agonism using monomeric Fab fragments of a human TrkA-specific mAb. In this study, treatment with Fab fragments increased phosphorylation of TrkA and protected cells from apoptotic death, suggesting that monomeric binding agents could induce activation of TrkA. Of interest is the observation that cross-linking Fabs using anti-Fab Abs could enhance survival responses. This suggests that receptor dimerization is of added benefit to cellular activity. Because our agonist TrkB Abs appear to activate the receptor by binding several discrete epitopes, it would be instructive to see if any of our Fab fragments are also functionally active. Furthermore, structural studies of Ab-bound TrkB using x-ray crystallography might reveal important conformational cues required for full receptor activation.

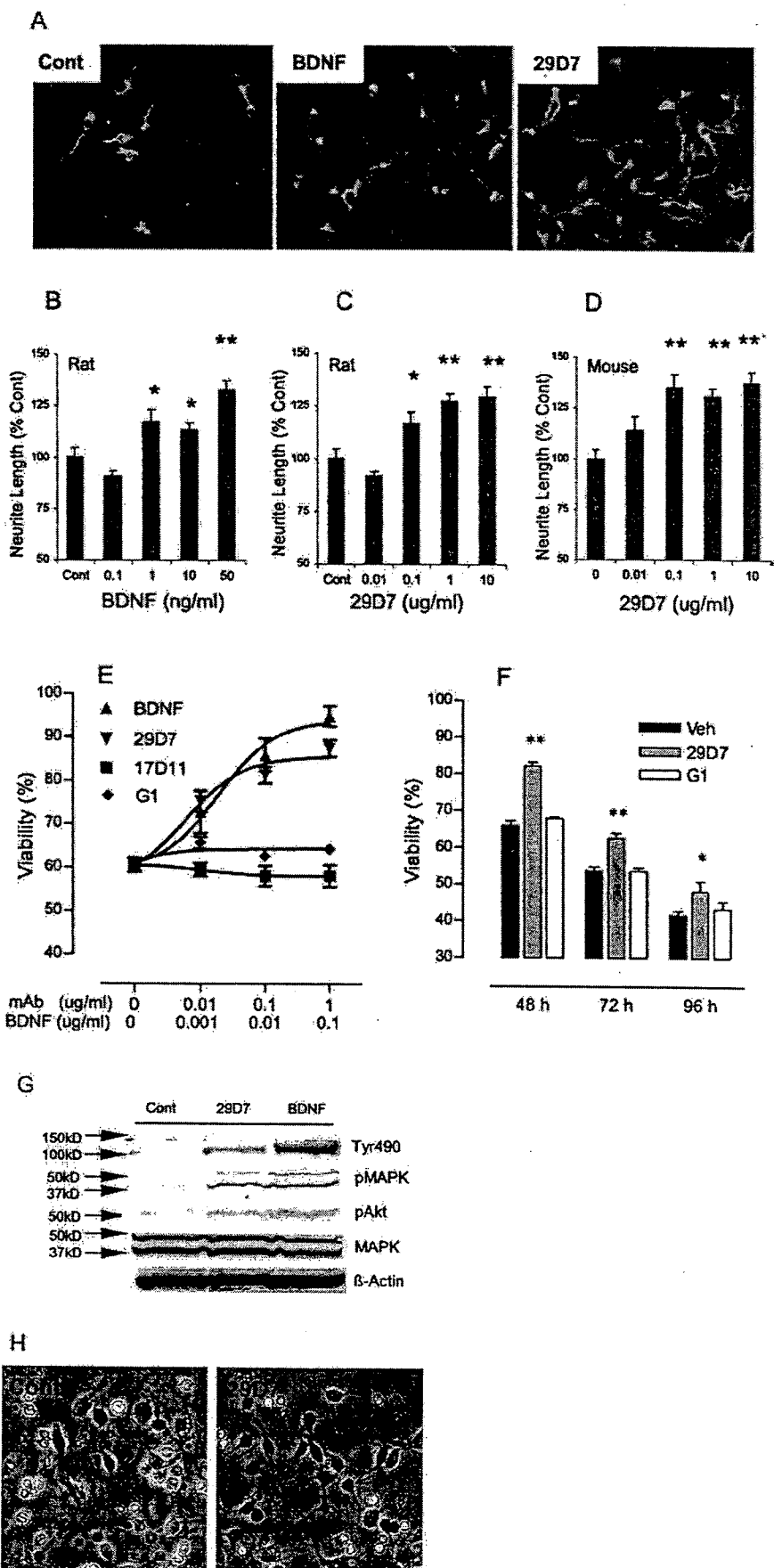


Figure 7. A TrkB mAb, 29D7, increases neurite outgrowth and neuronal survival in the primary neuronal cultures. **A**, Representative images of TuJ1 immunostaining of rat cerebellar neurons treated with a vehicle, BDNF, (10 ng/ml), or 29D7 (0.1 μ g/ml). **B**, **C**, Dissociated rat CGNs (postnatal day 7) were seeded onto 96-well plates in the presence of BDNF (**B**) or 29D7 (**C**) at indicated concentrations. **D**, Effects of 29D7 on mouse CGNs. After a 24 h treatment, cells were fixed and neurite outgrowth was visualized by TuJ1 immunostaining. Data represent changes in the total neurite lengths after indicated treatments compared with a vehicle-treated control. * $p < 0.05$, ** $p < 0.01$ compared with vehicle control determined by one-way ANOVA followed by Dunnett's *post hoc* test. **E**, Rat CGNs were plated in a standard Neurobasal medium. The next day, serum and potassium were removed with or without the addition of BDNF or indicated Abs. Neuronal survival was measured 24 h after injury, and data are presented as the percentage of viability of uninjured neurons. All treatment concentrations of BDNF and 29D7 resulted in statistically significant enhancement of neuronal survival ($p < 0.05$ compared with the vehicle control, one-way ANOVA followed by Dunnett's *post hoc* test). **F**, Rat CGNs treated with an indicated Ab (29D7 or control IgG1, 1 μ g/ml) or a vehicle (Veh) were subject to serum/potassium deprivation as in **E**, and the viability was measured at 48, 72, and 96 h after injury. * $p < 0.05$, ** $p < 0.01$ compared with vehicle control (one-way ANOVA followed by Dunnett's test). **G**, Rat CGNs on six-well plates were incubated with a vehicle, BDNF (100 ng/ml), or 29D7 (1 μ g/ml) for 30 min, and an equal amount (20 μ g) of proteins were resolved in the SDS-PAGE gel. Phosphorylation levels of TrkB, MAPK, and Akt kinases were probed using phospho-specific Abs, and the same samples were also probed for total MAPK protein and β -actin levels to verify equal loading. **H**, Representative images of postnatal cortical neurons treated with or without 29D7 (1 μ g/ml) for 48 h. The addition of 29D7 in culture medium increased the number of healthy neurons with intact processes (arrows). Cont, Control.

Our data from competition binding studies suggest that each of our five TrkB Abs recognizes a unique structural epitope of TrkB. We show that the binding of 7F5 or 11E1 to TrkB could be completely displaced by BDNF, suggesting that these Abs may recognize epitopes critical to TrkB–BDNF interactions. However, our data also suggest that 7F5 and 11E1 bind to unique epitopes, because even an excess amount of 11E1 could not completely compete with 7F5 binding onto TrkB. It is worth noting that 11E1 demonstrated the highest functional potency among our Abs in promoting neuritogenesis and cell survival in differentiated SH-SY5Y cells. This may suggest that the epitope recognized by 11E1 might be a critical domain regulating TrkB activation. Because the 11E1 epitope appears to be colocalized with that of BDNF, it can be postulated that both 11E1 and BDNF activate TrkB through mechanisms involving common binding hot spots, similar to those illustrated in the crystal structures of a TrkB dimmer complexed with NT-4 (Banfield et al., 2001).

Compared with 7F5 and 11E1, Abs 17D11 and 19E12 showed different binding properties in that they inhibited BDNF binding only by 40–60%, even at saturating doses. ELISA avidities of these Abs to TrkB were similar to those of the BDNF-blocking Abs (7F5 and 11E1); hence, this cannot account for the differential efficacy in BDNF displacement. It is reasonable to postulate that the binding sites of these Abs only partially overlap with the BDNF docking sites, or that the binding of these Abs to TrkB induced an unfavorable conformational change of the BDNF docking site, allowing partial access of BDNF to TrkB in the presence of bound Ab. Again, despite similar BDNF-blocking activities, the precise binding epitopes of these two Abs appear not to be the same, as the binding of 17D11 could not be completely displaced by 19E12.

We also characterized another Ab, 29D7, that appears to possess the most distinct binding properties. 29D7 binds strongly to both hTrkB and mTrkB, and it recognizes an epitope that is completely distinct from the BDNF docking site. Not surprisingly, 29D7 does not compete with either BDNF or any of our other Abs. That both BDNF and 29D7 can bind TrkB concomitantly led us to the hypothesis that the addition of 29D7 would not negate functional activities of BDNF. Indeed, we confirmed this by showing that BDNF-mediated neurite outgrowth was not affected by the presence of 29D7 (our unpublished data). Therefore, this substantiates the existence of epitopes outside of the BDNF docking sites that can be targeted for TrkB activation without interfering with the physiological role of BDNF. As a number of studies have shown, upregulation of BDNF expression after traumatic injuries to the brain (Comelli et al., 1992; Dugich-Djordjevic et al., 1992; Yang et al., 1996) might represent part of a self-repair mechanism, and it is important that this effect should not be altered by administering reagents such as 29D7 that are non-BDNF competing. Intriguingly, we observed that the combination of BDNF with 29D7 was no more efficacious in promoting neurite outgrowth than treatment with either agent alone. One reason for this may be that there exists only a limited pool of receptors that can be dimerized and activated by agonist binding. However, this cannot fully explain our results because no synergy was observed by dual treatment even at submaximal concentration of either BDNF or 29D7.

Among the five Abs, 29D7 shows the highest affinity toward mTrkB (13 pM ELISA-binding ED_{50}). Therefore, it was important to know whether 29D7 would demonstrate functional activities toward endogenous TrkB. Using primary neuronal cultures, we showed that 29D7 could robustly induce receptor phosphorylation as well as activation of downstream signaling cascades. Fur-

thermore, treatment with 29D7 increased the survival and growth of mouse and rat CGNs and rat cortical neurons, validating its functional effectiveness toward contextual neuronal systems. Surprisingly, 17D11, which also has a subnanomolar ELISA binding avidity to mTrkB, had no effects on either neurite growth or survival of mouse CGN. A lack of functional efficacy of 17D11 toward mTrkB is in contrast with its robust activity on hTrkB. This observation suggests that the 17D11 epitope on hTrkB is different from that on mTrkB and supports the notion that binding of a bivalent ligand itself may not be sufficient to induce receptor activation. Additional binding and structural analysis will be required to better understand this effect. To our knowledge, this is the first report that demonstrates isolation of human and rodent cross-reactive Abs using novel immunization techniques. By priming mice with antigens from both species, we successfully generated high-affinity cross-specific Abs. Because preclinical assessment of biological activity is critical to development of clinical candidates, this strategy might have a wide utility in isolating future therapeutic Abs.

In conclusion, we describe novel mAbs that selectively activate TrkB and thereby enhance neuronal survival and neurite outgrowth. Additional studies are in progress to address whether they have potential therapeutic use in ameliorating neurodegeneration associated with acute brain injury and/or promoting long-term repair.

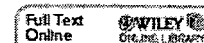
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Diabetes Obes Metab. 2003 Jan;5(1):27-37.

Brain-derived neurotrophic factor modulates glucagon secretion from pancreatic alpha cells: its contribution to glucose metabolism.

Hanyu O, Yamatani K, Ikarashi T, Soda S, Maruyama S, Kamimura T, Kaneko S, Hirayama S, Suzuki K, Nakagawa O, Nawa H, Aizawa Y.

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Abstract

AIM: Brain-derived neurotrophic factor (BDNF) reduces plasma glucose levels in obese db/db diabetic mice and is speculated to produce its effects via the hypothalamus, the regulatory centre of satiety and the autonomic nervous system. The potential effect of BDNF directly on peripheral endocrine organs, however, remains to be clarified. Here we report the effects of BDNF on hormonal secretion from pancreatic islets of Langerhans using their isolated culture.

METHODS AND RESULTS: In an immunohistochemical study, mouse pancreatic alpha cells were stained specifically with the anti-TrkB (a specific receptor for BDNF) antibody. After 7 days culture of isolated islets of the normal mouse pancreas, 10 ng/ml BDNF decreased the secretion of glucagon per 6 h significantly less than that of the control ($p = 0.016$). In contrast, there were no significant changes in insulin secretion or glucagon and insulin contents in the islets cultured under the same conditions. In vivo administration of BDNF (10 mg/kg/day) to normal mice for 7 days significantly decreased their food consumption ($p < 0.05$). The fasting plasma glucose levels were decreased on day 7 compared with day 1 more significantly in BDNF-treated mice ($p = 0.043$) than in pair-fed control mice ($p = 0.14$). In newborn BDNF-knockout mice, fasting plasma glucose levels increased in the order of homozygote, heterozygote and wild type ($p = 0.033$). No apparent immunohistochemical abnormality was observed for pancreatic glucagon in the BDNF-knockout mice.

CONCLUSION: These data suggest that BDNF affects glucose metabolism not only with its anorectic effect but also with modulated glucagon secretion from pancreatic alpha cells.

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Publication Types, MeSH Terms, Substances

LinkOut - more resources

Brain-Derived Neurotrophic Factor Improves Blood Glucose Control and Alleviates Fasting Hyperglycemia in C57BLKS-Lepr^{db}/lepr^{db} Mice

James R. Tonra, Michiko Ono, Xiaochun Liu, Karen Garcia, Carl Jackson, George D. Yancopoulos, Stanley J. Wiegand, and Vivien Wong

Systemic administration of brain-derived neurotrophic factor (BDNF) decreases nonfasted blood glucose in obese, non-insulin-dependent diabetic C57BLKS-Lepr^{db}/lepr^{db} (*db/db*) mice, with a concomitant decrease in body weight. By measuring percent HbA_{1c} in BDNF-treated and pair-fed animals, we show that the effects of BDNF on nonfasted blood glucose levels are not caused by decreased food intake but reflect a significant improvement in blood glucose control. Furthermore, once established, this effect can persist for weeks after cessation of BDNF treatment. Oral glucose tolerance tests were performed to examine the effects of BDNF on blood glucose control in the fasted state and after an oral glucose challenge. BDNF treatment normalized fasting blood glucose from initially hyperglycemic levels and also showed evidence for beneficial, although less marked, effects on the ability to remove exogenous glucose from blood. One means to lower fasting blood glucose is to reduce the glucose output of peripheral tissues that normally play a part in the maintenance of fasting hyperglycemia. Because the liver is the major endogenous source of glucose in blood during fasting, and because hepatic weight and glucose output are increased in type 2 diabetes, we evaluated the effects of BDNF on liver tissue. BDNF reduced the hepatomegaly present in *db/db* mice, in association with reduced liver glycogen and reduced liver enzyme activity in serum, supporting the possible involvement of liver tissue in the mechanism of action for BDNF. *Diabetes* 48:588–594, 1999

Brain-derived neurotrophic factor (BDNF) is a member of the nerve growth factor-related family of neurotrophins, which also includes neurotrophin-3 and neurotrophin 4/5 (1). These proteins exert trophic effects on specific populations of neurons that express their respective high-affinity receptors (2).

From Regeneron Pharmaceuticals (J.R.T., X.L., K.G., C.J., G.D.Y., S.J.W., V.W.), Tarrytown, New York; and Sumitomo Pharmaceuticals Research Center (M.O.), Discovery Laboratories II, Osaka, Japan.

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J.R.T., X.L., K.G., C.J., G.D.Y., S.J.W., and V.W. hold stock in Regeneron Pharmaceuticals.

ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; AUC, area under the curve; BDNF, brain-derived neurotrophic factor; ELISA, enzyme-linked immunosorbent assay; OGTT, oral glucose tolerance test; PLSD, paired least-squares difference.

In addition to promoting the survival of responsive neurons, the neurotrophins can affect such basic cellular processes as protein expression, cellular differentiation, and neurite extension (3–5). Recently it was reported that systemic administration of BDNF lowered nonfasted blood glucose in hyperglycemic C57BLKS-Lepr^{db}/lepr^{db} (*db/db*) mice, a model for obesity and non-insulin-dependent diabetes (6), but only if given before the mice were 23 weeks of age (7). BDNF decreased food intake and body weight in *db/db* mice, but pair-feeding to the reduced food intake level of BDNF-treated mice did not cause the same effects on blood glucose, suggesting that the effects of BDNF on body weight and blood glucose are separable. BDNF was also effective in reducing nonfasted blood glucose in mildly hyperglycemic *ob/ob* mice but did not affect blood glucose in nondiabetic mice or insulin-dependent diabetic rats (7).

We expanded on those studies to demonstrate the potential of BDNF as a novel hypoglycemic agent for the treatment of non-insulin-dependent diabetes. Results show not only that BDNF reduces blood glucose levels during treatment, but also that the effect continues for weeks after treatment cessation, even when BDNF is no longer detected in blood. Measurements of the percent HbA_{1c} (8) were used to demonstrate that the effects of BDNF on nonfasted blood glucose reflect a dramatic improvement in blood glucose control that is not dependent on the timing of blood glucose measurements relative to food intake. To gain insights into the mechanism of action of BDNF, oral glucose tolerance tests (OGTTs) were performed. Results from those tests and additional studies show that BDNF can normalize fasting blood glucose in hyperglycemic mice, and this effect may be related to effects of BDNF on the liver.

RESEARCH DESIGN AND METHODS

Mice. Male *db/db* mice on a C57BLKS/J background were obtained from the Jackson Laboratory (Bar Harbor, ME) at 4–8 weeks of age. Animals were originally housed three per cage but were single-housed 1 week before the start of neurotrophin treatment. All treatments were started at 10 weeks of age. Mice were given food (Purina Rodent Laboratory Chow 5001; Purina Mills, Richmond, IN) and water ad libitum, except for mice that were pair-fed daily to a BDNF-treated group (see below). Male heterozygous *db/+* and wild-type C57BL6 mice were used as nondiabetic, nonobese controls. All animal use in this study was conducted in compliance with approved institutional animal care and use protocols and according to NIH guidelines (*Guide for the Care and Use of Laboratory Animals*, NIH publication no. 86–23, 1985).

BDNF administration and pair-feeding. Mice were injected subcutaneously two (Monday and Thursday), three (Monday, Wednesday, and Friday), or five (Monday through Friday) times a week with recombinant human BDNF (Amgen, Thousand Oaks, CA, or Sumitomo, Osaka, Japan; no difference in efficacy with respect to nonfasted blood glucose, fasting blood glucose, and percent HbA_{1c}). A

pair-fed group of vehicle (phosphate-buffered saline)-treated *db/db* mice was included in some of the studies because BDNF treatment decreases food intake by about 50% in *db/db* mice (results not shown) compared with vehicle-treated mice (7). Mice in the pair-fed group were fed the average amount of food consumed by BDNF-treated mice over the previous 24 h. Food intake was estimated by weighing the food remaining from the previous day. Pair-fed animals received their daily ration of food between 0900 and 1100, always after nonfasted blood glucose measurements. At death (see below), trunk blood and tissues were collected in the morning before pair-feeding for that day. Measurements of fasted blood glucose and glycated hemoglobin were made to overcome the possible dependence of certain measures of blood glucose control (that is, nonfasted blood glucose) on the timing of the measurements relative to food consumption.

Blood and tissue assays. Nonfasted blood glucose was measured in blood collected from the tip of the tail between 0800 and 1200 using a Glucometer 3 Blood Glucose Meter (Bayer, Elkhart, IN) (glucose oxidase method). Percent HbA_{1c} (8) was measured with a Diastat Analyzer (Biorad, Hercules, CA). Insulin, glucagon (Linco, St. Charles, MO), and corticosterone (ICN, Costa Mesa, CA) levels were assayed in serum by radioimmunoassay. To minimize the effects of euthanasia on levels of the stress hormone corticosterone, mice were killed by decapitation (9) after acclimation in an undisturbed room for 1–2 h. No effect of BDNF treatment on nonfasted corticosterone was detected (results not shown). Alanine aminotransferase (ALT/SGPT, coupled ALT/MDH method [10]), aspartate aminotransferase (AST/SGOT, coupled AST/MDH method [10]) and glucose (hexokinase method) were also assayed in serum using the Monarch blood chemistry analyzer (Instrumentation Laboratory, Lexington, MA). BDNF was assayed by enzyme-linked immunosorbent assay (ELISA) (Promega, Madison, WI) in plasma. Liver (left anterior lobe), heart, and fat (white adipose tissue; left and right epididymal fat pads combined) were dissected at death and weighed.

Oral glucose tolerance test. After an 18- to 20-h fast starting at 1430, animals were bled from the tail for a baseline (time 0) measurement. Afterward, animals were administered 89 mg D-glucose (Sigma, St. Louis, MO) dissolved in 0.2 ml distilled water (~2.2 g/kg body wt) through a feeding needle (VWR, Plainfield, NJ). Blood was drawn from the tail 20, 60, and 210 min after glucose administration. The area under the blood glucose curve (AUC) was calculated after subtracting the fasting blood glucose value from all glucose measurements. AUC is reported for calculations from 0 to 60 min after glucose administration, but results were the same for calculations from 0 to 210 min.

Glycogen determination. Liver glycogen was determined using a protocol similar to that previously described (11). Glycogen was isolated from liver (stored at -80°C) by ethanol precipitation after the tissue was homogenized in 20 mM Tris-HCl with 0.1% Triton-X100, pH 8.6. Glycogen was hydrolyzed enzymatically with amyloglucosidase (Sigma) in 0.4 mol/l sodium acetate buffer, pH 4.8, followed by glucose determination with a Sigma glucose diagnostic kit (Glucose HK 20, hexokinase method).

Data analysis. Differences in means were tested using analysis of variance (ANOVA) with Fisher's paired least-squares difference (PLSD) as a post hoc test (Statview; Abacus, Berkeley, CA). Differences in blood glucose during the OGTTs were evaluated with a repeated-measures ANOVA followed by Fisher's PLSD as a post hoc test (Statview). $P < 0.05$ was considered statistically significant.

RESULTS

Lasting effects of BDNF on nonfasted blood glucose. As shown previously (7), BDNF decreased nonfasted blood glucose in *db/db* mice (Fig. 1A). BDNF administered five times a week, starting at 10 weeks of age, normalized nonfasted blood glucose (nondiabetic ~130 mg/dl) (Table 1) after 1 week of treatment, and glucose remained at this level through 4 weeks of treatment (Fig. 1A, black bar). Pair-feeding did not affect nonfasted blood glucose. This result differs from a previous result that reported a small but significant decrease in nonfasted blood glucose after 8 weeks of pair-feeding (7), possibly due to the use of females rather than males or the different source of *db/db* mice (Clea Japan) by Ono et al. (7).

After treatment was stopped, blood glucose in BDNF-treated animals remained below pretreatment levels for an additional 4 weeks, and lower than that of vehicle-treated and pair-fed *db/db* controls for 12 weeks (Fig. 1A). In contrast, body weights for BDNF-treated *db/db* mice were depressed during treatment but increased rapidly after treatment was stopped, surpassing those of vehicle-treated *db/db* mice 8

weeks before blood glucose values were equivalent in the two groups (Fig. 1B).

One possible explanation for the lasting effect of BDNF on blood glucose is that BDNF has a prolonged plasma half-life. To test this possibility, BDNF was injected three times a week for 2 weeks at 30 or 50 mg/kg and then treatment was stopped. Plasma BDNF was measured by a sensitive two-site ELISA at 3, 6, 10, and 17 days after the final treatment. BDNF was detectable in plasma for up to 6 days after the last injection, but was not detectable by 10 days (Fig. 2B). In contrast, the effect on blood glucose was maintained through 17 days after the last BDNF injection (Fig. 2A). These data indicate that BDNF treatment results in a lasting improvement in the ability of *db/db* mice to maintain blood glucose near nondiabetic levels that, once established, can persist for weeks without additional BDNF (Fig. 1A).

Percent HbA_{1c}. Although BDNF treatment decreases nonfasting blood glucose, the decrease in food intake observed during BDNF treatment suggested that the effect may depend on the timing of glucose measurements relative to food consumption. To address this issue, we evaluated the percent of hemoglobin nonenzymatically glycated (percent HbA_{1c}), which is an integrated measure of blood glucose regulation during the weeks before measurement (8). We found that after 4 weeks treatment, starting at 10 weeks of age, percent HbA_{1c} in BDNF-treated *db/db* mice was 45% below that of vehicle-treated *db/db* mice and similar to percent HbA_{1c} in nondiabetic controls (Fig. 3). Pair-feeding to the reduced food intake of BDNF-treated mice had no effect on percent HbA_{1c}. In addition, treatment of *db/db* mice for 4 weeks with a control protein, cytochrome c (12) (30 mg/kg), did not alter percent HbA_{1c} (results not shown). Thus, BDNF treatment dramatically improved blood glucose control, and this effect was specific and not caused by alterations in food intake.

Effects of BDNF are maintained when treatment frequency is reduced. The lasting effects of BDNF on blood glucose control (Fig. 1A) and the presence of BDNF in blood for days after the last injection (Fig. 2B) suggested that efficacy might be maintained with less frequent administration of BDNF. Since decreasing treatment frequency would be both experimentally and clinically relevant, we examined the efficacy of BDNF when administered two, three, or five times a week. We found that less frequent dosing of BDNF (two and three times a week) had effects on percent HbA_{1c} that were similar, if not identical, to dosing at five times a week (Table 1). Pair-feeding caused an equivalent reduction in body weight compared to the three times a week BDNF treatment, unlike pair-feeding to the five times a week BDNF treatment (Fig. 1B), but percent HbA_{1c} was not affected by pair-feeding.

Oral glucose tolerance test. To gain insights into the mechanism of action of BDNF, we examined the effects of BDNF on blood glucose control in the fasted state and after administration of a standard oral glucose load by utilizing an OGTT. Ten untreated *db/db* mice were randomly selected from a larger group of mice and an OGTT was performed at 10 weeks of age (Fig. 4). Treatment of the remaining mice in the group was started 1 day before the test and continued for 4.5 weeks (three times a week), at which time an OGTT was performed (Fig. 4).

Blood glucose levels during the OGTTs (Fig. 4A) were significantly different in the different groups tested ($P < 0.0001$;

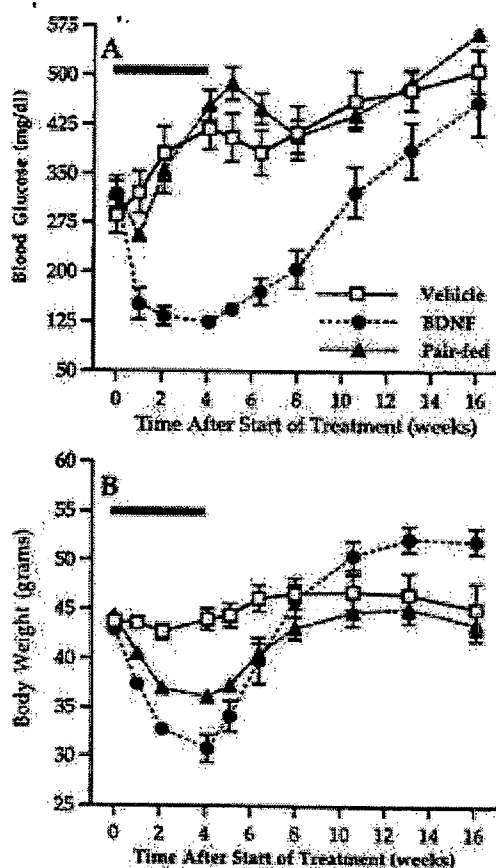


FIG. 1. Recovery after BDNF treatment cessation. Time course of non-fasted blood glucose (A) and body weight during and after BDNF treatment (five times a week, 30 mg/kg) (B) in *db/db* mice. The black bar represents the duration of BDNF treatment (4 weeks). Mean $\pm$ SE is plotted for $n = 9$ –10 per group.

effect of treatment group). As reported previously (7), BDNF-treated mice had lower blood glucose levels during an OGTT compared with pair-fed mice ($P < 0.0001$). In addition, BDNF improved the OGTT results when compared with the pretreatment test performed at 10 weeks of age ($P < 0.0001$).

TABLE 1
Effects of BDNF treatment on percent HbA_{1c} and tissue weights

Treatment	Strain	<i>n</i>	% HbA _{1c}	Blood glucose (mg/dl)	Body weight (g)	Heart (mg)	Fat (g)	Liver (mg)
None	<i>db/+</i>	5	3.3 $\pm$ 0.1	131 $\pm$ 4	30.1 $\pm$ 0.5	147 $\pm$ 14	0.76 $\pm$ 0.06	443 $\pm$ 17
Vehicle	<i>db/db</i>	7	8.4 $\pm$ 0.2*	476 $\pm$ 44*	42.9 $\pm$ 1.9*	151 $\pm$ 11	2.37 $\pm$ 0.04*	848 $\pm$ 58*
Two times per week BDNF (30 mg/kg)	<i>db/db</i>	7	5.2 $\pm$ 0.4*†	140 $\pm$ 15†	35.7 $\pm$ 1.3*†	123 $\pm$ 5*†	1.99 $\pm$ 0.06*†	563 $\pm$ 20*†
Three times per week BDNF (30 mg/kg)	<i>db/db</i>	7	4.3 $\pm$ 0.2*†	116 $\pm$ 4.8†	34.2 $\pm$ 1.1*†	121 $\pm$ 5*†	1.99 $\pm$ 0.05*†	482 $\pm$ 29†
Five times per week BDNF (30 mg/kg)	<i>db/db</i>	7	4.3 $\pm$ 0.2*†	115 $\pm$ 6.6†	28.8 $\pm$ 1.3*†	115 $\pm$ 5*†	1.59 $\pm$ 0.09*†	410 $\pm$ 11†
Pair-fed to three times per week BDNF	<i>db/db</i>	6	8.5 $\pm$ 0.3*	545 $\pm$ 17*†	32.1 $\pm$ 1.4*†	125 $\pm$ 4†	1.75 $\pm$ 0.1*†	600 $\pm$ 23*†

Data are means $\pm$ SE. Percent HbA_{1c} and glucose were measured on the last day of a 5-week treatment. Animals were killed 3 days after the final treatment, and tissue weights were obtained. * $P < 0.05$ vs. heterozygous mice; † $P < 0.05$ vs. vehicle-treated *db/db* mice, data analysis by ANOVA followed by Fisher's PLSD.

This effect is observed to be due to a lowering of fasting blood glucose (time 0) from the hyperglycemic levels observed at 10 weeks of age to the normal level observed in nondiabetic heterozygous mice ($P < 0.0001$ for BDNF-treated *db/db* vs. *db/db* at 10 weeks; one-way ANOVA).

The ability to respond to an oral glucose challenge and clear the exogenous glucose load from blood was evaluated by comparing the areas under the OGTT curves. To control for effects of treatment on fasting blood glucose, AUC was calculated after shifting the OGTT curve of each animal so that all mice started from a common baseline at time 0. AUC (Fig. 4B) was not significantly reduced by BDNF treatment compared with the 10-week-old *db/db* mice ($P = 0.26$) but was significantly improved compared with vehicle-treated and pair-fed controls after 4.5 weeks of treatment ($P < 0.006$ for both). Compared with nondiabetic heterozygous mice, AUC in BDNF-treated *db/db* mice was elevated ($P < 0.01$). Thus the ability to respond to an oral glucose load showed signs of improvement with BDNF treatment. Compared with the pretreatment condition, however, BDNF did not significantly improve the ability to remove exogenous glucose from blood, in spite of a normalization of fasting blood glucose (Fig. 4A, time 0). Furthermore, in a separate study, BDNF (30 mg/kg, three times a week for 4.5 weeks) normalized fasting blood glucose but did not significantly improve the response to an oral glucose load (results not shown). The cause of the differential effects of BDNF on the response to a glucose load is unknown. However, results demonstrate that the normalizing effects of BDNF on fasting blood glucose do not require an improvement in the ability to remove exogenous glucose from blood.

Insulin and glucagon are potent regulators of blood glucose, so BDNF could affect fasting blood glucose by altering their release. Two weeks of daily BDNF treatment (20 mg/kg body wt) starting at 7 weeks of age has been reported to decrease nonfasted plasma insulin levels in *db/db* mice (7). Our results agree with this finding, in that 4 days of BDNF treatment lowered nonfasted serum insulin compared with vehicle-treated *db/db* mice (Table 2). To control for differential food intake before measurement, we evaluated the effects of 4.5 weeks of BDNF treatment (30 mg/kg, three times a week) on insulin and glucagon release in the fasted state, when blood glu-

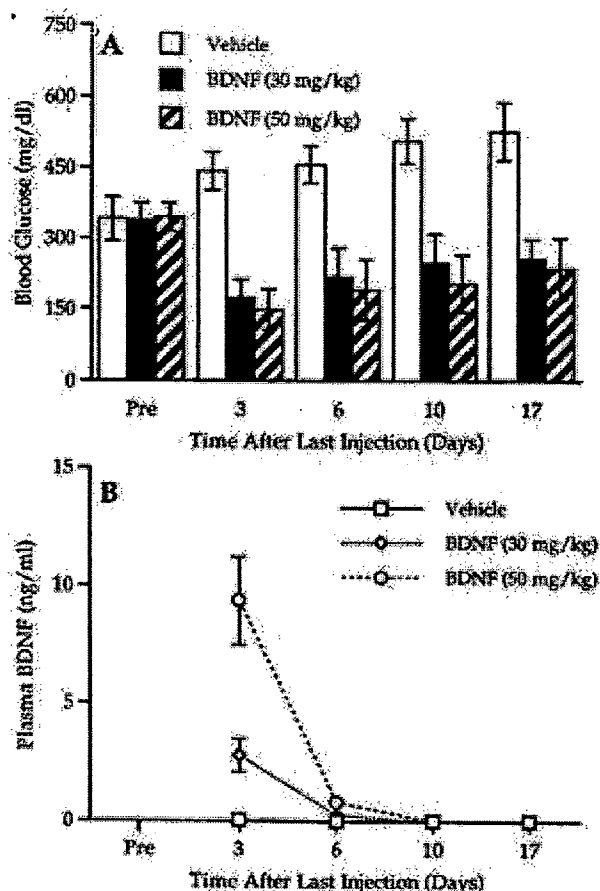


FIG. 2. Lack of a correlation between blood glucose and plasma BDNF after treatment cessation. Nonfasted blood glucose (A) and plasma BDNF (B) in *db/db* mice after 2 weeks of treatment three times a week at the BDNF dosage indicated. Measurements were made 3, 6, 10, and 17 days after the final injection. Blood glucose was also measured 3 days before the start of treatment (Pre). Mean $\pm$ SE is plotted for $n = 5-6$ per group.

cose was shown to be lower in BDNF-treated *db/db* mice (Fig. 4, time 0). Fasting insulin and glucagon were elevated by 42 and 53% ($P < 0.0003$ for both), respectively, in vehicle-treated *db/db* mice compared with heterozygous mice. BDNF treatment or pair-feeding did not affect fasting insulin or glucagon levels ($P > 0.25$ for both versus vehicle, results not shown). Thus BDNF does not affect fasting blood glucose by altering pancreatic insulin or glucagon secretion, although changes in insulin responsiveness may be involved (see DISCUSSION).

Liver weight and glycogen. Through gluconeogenesis and glycogenolysis, the liver is the major source of glucose during fasting (11,13-15). The finding that BDNF normalizes fasting blood glucose compared with pretreatment levels, without a commensurate marked improvement in the ability to remove exogenous glucose from blood (Fig. 4), suggests that decreased glucose output by the liver might be part of the mechanism of action for BDNF. Liver weight is disproportionately increased in *db/db* mice, and this weight increase is associated with elevated hepatic glucose output (6,11). Consistent with previous studies, we found that the wet weights of liver and epididymal fat pad (6,11,16), but not heart (16), were elevated in *db/db* mice compared with age-matched heterozygous mice (Table 1). Five weeks of BDNF treatment

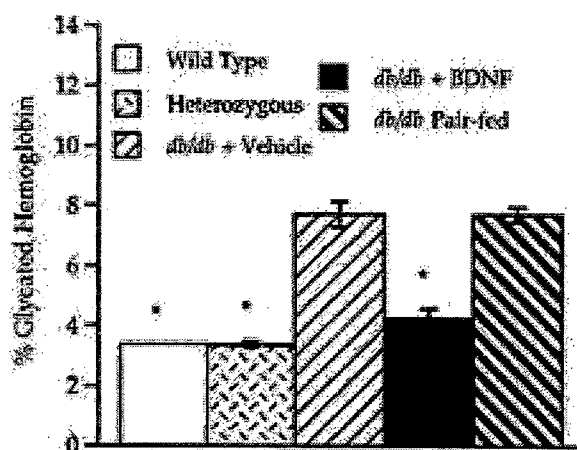


FIG. 3. Effect of BDNF on % HbA_{1c}. Mice were treated five times a week for 4 weeks (BDNF 30 mg/kg), and % HbA_{1c} was measured 1 week after treatment cessation. Wild-type refers to C57BL/6 age- and sex-matched mice. Pair-fed animals were fed daily the average amount of food consumed by the BDNF-treated mice the previous day. Mean $\pm$ SE is plotted for $n = 5-10$ mice per group. * $P < 0.0001$ vs. vehicle-treated *db/db* mice by ANOVA followed by Fisher's PLSD.

or pair-feeding lowered total body weight as well as the wet weight of liver, heart, and fat. Of significance from this study is the finding that, although the BDNF-induced decrease in the weight of heart and fat could be mimicked by pair-feeding, the decrease in liver weight was significantly greater in BDNF-treated animals than in pair-fed animals. In fact, BDNF treatment at three or five times a week (30 mg/kg) resulted in near normal (heterozygous) liver weights. When liver weight is expressed as a percentage of body weight (Fig. 5), it is seen that the excess weight of the liver (hepatomegaly) in *db/db* mice, in relation to body weight (6), was not observed in BDNF-treated animals but was still present after pair-feeding.

Since glycogen is a stored source of glucose used by the liver during periods of fasting, decreased glycogen stores in BDNF-treated mice during periods of free food access could result in decreased glucose output during periods of fasting, resulting in lower fasting blood glucose (Fig. 4). In support of this, total glycogen content of the liver in the nonfasted condition was 36% lower in BDNF-treated mice compared with vehicle-treated controls (Table 3). Glycogen concentration was not affected by BDNF (33.6 ± 2.7 for BDNF vs. 34.4 ± 2 mg glycogen/g tissue for vehicle). These values at 14 weeks of age are comparable to those previously reported for 8-week-old *db/db* mice (51 and 64 mg glycogen/g liver weight) (6,11). Glycogen concentration in the liver of pair-fed mice is reduced by 42% compared with vehicle- and BDNF-treated mice (Table 3). Since pair-fed animals are hyperglycemic at the time of tissue sampling (data not shown; see Fig. 1A), this finding suggests that pair-fed animals are actively maintaining hyperglycemia when food access is restricted (see DISCUSSION).

Liver enzyme activities in serum. We also examined the serum activities of two enzymes released into blood by the liver, ALT and AST. In mice, ALT is found predominantly in liver, whereas AST is found in cardiac muscle, liver, blood vessels, and brain (17). Serum ALT activity was elevated in *db/db* mice compared with heterozygous mice (Table 2). BDNF

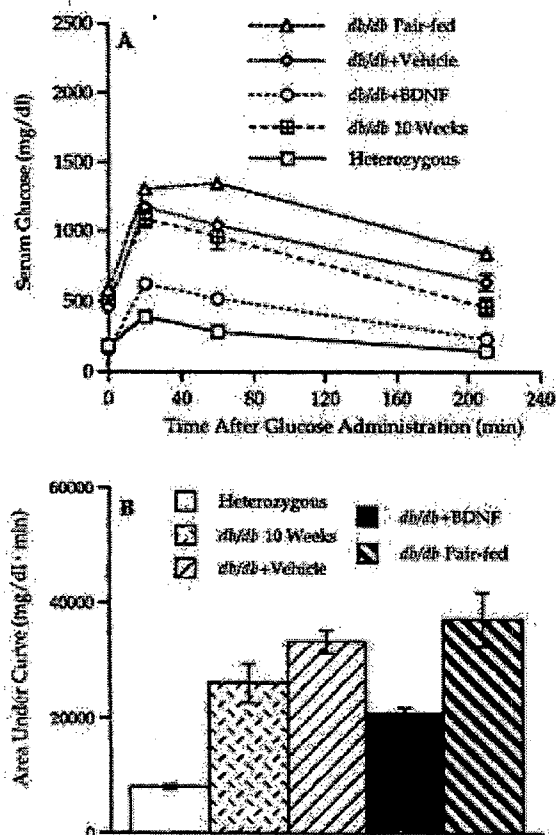


FIG. 4. Effect of BDNF on OGTTs. Mice were randomized into groups by body weight and nonfasted blood glucose at 9 weeks of age so that group means were not significantly different and were within 1 g of each other for body weight and 45 mg/dl for blood glucose. The oral glucose tolerance of one group from this lot was tested at 10 weeks of age (*db/db* 10 weeks). The remaining mice, including a group of age-matched heterozygous mice, were treated three times a week for 4.5 weeks (BDNF 30 mg/kg), at which time an OGTT was administered. Animals were fasted for 18 h before blood collection for fasting blood glucose (reported at Time 0). A standard glucose load (123 mg) was subsequently gavaged into the stomach, and capillary blood was sampled from the tail at 20, 60, and 210 min after gavage. A: Blood glucose values during the OGTT. B: Area under the OGTT curve (AUC) from 0 to 60 min after glucose administration. Before calculation of AUC, all curves were adjusted to a common baseline at time 0 by subtracting the initial glucose from all glucose measurements. Mean $\pm$ SE is plotted for $n = 5$ heterozygous mice and $n = 9-10$ *db/db* mice per group.

treatment for 4 consecutive days, but not pair-feeding, lowered ALT in both mutant and heterozygous mice. These changes in ALT appeared to parallel changes in liver weights. No difference in AST activity was observed between *db/db* and heterozygous mice, and no effect of BDNF treatment was detected, although a trend for increased AST was observed in BDNF-treated *db/db* mice (Table 2).

In humans, the ratio ALT/AST (DeRitis ratio), or its reciprocal, has been reported to differentiate between certain pathologies (18); for example, ALT/AST is elevated in morbidly obese humans with excess accumulation of triglycerides in hepatocytes (fatty liver) (19,20). In our study, ALT/AST was higher in *db/db* mice than in heterozygous mice (Table 2), consistent with the presence of fatty liver in *db/db* mice (21). After only 4 days of treatment, BDNF lowered the ALT/AST ratio in *db/db* mice. BDNF had a similar but less dramatic effect on the ALT/AST ratio in heterozygous mice

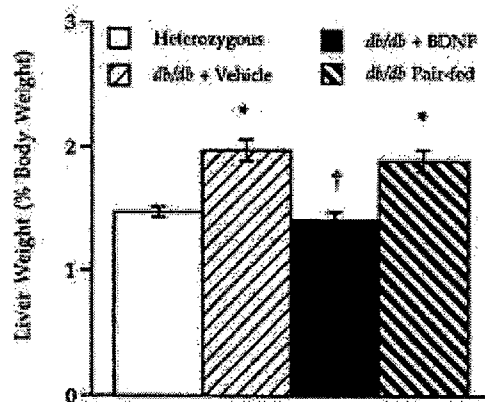


FIG. 5. Effect of BDNF on liver weight when expressed as a percentage of body weight. Mice were treated three times a week for 4 weeks (BDNF 30 mg/kg). Values are $100 \times (\text{liver wt/body wt})$. Means $\pm$ SE are plotted for $n = 5-7$ per group. * $P < 0.05$ vs. untreated heterozygous mice; † $P < 0.05$ vs. vehicle-treated and pair-fed *db/db* mice by ANOVA followed by Fisher's PLSD.

(Table 2). The same effect on ALT/AST in *db/db* mice was observed after 4 weeks of BDNF treatment (30 mg/kg, three times a week, results not shown).

DISCUSSION

The present study demonstrates that BDNF may have significant potential as a hypoglycemic agent for the treatment of non-insulin-dependent diabetes. BDNF treatment of *db/db* mice resulted in percent HbA_{1c} and fasting blood glucose values that were near those of nondiabetic mice. Since pair-feeding *db/db* mice to BDNF-treated mice had no effect on these measurements, our results show that the effects of BDNF on blood glucose are not caused by alterations in food intake. Of potential clinical importance for increasing patient compliance is the finding that BDNF is effective when administered as infrequently as two times a week. Furthermore, the improvement in blood glucose control compared with the pretreatment condition can last for weeks after treatment cessation, even when BDNF is no longer present in blood. Therefore lasting physiologic changes induced by BDNF, rather than pharmacokinetic properties of BDNF, result in maintained improvement in blood glucose control.

Improved blood glucose control in BDNF-treated animals is related to a dramatic reduction in fasting blood glucose. Beneficial effects of BDNF on the response to an oral glucose challenge probably contribute to improved blood glucose control, but the dramatic effect of BDNF on fasting blood glucose did not require an improvement in the ability to remove exogenous glucose from blood. This indicates that BDNF can reduce the glucose output of peripheral tissues that maintain fasting hyperglycemia. Thus the effect of BDNF on fasting hyperglycemia, as well as the effects on liver weight, liver glycogen, and liver enzyme activities in serum, suggests that reduced glucose output by the liver may be part of the mechanism of action for BDNF.

Reducing hepatic glucose production has previously been recognized as a means to treat diabetes, because hepatic glucose output is markedly elevated in type 2 diabetic patients (22), as well as *db/db* mice (11). This increase in hepatic glu-

TABLE 2
Effects of BDNF on serum enzyme activities and insulin

Treatment	Strain	n	ALT/(IU/l)*†	AST/(IU/l)	ALT/AST*†‡	Insulin (ng/ml)†
Vehicle	<i>db/db</i>	11	80.3 ± 4.3	126.5 ± 7.4	0.65 ± 0.04	9.4 ± 2.6
BDNF (30 mg/kg)	<i>db/db</i>	11	61.2 ± 2.1	164.5 ± 15.6	0.40 ± 0.03	3.7 ± 0.4
Pair-fed to BDNF	<i>db/db</i>	10	70.9 ± 7.2	106.3 ± 14.3	0.71 ± 0.06	12.2 ± 2.8
Vehicle	Heterozygous	7	40.7 ± 1.0	141.3 ± 6.7	0.29 ± 0.01	0.7 ± 0.2
BDNF (30 mg/kg)	Heterozygous	7	34 ± 1.1	138.6 ± 11.1	0.26 ± 0.02	1.3 ± 0.5
Pair-fed to BDNF	Heterozygous	6	45.8 ± 1.7	140.8 ± 14.3	0.34 ± 0.03	2.0 ± 1.1

Data are means ± SE. Mice were treated daily for 4 days and killed by decapitation on the 5th day, 1 h after food removal. * $P < 0.02$ effect of treatment; † $P < 0.0001$ effect of strain; ‡ $P < 0.03$ interaction between treatment and strain (two-way ANOVA).

cose production is crucial to the maintenance of fasting hyperglycemia (15,22) and is observed in spite of high serum insulin levels in *db/db* mice (6), indicating relative insensitivity of the liver to insulin. Such conditions, also reported in non-insulin-dependent diabetic patients (22), are thought to reflect an impaired feedback loop between the pancreatic β -cell and the liver (15). Both impaired β -cell glucose sensitivity and impaired hepatic insulin sensitivity have been postulated to result in increased glucose output by liver and hyperinsulinemia in type 2 diabetic patients (15,22). Fasting hyperinsulinemia was unaffected by BDNF in spite of a normalization in fasting blood glucose, indicating that in fasted condition the β -cell was still relatively glucose insensitive after BDNF treatment, while the insulin sensitivity of the liver may have been increased in BDNF-treated mice.

The involvement of insulin in the mechanism of action of BDNF is supported by lack of effect of BDNF on blood glucose in older *db/db* mice that have reduced insulin levels (6) and in hypoinsulinemic streptozotocin-treated diabetic rats (7). However, BDNF treatment resulted in significantly lower fasting blood glucose in younger hyperglycemic, hyperinsulinemic *db/db* mice without affecting fasting hyperinsulinemia. This finding suggests that in the fasted condition, if insulin is involved in the mechanism of action of BDNF, it is through an increase in insulin sensitivity rather than increased release (7). The finding that the ALT/AST ratio is decreased by BDNF may support this possibility because hepatic steatosis in patients with hypertriglyceridemia is linked to a decrease in insulin sensitivity (23). Alleviation of fatty liver, indicated by a reduction in the ALT/AST ratio (19,20), could therefore be associated with increased insulin sensitivity.

It is interesting that reduction of food intake in *db/db* mice pair-fed to BDNF-treated mice did not improve blood glucose levels through a decrease in the amount of ingested glucose.

An explanation for this result is that pair-fed animals compensate for decreased food intake with increased glucose output by peripheral tissues to maintain hyperglycemic conditions and elevated HbA_{1c}, but BDNF-treated animals consuming equivalent amounts of food do not. This is supported by the decreased glycogen concentration in the liver of pair-fed animals, which suggests active glycogenolysis to compensate for the effects of reduced food intake on blood glucose. Thus simply reducing the food intake of *db/db* mice (pair-feeding) does not improve blood glucose levels when started at 10 weeks of age because of compensating mechanisms to maintain elevated blood glucose levels. In this regard, when *db/db* mouse liver is perfused with recirculating medium containing various initial concentrations of glucose, hepatic glucose uptake and output are adjusted to change the perfusate glucose concentration toward the physiologic, hyperglycemic level normally observed in *db/db* mice (11). This is in contrast to liver in wild-type mice, which adjusts hepatic glucose uptake and output to change the perfusate glucose concentration toward normoglycemic levels.

Based on the predominantly neural localization of the high-affinity BDNF tyrosine kinase receptor, trk B, effects of BDNF probably involve the nervous system to some extent, although non-neuronal tissue (such as spleen) can also express trk B mRNA (24,25). As previously suggested (7), BDNF may act on trk B-expressing neurons in the hypothalamus (26,27), an area implicated in the control of body weight, food intake, and metabolism (28). Additionally, BDNF could act on the autonomic nervous system, since anatomic changes in preganglionic and postganglionic sympathetic neurons have been reported in mice overexpressing BDNF in the superior cervical ganglion (29). Neural effects of BDNF could be transmitted to peripheral tissues, such as the liver, through the autonomic nervous system or endocrine pathways (13,14).

TABLE 3
Effects of BDNF on liver glycogen

Treatment	Total glycogen (mg)	Glycogen concentration (mg/g wet wt)
Vehicle	25.0 ± 1.9	34.4 ± 2.0
BDNF (30 mg/kg)	15.0 ± 1.9*	33.6 ± 2.7
Pair-fed to BDNF	11.6 ± 2.1*	20.1 ± 3.3†

Data are means ± SE. $n = 9$ per group. Mice were treated three times a week for 4.5 weeks. The left anterior lobe of the liver was removed and stored frozen at -80°C until assayed for glycogen. * $P < 0.001$ vs. vehicle, † $P < 0.001$ vs. BDNF and vehicle (ANOVA).

In conclusion, the present results demonstrate that BDNF treatment results in dramatically improved blood glucose control in non-insulin-dependent diabetic mice, and this effect is not caused by decreased food intake. Our results indicate that BDNF can alleviate the fasting hyperglycemia in *db/db* mice that contributes to the development of diabetes. In this regard, effects on the liver suggest that this tissue may be involved in the mechanism of action for BDNF.

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Brain-derived neurotrophic factor reduces blood glucose level in obese diabetic mice but not in normal mice.

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Abstract

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family. However, it is not yet known if BDNF works on the endocrine system itself. Here we report that BDNF improves hyperglycemia in obese diabetic animals. BDNF reduced the blood glucose level in obese db/db diabetic mice in which the effect of BDNF was age-dependent and high under the condition of hyperinsulinemia, while BDNF showed no effect on non-diabetic db/m mice. These results suggest that BDNF ameliorates insulin resistance by enhancing insulin action in peripheral tissues. Furthermore, BDNF was found to reduce the plasma insulin level in db/db mice. Among the neurotrophin family, NT-3 also reduced the blood glucose level in db/db mice. These results provide a novel insight that neurotrophin functions on the endocrine system as well as the nervous system.

PMID: 9299565 [PubMed - indexed for MEDLINE]

MeSH Terms, Substances

LinkOut - more resources



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07H 21/02, 21/04, C12Q 1/68, C12P 19/34	A1	(11) International Publication Number: WO 96/01837 (43) International Publication Date: 25 January 1996 (25.01.96)
(21) International Application Number: PCT/US95/08180 (22) International Filing Date: 29 June 1995 (29.06.95) (30) Priority Data: 08/271,454 7 July 1994 (07.07.94) US (71) Applicant: THE CHILDREN'S HOSPITAL OF PHILADELPHIA [US/US]; 34th and Civic Center Boulevard, Philadelphia, PA 19104 (US). (72) Inventors: BRODEUR, Garrett, M.; 2010 Spruce Street, Philadelphia, PA 19104 (US). NAKAGAWARA, Akira; Devine Tracey Hotel, 20 South 36th Street, Philadelphia, PA 19104 (US). (74) Agents: JOHNSON, Philip, S. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris, One Liberty Place - 46th floor, Philadelphia, PA 19103 (US).	(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>	
(54) Title: COMPOSITIONS AND METHODS OF MAKING AND USING HUMAN FULL LENGTH TRK-B		
(57) Abstract The amino acid sequence of the full length version of the human neurotrophic factor receptor <i>TRK B</i> and a nucleotide sequence encoding it are disclosed. Transformed host cells and transgenic animals containing nucleic acid molecules encoding full length human TRK-B are disclosed. Probes, primers and antisense molecules identical and complementary to the nucleotide sequence that encodes full length human TRK-B, particularly the intracellular portion of the receptor are disclosed. Monoclonal antibodies that bind to the full length human TRK-B protein are disclosed. Methods of using and kits which include probes, primers, antisense molecules and monoclonal antibodies are disclosed.		

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CLAIMS

1. An isolated and pure nucleic acid molecule having a nucleotide sequence of SEQ ID NO:1.
2. A recombinant vector comprising a nucleic acid molecule having a nucleotide sequence of SEQ ID NO:1.
3. The recombinant vector of claim 2 wherein said recombinant vector is an expression vector and said nucleotide sequence of SEQ ID NO:1 is operably linked to regulatory sequences.
4. A transformed host cell comprising a recombinant vector according to claim 3.
5. An isolated and pure nucleic acid molecule consisting of 5-200 nucleotides and comprising a nucleotide sequence of at least 5 nucleotides which are identical or complementary to a nucleotide sequence in nucleotides 1-158 or 1749-3707 of SEQ ID NO:1.
6. The nucleic acid molecule of claim 5 consisting of 5-100 nucleotides.
7. The nucleic acid molecule of claim 5 consisting of 8-50 nucleotides.
8. The nucleic acid molecule of claim 5 consisting of 10-20 nucleotides.
9. The nucleic acid molecule of claim 5 consisting of 12-16 nucleotides.
10. The nucleic acid molecule of claim 5 comprising a nucleotide sequence of at least 5 nucleotides which are identical to a nucleotide sequence in nucleotides 1-158 or 1749-3707 of SEQ ID NO:1.

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11. A pharmaceutical composition comprising:
a) a nucleic acid molecule according to claim
10; and,
b) a pharmaceutically acceptable carrier or
5 diluent.

12. The nucleic acid molecule of claim 5 consisting of a nucleotide sequence of at least 5 nucleotides, said nucleotide sequence being identical to a nucleotide sequence in nucleotides 1-158 or 1749-3707 of SEQ ID NO:1.

10 13. The nucleic acid molecule of claim 5 comprising a nucleotide sequence of at least 5 nucleotides which are complementary to a nucleotide sequence in nucleotides 1-158 or 1749-3707 of SEQ ID NO:1.

14. The nucleic acid molecule of claim 5 consisting of a
15 nucleotide sequence which is complementary to a nucleotide sequence a nucleotides 1-158 or 1749-3707 of SEQ ID NO:1.

15. Essentially pure and isolated protein having an amino acid sequence encoding by nucleotides 352-2817 of SEQ ID NO:1.

16. A monoclonal antibody which specifically binds to a
20 protein according to claim 15 at an epitope within the amino acid sequence encoded by nucleotides 1750-2817 of SEQ ID NO:1.

17. A method of determining whether a human neuroblastoma cell has a full length TRK-B receptor protein comprising:
a) contacting said cell with an antibody according
25 to claim 16; and,
d) detecting whether said antibody binds to a protein in said cell.

18. A kit for detecting human neuroblastoma having full length TRK-B protein comprising:

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- a) a first container containing a monoclonal antibody according to claim 16; and,
- b) a second container containing a peptide which has an epitope recognized by said monoclonal antibody;
- 5 wherein, said monoclonal antibody is a detectably labelled antibody or said kit further comprises a third container which contains detectably labelled antibodies that bind to said monoclonal antibody.
19. The kit of claim 18 wherein said monoclonal antibody
10 is a detectably labelled antibody.
20. The kit of claim 18 further comprising a third container which contains detectably labelled antibodies that bind to said monoclonal antibody.
21. A pharmaceutical composition comprising:
15 a) a monoclonal antibody according to claim 16; and,
b) a pharmaceutically acceptable carrier or diluent.
22. A set of PCR primers comprising a first
20 oligonucleotide primer and a second oligonucleotide primer, each of which is 8 to 20 nucleotides in length, wherein said first primer and said second primer each comprise a nucleotide sequence identical or complementary to a fragment of SEQ ID NO:1 and at least one of said primers consists of a nucleotide
25 sequence identical or complementary to a fragment of a nucleotide sequence unique to full length human TRK-B.
23. A kit for detecting human neuroblastoma having full length TRK-B protein comprising:
a) a first container comprising a set of PCR primers
30 according to claim 22; and
b) a second container comprising a DNA molecule equal in size to a DNA molecule that is amplified by PCR using

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said primers and cDNA generated from an RNA transcription product of a neuroblastoma cell having full length TRK-B protein.

24. A method of determining whether a human neuroblastoma
5 cell has a full length TRK-B receptor protein comprising:
detecting the presence of an RNA molecule that encodes
said full length TRK-B receptor intracellular domain.

25. The method of claim 24 wherein the presence of said
RNA sequence is detected by means selected from the group
10 consisting of:

- a) PCR amplification; and
- b) oligonucleotide hybridization.

26. The method of claim 25 wherein the presence of said
RNA sequence is detected by PCR amplification.

15 27. The method of claim 26 wherein the presence of said
RNA sequence is detected by PCR amplification using cDNA
generated from RNA of said cell; and,

PCR primers comprising a first oligonucleotide primer
and a second oligonucleotide primer, each of which is 8 to 20
20 nucleotides in length, wherein said first primer and said
second primer each comprise a nucleotide sequence identical or
complementary to a fragment of SEQ ID NO:1 and at least one of
said primers consists of a nucleotide sequence identical or
complementary to a fragment of a nucleotide sequence unique to
25 full length human TRK-B.

28. The method of claim 25 wherein the presence of said
RNA sequence is detected by oligonucleotide hybridization.

29. The method of claim 28 wherein the presence of said
RNA sequence is detected by oligonucleotide hybridization
30 comprising the steps of:

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- a) fixing RNA from cells to be tested to a solid phase;
- b) contacting a labelled oligonucleotide probe and said RNA, said probe consisting of 5-200 nucleotides and comprising a nucleotide sequence of at least 5 nucleotides which are identical to at least 5 nucleotides of unique nucleotide sequences of full length TRK-B, and
- c) detecting whether said probe hybridizes to said RNA.
- 10 30. The method of claim 28 wherein the presence of said RNA sequence is detected by oligonucleotide hybridization comprising the steps of:
- a) generating cDNA from RNA from a cell to be tested;
- 15 b) fixing said cDNA to a solid phase;
- c) contacting a labelled oligonucleotide probe and said cDNA, said probe consisting of 5-200 nucleotides and comprising a nucleotide sequence of at least 5 nucleotides which are complementary to at least 5 nucleotides of unique
- 20 nucleotide sequences of full length TRK-B; and
- d) detecting whether said probe hybridizes to said cDNA.
31. A kit for detecting human neuroblastoma having full length TRK-B protein comprising:
- 25 a) a first container containing a labelled probe having 5-200 nucleotides and comprising a nucleotide sequence of at least 5 nucleotides which are identical or complementary to at least 5 nucleotides of unique nucleotide sequences of full length TRK-B; and
- 30 b) a second container containing a DNA molecule that comprises a DNA sequence complementary to said probe.
32. The kit of claim 31 wherein said probe 5-100 nucleotides.

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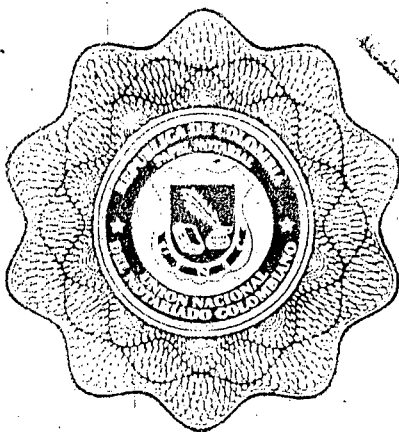
33. The kit of claim 31 wherein said probe has 8-50 nucleotides.

34. The kit of claim 31 wherein said probe has 10-20 nucleotides.

5 35. The kit of claim 31 wherein said probe has 12-16 nucleotides.

36. The kit of claim 31 wherein said probe comprises a nucleotide sequence of at least 5 nucleotides which are identical to at least 5 nucleotides of unique nucleotide
10 sequences of full length TRK-B; and

37. The kit of claim 31 wherein said probe comprises a nucleotide sequence of at least 5 nucleotides which are complementary to at least 5 nucleotides of unique nucleotide
15 sequences of full length TRK-B.



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AA 8567146

REPUBLICA DE COLOMBIA

NOTARIA 33 DE BOGOTA D.C.

ESCRITURA PUBLICA NUMERO: 1 6 5 8

- - MIL SEISCIENTOS CINCUENTA Y OCHO - -

FECHA DE OTORGAMIENTO: CUATRO (4) DE

JULIO - - - - -

DEL AÑO DOS MIL DOS (2002). -----

ACTO: -----

1.- PODER ESPECIAL.-----

OTORGADO POR: LAFRANCOL S.A., con Nit. No. - - -

890.301.463-8 - - - - -

a favor de: JOSE LUIS REYES VILLAMIZAR, con C.C.No.

79.152.473 de Usaquén - Bogotá.-----

DIANA BEATRIZ LOPEZ

NOTARIA TREINTA Y TRES (33) DE BOGOTA D.C.

EN LA CIUDAD DE BOGOTA, DISTRITO CAPITAL,

DEPARTAMENTO DE CUNDINAMARCA, REPUBLICA DE COLOMBIA,

ANTE MI, DIANA BEATRIZ LOPEZ, - - - - -

NOTARIA TREINTA Y TRES (33) - - - - -

DE ESTE CIRCULO SE OTORGA LA ESCRITURA PUBLICA QUE

SE CONSIGNA EN LOS SIGUIENTES TERMINOS:-----

COMPARECIO: JUAN MARIA RENDON, mayor de edad,

vecino de Bogotá, D.C, identificado con la cédula de

ciudadanía número 174254000C. después de

Expedida en Bogotá - confrontación con el

Y manifestó:-----

PRIMERO: Que obra en su carácter de representante

Legal de en nombre y en representación de LAFRANCOL

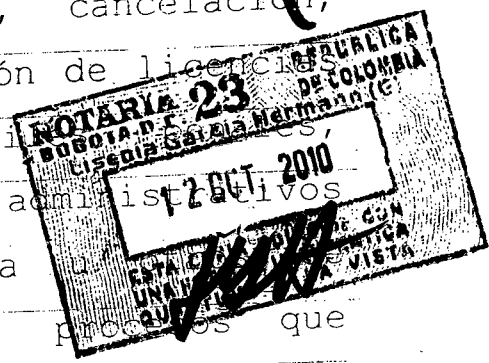
S.A, sociedad comercial organizada existente

conforme a las leyes colombianas con domicilio y

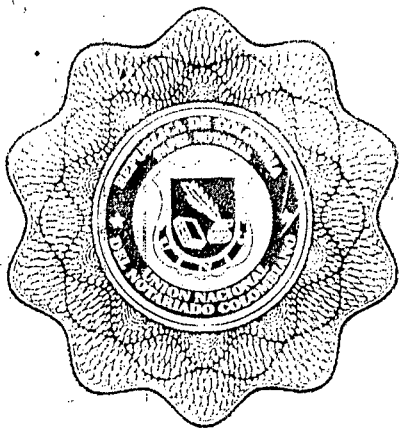
sede principal en Cali - Valle constituida mediante

escritura pública número

De la Notaría
De fecha
Reformada en distintas oportunidades, según consta en el certificado expedido por la Cámara de Comercio de Cali - Valle.-----
SEGUNDO: Que en tal carácter, por este público instrumento, concede PODER ESPECIAL, PERO AMPLIO Y SUFICIENTE a JOSE LUIS REYES VILLAMIZAR , mayor de edad, vecino de Bogotá, D.C, identificado con la cédula de ciudadanía número 79.152.473 de Usaquén, abogado en ejercicio, portador de la T.P.No. 446 55 del C.S. de la J., domiciliado en Bogotá, D.C, República de Colombia, con dirección en la carrera 17 No. 88-23, Oficina 205, de la misma ciudad, como su representante con poder especial, amplio y suficiente, para obtener la protección los derechos de propiedad industrial y de libre competencia de Lafrancol S.A., a cuyo efecto lo autorizo, par que nos represente ante cualesquiera entidad o persona, ejerza o no jurisdicción, especialmente ante las el orden administrativo, contencioso, administrativo y judicial, en todo lo concerniente a los siguientes asuntos:-----
a.- Obtención, formulación de oberservaciones y oposición a patentes de invención, modelos y dibujos industriales y el registro de licencias de uso de esos derechos.-----
b.- Las acciones de oposición, cancelación, nulidad, medidas cautelares, concesión de licencias y en general los procesos civiles, administrativos o contencioso-administrativos relacionados con estos derechos de diferente naturaleza, acciones y procesos que



DE
NOTARIA
BOGOTÁ D.C.
12 OCT 2010



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promueva Lafrancol S.A., o se promuevan contra esta y.-----

c.- Para que en todos los asuntos arriba determinados, tanto en los términos señalados por los artículos 42 y complementarios

de la decisión 486 de la Comunidad Andina como del artículo 70 del C.P.C. pueda sustituir este mandato, transigir, conciliar, admitir los hechos del proceso, desistir, cancelar, recibir, renunciar y ratificar los actos de agentes oficiosos y ceder a título gratuito y/o oneroso, licenciar, inscribir contratos de cesión y de licencia, inscripción de prendas, levantamiento de prendas, inscribir derechos de retención y en general todos los asuntos que se deban tramitar ante la Superintendencia de Industria y Comercio.-----

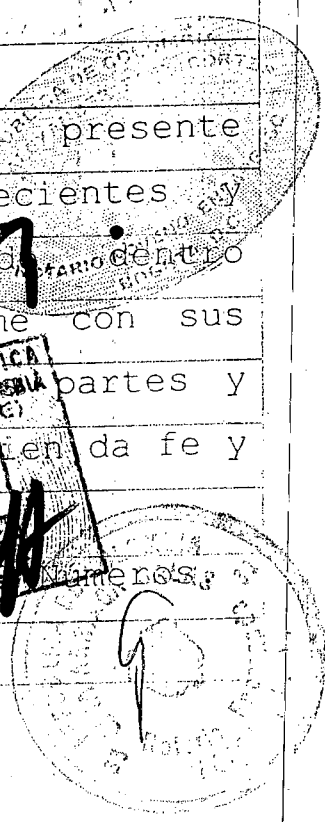
Manifestamos que de la veracidad de las declaraciones del contenido y autenticidad de los documentos anexos en éste instrumento público sólo respondemos los comparecientes como NOTARIO NOVENO DE BOGOTÁ, D.C. después de la copia por confrontación, autenticó esta copia por corresponder a la copia auténtica del documento, de la cual se ha tomado y que se ha tenido a la vista Bogotá, D.C.

HASTA AQUI LA MINUTA PRESENTADA

OTORGAMIENTO Y AUTORIZACION: Leído el presente instrumento Público por los comparecientes y advertidos de la formalidad de su contenido dentro del término legal, lo hallaron conforme con sus intenciones y lo aprobaron en todas sus partes y firmaron junto con la Suscrita, quien da fe y lo autoriza. -----

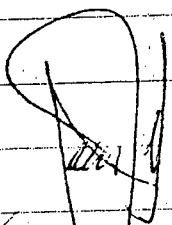
Se utilizaron las hojas de papeles numerados AA 8567146, 8567152.

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BOGOTÁ D.C. DE COLOMBIA
12 OCT. 2010
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RESOLUCION No. 4188 del 28 de Diciembre del 2001


JUAN MARIA RENDON

C.C. No. 17.125.100 de Bogotá

LAFRANCOL S.A.

Nit. No. 890.305.463-8

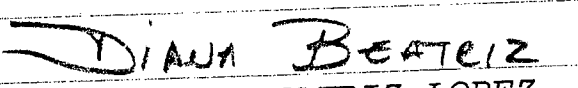
TEL: 622-04-66.

ACEPTO :


JOSE LUIS REYES VILLAMIZAR

C.C. No. 29.152.473

TEL: 621-2631


DIANA BEATRIZ LOPEZ

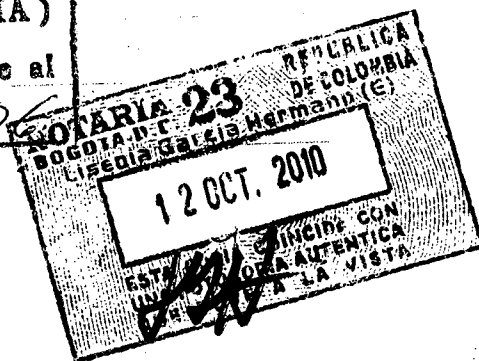
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n.c.a.

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CERTIFICADO NACIONAL

CAMARA DE COMERCIO DE CALI

FECHA: 2010/10/12

HORA: 08:41:09

OPERACION: 09TYU1012006

PAGINA: 1

CERTIFICA:

NOMBRE: LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.

SIGLA : LAFRANCOL S.A.

DOMICILIO: CALI VALLE

DIRECCION COMERCIAL : ---K 1 46 84

DIRECCION NOTIFICACION JUDICIAL: ---K 1 46 84

CIUDAD: CALI

MATRICULA MERCANTIL NRO. 5233--4 FECHA MATRICULA : 02 DE OCTOBER DE 1958

DIRECCION ELECTRONICA : lafrancolsa@lafrancol.co

DIRECCION WEB : www.lafrancol.com

AFILIADO

CERTIFICA:

NIT : 890301463-8

CERTIFICA:

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

CERTIFICA:

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

CERTIFICA:

REFORMAS

DOCUMENTO	FECHA.DOC	ORIGEN	FECHA.INS	NRO.
INS LIBRO				
E.P. 1104	30/06/1959	NOTARIA CUARTA DE CALI	07/07/1959	
19467				
E.P. 2309	26/12/1959	NOTARIA CUARTA DE CALI	29/12/1959	
20089				
E.P. 1725	24/07/1962	NOTARIA CUARTA DE CALI	26/07/1962	
24528				
E.P. 1824	31/07/1963	NOTARIA CUARTA DE CALI	14/08/1963	

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CERTIFICADO NACIONAL

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26360

E.P. 2422 25/10/1963 NOTARIA CUARTA DE CALI 28/10/1963

26717

E.P. 1720 16/09/1964 NOTARIA CUARTA DE CALI 22/09/1964

28406

E.P. 101 31/01/1966 NOTARIA CUARTA DE CALI 01/02/1966

31039

E.P. 552 21/03/1968 NOTARIA CUARTA DE CALI 01/04/1968

35711

E.P. 4964 12/12/1972 NOTARIA CUARTA DE CALI 16/01/1973

2975 IX

E.P. 1956 30/05/1973 NOTARIA CUARTA DE CALI 13/06/1973

4337 IX

E.P. 4964 12/12/1972 NOTARIA CUARTA DE CALI 28/11/1973

5994 IX

E.P. 5579 12/11/1973 NOTARIA CUARTA DE CALI 28/11/1973

5995 IX

E.P. 4406 30/09/1974 NOTARIA CUARTA DE CALI 28/11/1974

10887 IX

E.P. 192 03/02/1978 NOTARIA CUARTA DE CALI 14/02/1978

25355 IX

E.P. 3479 07/09/1979 NOTARIA CUARTA DE CALI 19/09/1979

34204 IX

E.P. 4274 12/12/1984 NOTARIA NOVENA DE CALI 19/12/1984

73088 IX

E.P. 1155 21/05/1985 NOTARIA SEPTIMA DE CALI 03/06/1985

76917 IX

E.P. 1553 20/06/1986 NOTARIA SEPTIMA DE CALI 08/07/1986

85997 IX

E.P. 1458 09/06/1987 NOTARIA SEPTIMA DE CALI 24/12/1987

3508 IX

E.P. 7222 14/12/1988 NOTARIA TERCERA DE CALI 19/12/1988

13847 IX

E.P. 4530 04/09/1989 NOTARIA TERCERA DE CALI 07/09/1989

21508 IX

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CERTIFICADO NACIONAL

CAMARA DE COMERCIO DE CALI

FECHA: 2010/10/12

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OPERACION: 09TYU1012006

PAGINA: 4

GRANEL. EN DESARROLLO DE SU OBJETO SOCIAL PODRÁ LA SOCIEDAD ADQUIRIR Y VENDER BIENES RAÍCES Y MUEBLES, DARLOS Y TOMARLOS EN ARRENDAMIENTO, GARANTIZAR CON HIPOTECA SOBRE LOS BIENES RAÍCES O CON PRENDA SOBRE LOS BIENES MUEBLES, OBLIGACIONES A CARGO DE LA SOCIEDAD; DAR Y RECIBIR A MUTUO COMERCIAL DINERO Y CELEBRAR LOS CONTRATOS COMERCIALES DE CAMBIO EN TODAS SUS MANIFESTACIONES, FUNDAR EMPRESAS QUE TENGAN O NO LA MISMA FINALIDAD SOCIAL O ASOCIARSE CON ELLAS.

CERTIFICA:

LA SOCIEDAD TIENE LOS SIGUIENTES ORGANOS PRINCIPALES: 1) LA ASAMBLEA GENERAL DE ACCIONISTAS; 2) LA JUNTA DIRECTIVA; 3) LOS REPRESENTANTES LEGALES. .
FUNCIONES DE LA ASAMBLEA GENERAL DE ACCIONISTAS ENTRE OTRAS:

A) DECLARAR ORDINARIA O EXTRAORDINARIAMENTE DISUELTA Y EN ESTADO DE LIQUIDACION A LA SOCIEDAD. B) AUTORIZAR LA VENTA, TRANSFERENCIA, ACTOS DISPOSITIVOS, DE ADMINISTRACION, O ARRIENDO, HIPOTECAR, GRAVAR, TOTAL O PARCIALMENTE LOS BIENES QUE COMPONEN SU PATRIMONIO; C) DECRETAR EL AUMENTO DE CAPITAL AUTORIZADO; D) DECRETAR LA PRORROGA DEL TERMINO DE DURACION DE LA SOCIEDAD; E) REMOVER EN CUALQUIER MOMENTO AL PRESIDENTE EJECUTIVO, A LOS MIEMBROS DE LA JUNTA DIRECTIVA O AL REVISOR FISCAL Y SUS RESPECTIVOS SUPLENTE; F) REALIZAR CUALQUIER MODIFICACION ESTATUTARIA; G) DISPONER LA ESCISION, FUSION O TRANSFORMACION DE LA SOCIEDAD; H) NOMBRAR A LOS MIEMBROS DE LA JUNTA DIRECTIVA Y EL REVISOR FISCAL DE LA SOCIEDAD Y SUS RESPECTIVOS SUPLENTE; M) AUTORIZAR LA READQUISICION DE ACCIONES POR PARTE DE LA COMPAÑIA, TOMADAS DE LAS RESERVAS QUE PARA TAL EFECTO SE CONSTITUYEN O DE LAS UTILIDADES LIQUIDAS, SIEMPRE QUE LAS ACCIONES SE ENCUENTREN LIBERADAS Y DETERMINAR, CON ARREGLO A LA LEY, LA DESTINACION POSTERIOR QUE DEBERA DARSE A ESAS ACCIONES; S) NOMBRAR Y REMOVER EL LIQUIDADOR DE LA SOCIEDAD Y SU SUPLENTE, FIJAR SU REMUNERACION Y CONSIDERAR, APROBAR O IMPROBAR SUS CUENTAS.

LA JUNTA DIRECTIVA. SE COMPONE DE TRES (3) MIEMBROS QUE SERÁN ELEGIDOS POR LA ASAMBLEA GENERAL DE ACCIONISTAS. SUS SUPLENTE SERAN PERSONALES.

FUNCIONES DE LA JUNTA DIRECTIVA ENTRE OTRAS:

A) CONVOCAR A LA ASAMBLEA GENERAL DE ACCIONISTAS A REUNIONES ORDINARIAS Y EXTRAORDINARIAS, SIEMPRE QUE LO EXIJAN LAS NECESIDADES IMPREVISTAS O URGENTES DE LA SOCIEDAD O LO SOLICITEN ACCIONISTAS QUE REPRESENTEN NO MENOS DEL VEINTE Y CINCO POR CIENTO (25%) DE LAS ACCIONES SUSCRITAS Y PAGADAS;
B) CREAR LOS CARGOS QUE JUZGUE NECESARIOS PARA LA BUENA MARCHA DE LA EMPRESA,

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CERTIFICADO NACIONAL

CAMARA DE COMERCIO DE CALI

FECHA: 2010/10/12

HORA: 08:41:09

OPERACION: 09TYU1012006

PAGINA: 5

SEÑALAR SUS FUNCIONES, FIJAR SUS ASIGNACIONES O LA FORMA DE RETRIBUCIÓN, NOMBRAR Y REMOVER LIBREMENTE A LOS EMPLEADOS DE LA SOCIEDAD, EXCEPTO AQUELLOS CUYO NOMBRAMIENTO O REMOCIÓN CORRESPONDA A LA ASAMBLEA GENERAL DE ACCIONISTAS A LAS PERSONAS QUE DEBEN CORRESPONDERLOS O LOS DESEMPEÑEN.

C) NOMBRAR, REMOVER Y FIJAR ASIGNACIONES AL PRESIDENTE EJECUTIVO, VICEPRESIDENTES Y ADMINISTRADORES QUE REPORTEN DIRECTAMENTE A LA PRESIDENCIA EJECUTIVA.

E) CONSIDERAR Y ANALIZAR LOS BALANCES DE PRUEBA, LO MISMO QUE APROBAR PREVIAMENTE A LA CELEBRACIÓN DE LA ASAMBLEA GENERAL DE ACCIONISTAS, LOS BALANCES DE PRUEBA, Y APROBAR PREVIAMENTE EL BALANCE GENERAL DE FIN DE EJERCICIO, EL INFORME DE LA ADMINISTRACIÓN Y EL PROYECTO SOBRE DISTRIBUCIÓN DE UTILIDADES O CANCELACIÓN DE PÉRDIDAS QUE SE PROPONDRÁN A LA ASAMBLEA GENERAL DE ACCIONISTAS EN SUS REUNIONES ORDINARIAS. PRESENTAR, CONJUNTAMENTE CON EL PRESIDENTE EJECUTIVO, A LA ASAMBLEA GENERAL DE ACCIONISTAS LOS BALANCES, CUENTAS E INVENTARIOS DE LA COMPAÑÍA Y PROPONER LA DISTRIBUCIÓN DE UTILIDADES.

F) FIJAR Y REGLAMENTAR CUANDO LO ESTIME CONVENIENTE EL PAGO DE INTERESES SOBRE LOS DIVIDENDOS EXIGIBLES NO COBRADOS POR LOS ACCIONISTAS.

G) DETERMINAR LA APLICACIÓN QUE DEBA DARSE A LAS UTILIDADES QUE CON EL CARÁCTER DE RESERVA DE INVERSIÓN, HAYAN SIDO APROPIADAS POR LA ASAMBLEA GENERAL DE ACCIONISTAS, PARA EL APROVECHAMIENTO DE INCENTIVOS ESTABLECIDOS POR LAS LEYES FISCALES.

H) AUTORIZAR POR VÍA GENERAL, LIBERALIDADES, BENEFICIOS O PRESTACIONES DE CARÁCTER EXTRALEGAL A FAVOR DEL PERSONAL DE LA SOCIEDAD.

I) DECIDIR EN CASO DE MORA DE UN ACCIONISTA, PARA EL PAGO DE INSTALAMENTOS PENDIENTES SOBRE ACCIONES QUE HUBIERE SUSCRITO, EL ARBITRIO DE INDEMNIZACIÓN QUE DEBA EMPLEARSE POR EL PRESIDENTE EJECUTIVO, ENTRE LOS VARIOS AUTORIZADOS POR LA LEY;

J) AUTORIZAR AL PRESIDENTE EJECUTIVO, PARA QUE SOLICITE, DE ACUERDO CON LA LEY VIGENTE, LLEGADO EL CASO, QUE SE ADMITA A LA COMPAÑÍA EN LA CELEBRACIÓN DE CONCORDATO PREVENTIVO CON SUS ACREEDORES, O REESTRUCTURACION EMPRESARIAL.

Q) CREAR LOS COMITÉS QUE ESTIME CONVENIENTES PARA ASESORAR AL PRESIDENTE EJECUTIVO Y REPRESENTANTES LEGALES DE LA SOCIEDAD EN DETERMINADOS ASUNTOS, FIJARLES SUS FUNCIONES Y SEÑALAR LA REMUNERACIÓN DE SUS MIEMBROS;

R) AUTORIZAR AL TERCER REPRESENTANTE LEGAL QUE ES EL PRESIDENTE EJECUTIVO, AL

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CUARTO Y QUINTO REPRESENTANTES LEGALES PARA COMPROMETER A LA SOCIEDAD O A LOS NEGOCIOS SOCIALES EN CUANTÍA SUPERIOR A SEIS MIL SALARIOS MINIMOS MENSUALES LEGALES VIGENTES (6,000 SMMLV) PARA CELEBRAR CONTRATOS COMERCIALES DE COMPRAVENTA CON LA NACIÓN (MINISTERIOS Y DEPARTAMENTOS ADMINISTRATIVOS), ENTIDADES DESCENTRALIZADAS (ESTABLECIMIENTOS PÚBLICOS, EMPRESAS INDUSTRIALES Y COMERCIALES DEL ESTADO, SOCIEDADES DE ECONOMÍA MIXTA), EMPRESAS SOCIALES CIVILES Y COMERCIALES, CUALQUIERA QUE SEA SU NATURALEZA CON FACULTADES PARA EXIGIR O SUSCRIBIR LOS DOCUMENTOS Y GARANTÍAS NECESARIOS HASTA LA CUANTÍA ANTES MENCIONADA Y REPRESENTAR A LA COMPAÑÍA EN CUALQUIER TRÁMITE O PROCESO JUDICIAL QUE SE ADELANTE ANTE LAS JURISDICCIONES CIVIL, LABORAL, COMERCIAL, PENAL Y CONTENCIOSO ADMINISTRATIVA EN CALIDAD DE DEMANDANTES O DEMANDADOS Y CUALQUIER OTRO TRÁMITE EN EL QUE SE REQUIERA REPRESENTAR A LA COMPAÑÍA ANTE ENTIDADES Y ORGANISMOS DEL ESTADO DE CUALQUIER ORDEN O ENTIDADES DE NATURALEZA PRIVADA.

S) AUTORIZAR AL TERCER Y CUARTO REPRESENTANTE LEGAL PARA ADQUIRIR ACTIVOS FIJOS SUPERIOR A DOSCIENTOS UNO SALARIOS MÍNIMOS MENSUALES LEGALES VIGENTES (201 SMMLV), CRÉDITOS CON ENTIDADES FINANCIERAS SUPERIORES A UN MILLÓN QUINIENTOS MIL DÓLARES AMERICANOS (US\$1.500.0000) Y DEFINIR LAS ATRIBUCIONES AL TERCER Y CUARTO REPRESENTANTE LEGAL CORRESPONDIENTE A OTRAS COMPRAS POR CONCEPTO DE ASESORÍAS Y GASTOS DIFERENTES A LOS ANTERIORES.

T) AUTORIZAR AL TERCER Y CUARTO REPRESENTANTE LEGAL DE LA COMPAÑÍA PARA QUE CELEBREN CONTRATOS DE SOCIEDAD O PARTICIPEN EN OTRAS COMPAÑÍAS COMERCIALES.

U) AUTORIZAR AL TERCER Y CUARTO REPRESENTANTE LEGAL PARA QUE SUSCRIBAN TODA CLASE DE DOCUMENTOS PÚBLICOS O PRIVADOS Y CON EL PROPÓSITO DE INSTRUMENTAR CUALQUIER TIPO DE ACTOS, NEGOCIOS JURÍDICOS, OPERACIONES DE CUALQUIER ÍNDOLE, Y SIEMPRE Y CUANDO SUPERE LA CUANTÍA PREVISTA PARA LA REALIZACIÓN DE ACTOS POR PARTE DEL REPRESENTANTE LEGAL DE LA COMPAÑÍA.

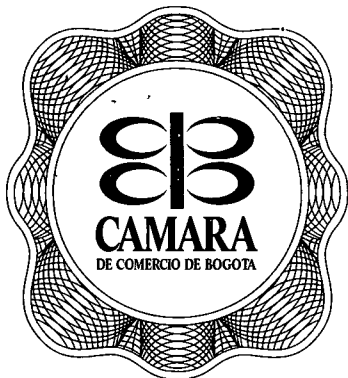
V) SOBRE LAS BASES QUE SEÑALE LA ASAMBLEA GENERAL DE ACCIONISTAS, REGLAMENTAR LA EMISIÓN Y SUSCRIPCIÓN DE BONOS.

W) AUTORIZAR LA CONSTITUCIÓN O SUPRESIÓN DE SUCURSALES O AGENCIAS.

BB) AUTORIZAR LA NOVACIÓN O CUALQUIER FORMA EXTINTIVA DE OBLIGACIONES.

CC) AUTORIZAR LA ENAJENACIÓN A CUALQUIER TÍTULO RELACIONADA CON MAQUINARIA INDUSTRIAL, TERRENOS, EDIFICIOS E INTANGIBLES Y APROBAR DONACIONES. IGUALMENTE AUTORIZARÁ LA CONSTITUCIÓN DE GARANTÍAS HIPOTECARIAS, PRENDARIAS O DE CUALQUIER OTRA ÍNDOLE SOBRE DICHOS ACTIVOS.

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DD) EMITIR ACCIONES DEL CAPITAL AUTORIZADO POR SUSCRIBIR.
REPRESENTACION LEGAL. ADMINISTRACION. LA ADMINISTRACIÓN DE LA SOCIEDAD
ESTA A CARGO DE LOS ACCIONISTAS, QUIENES LA DELEGAN EN EL PRESIDENTE
EJECUTIVO, TERCER REPRESENTANTE LEGAL DE LA COMPAÑÍA, ASI COMO LA
ASI COMO LA GESTIÓN Y EJECUCIÓN DE LOS NEGOCIOS Y ASUNTOS SOCIALES. EL
PRESIDENTE EJECUTIVO DE LA SOCIEDAD, SERÁ DESIGNADO POR LA JUNTA DIRECTIVA
PARA PERÍODOS DE UN (1) AÑO, SIN PERJUICIO DE SU LIBRE REMOCIÓN Y REELECCIÓN
INDEFINIDA.

EL PRESIDENTE Y VICEPRESIDENTE DE LA JUNTA DIRECTIVA, SERÁ TAMBIÉN
REPRESENTANTE LEGAL PRIMERO Y SEGUNDO DE LA SOCIEDAD. LAS ATRIBUCIONES
INHERENTES A LA CALIDAD DE REPRESENTANTES LEGALES, DE ESTOS CARGOS Y EN
ESPECIAL LAS DISPOSITIVAS, SERÁN EJERCIDAS SIN LAS LIMITACIONES ESTABLECIDAS
EN ÉSTOS ESTATUTOS.

EL CUARTO Y QUINTO REPRESENTANTE LEGAL, SERÁN REPRESENTANTES LEGALES DE LA
SOCIEDAD QUIENES SON ELEGIDOS POR LA JUNTA DIRECTIVA CON SUS LIMITACIONES EN
SUS FUNCIONES.

LAS ATRIBUCIONES INHERENTES A LA CALIDAD DEL CUARTO REPRESENTANTE LEGAL, Y EN
ESPECIAL LAS DISPOSITIVAS, SERÁN EJERCIDAS CON SUJECCIÓN A LAS LIMITACIONES
ESTABLECIDAS EN ÉSTOS ESTATUTOS PARA EL PRESIDENTE EJECUTIVO QUIEN ES EL
TERCER REPRESENTANTE LEGAL.

EXISTIRÁ UN QUINTO REPRESENTANTE LEGAL DE LA SOCIEDAD QUE TENDRÁ LIMITACIÓN EN
SUS FUNCIONES CUANDO SE TRATE DE COMPROMETER A LA SOCIEDAD O A LOS NEGOCIOS
SOCIALES EN CUANTÍA NO SUPERIOR A SEIS MIL SALARIOS MINIMOS MENSUALES LEGALES
VIGENTES (6,000 SMLV) PARA CELEBRAR CONTRATOS COMERCIALES DE COMPRAVENTA CON
LA NACIÓN (MINISTERIOS Y DEPARTAMENTOS ADMINISTRATIVOS), ENTIDADES
DESCENTRALIZADAS (ESTABLECIMIENTOS PÚBLICOS, EMPRESAS INDUSTRIALES Y
COMERCIALES DEL ESTADO, SOCIEDADES DE ECONOMÍA MIXTA), EMPRESAS SOCIALES
CIVILES Y COMERCIALES, CUALQUIERA QUE SEA SU NATURALEZA CON FACULTADES PARA
EXIGIR O SUSCRIBIR LOS DOCUMENTOS Y GARANTÍAS NECESARIOS HASTA LA CUANTÍA
ANTES MENCIONADA Y REPRESENTAR A LA COMPAÑÍA EN CUALQUIER TRÁMITE O PROCESO

JUDICIAL QUE SE ADELANTE ANTE LAS JURISDICCIONES CIVIL, LABORAL, COMERCIAL,
PENAL Y CONTENCIOSO ADMINISTRATIVA EN CALIDAD DE DEMANDANTES O DEMANDADOS Y
CUALQUIER OTRO TRÁMITE EN EL QUE SE REQUIERA REPRESENTAR A LA COMPAÑÍA ANTE
ENTIDADES Y ORGANISMOS DEL ESTADO DE CUALQUIER ORDEN O ENTIDADES DE NATURALEZA
PRIVADA.

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FUNCIONES DEL PRESIDENTE EJECUTIVO. EL PRESIDENTE EJECUTIVO DE LA SOCIEDAD ES UN MANDATARIO CON REPRESENTACIÓN, INVESTIDO DE FACULTADES EJECUTIVAS Y ADMINISTRATIVAS, Y COMO TAL, TIENE A SU CARGO LA TERCERA REPRESENTACIÓN LEGAL, LA GESTIÓN COMERCIAL Y FINANCIERA, LA RESPONSABILIDAD DE LA ACCIÓN ADMINISTRATIVA, LA COORDINACIÓN Y SUPERVISIÓN GENERAL DE LA EMPRESA, LAS CUALES CUMPLIRÁ CON ARREGLO A LAS NORMAS DE ESTOS ESTATUTOS Y LAS DISPOSICIONES LEGALES, Y CON SUJECCIÓN A LAS ÓRDENES E INSTRUCCIONES DE LA JUNTA DIRECTIVA. ADEMÁS DE LAS FUNCIONES ANTES INDICADAS, CORRESPONDE AL PRESIDENTE EJECUTIVO DE LA SOCIEDAD: A) COMPROMETER A LA SOCIEDAD O A LOS NEGOCIOS SOCIALES EN CUANTÍA NO SUPERIOR A SEIS MIL SALARIOS MÍNIMOS MENSUALES LEGALES VIGENTES (6,000 SMLV) PARA CELEBRAR CONTRATOS COMERCIALES DE COMPRAVENTA CON LA NACIÓN (MINISTERIOS Y DEPARTAMENTOS ADMINISTRATIVOS), ENTIDADES DESCENTRALIZADAS (ESTABLECIMIENTOS PÚBLICOS, EMPRESAS INDUSTRIALES Y COMERCIALES DEL ESTADO, SOCIEDADES DE ECONOMÍA MIXTA), EMPRESAS SOCIALES CIVILES Y COMERCIALES, CUALQUIERA QUE SEA SU NATURALEZA CON FACULTADES PARA EXIGIR O SUSCRIBIR LOS DOCUMENTOS Y GARANTÍAS NECESARIOS HASTA LA CUANTÍA ANTES MENCIONADA Y REPRESENTAR A LA COMPAÑÍA EN CUALQUIER TRÁMITE O PROCESO JUDICIAL QUE SE ADELANTE ANTE LAS JURISDICCIONES CIVIL, LABORAL, COMERCIAL, PENAL Y CONTENCIOSO ADMINISTRATIVA EN CALIDAD DE DEMANDANTES O DEMANDADOS Y CUALQUIER OTRO TRÁMITE EN EL QUE SE REQUIERA REPRESENTAR A LA COMPAÑÍA ANTE ENTIDADES Y ORGANISMOS DEL ESTADO DE CUALQUIER ORDEN O ENTIDADES DE NATURALEZA PRIVADA; ADQUISICIÓN DE ACTIVOS FIJOS NO SUPERIOR A DOSCIENTOS UNO SALARIOS MÍNIMOS MENSUALES LEGALES VIGENTES (201 SMLV); OBTENER CRÉDITOS CON ENTIDADES FINANCIERAS NO SUPERIORES A UN MILLÓN QUINIENTOS MIL DÓLARES AMERICANOS (US\$1.500.0000) Y CONTRATAR OTRAS COMPRAS POR CONCEPTO DE ASESORÍAS Y GASTOS DIFERENTES A LOS ANTERIORES PREVIA APROBACIÓN DE LA JUNTA DIRECTIVA. B) CUMPLIR Y HACER CUMPLIR LAS DECISIONES DE LA ASAMBLEA GENERAL DE ACCIONISTAS Y DE LA JUNTA DIRECTIVA Y HACER USO DE LA RAZÓN SOCIAL. C) REPRESENTAR LEGALMENTE A LA SOCIEDAD CONFORME A LA LEY ANTE TODAS LAS AUTORIDADES CIVILES, JUDICIALES, MILITARES, SANITARIAS, DE POLICÍAS ADUANALES, POSTALES, ETC. D) ORGANIZAR LOS SERVICIOS Y LAS OFICINAS DE LA SOCIEDAD, LA CONTABILIDAD Y APLICAR LOS ESTATUTOS Y REGLAMENTOS. E) LLEVAR A CABO LA GESTIÓN COMERCIAL Y FINANCIERA. F) TENDRÁ LA PLENA RESPONSABILIDAD DE LA ACCIÓN ADMINISTRATIVA, LA COORDINACIÓN Y LA SUPERVISIÓN GENERAL DE LA SOCIEDAD, LAS CUALES CUMPLIRÁ CON ARREGLO A LAS NORMAS DE ESTOS ESTATUTOS. G)

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COMPARECER EN LOS JUICIOS, TRANSIGIR Y COMPROMETER LOS NEGOCIOS SOCIALES DE CUALESQUIERA NATURALEZA QUE FUEREN, INTERPONER TODO GÉNERO DE RECURSOS Y CONSTITUIR APODERADOS QUE LO REPRESENTEN JUDICIAL O EXTRAJUDICIALMENTE. I) HACER DEPÓSITOS EN BANCOS Y AGENCIAS BANCARIAS, FIRMAR CHEQUES, LETRAS, PAGARÉS, GIROS, LIBRANZAS Y CUALQUIER OTRO DOCUMENTO, ASÍ COMO NEGOCIAR ESTOS INSTRUMENTOS, TENERLOS, COBRARLOS Y PAGARLOS, DESCARGARLOS, ETC. J) NOMBRAR Y REMOVER LIBREMENTE A LOS EMPLEADOS DISTINTOS DE LOS QUE LA JUNTA DIRECTIVA TENGA ATRIBUCIONES. K) PRESENTAR A LA ASAMBLEA GENERAL DE ACCIONISTAS, EN SUS REUNIONES ORDINARIAS, EL INFORME SOBRE LA FORMA COMO HAYA LLEVADO A CABO SU GESTIÓN E INNOVACIONES QUE CONVENGA INTRODUCIR PARA EL MEJOR SERVICIO DE SUS INTERESES. L) SUSCRIBIR TODA CLASE DE DOCUMENTOS PÚBLICOS O PRIVADOS, CUANDO LA JUNTA DIRECTIVA LO AUTORICE Y CON EL PROPOSITO DE INSTRUMENTAR CUALQUIER TIPO DE ACTOS, NEGOCIOS JURÍDICOS, OPERACIONES DE CUALQUIER ÍNDOLE, Y SIEMPRE Y CUANDO NO SUPERE LA CUANTÍA PREVISTA EN ESTOS ESTATUTOS COMO LÍMITE DEL EJERCICIO DE SUS FUNCIONES, Y POR ENCIMA DE LA CUAL REQUIERE AUTORIZACIÓN DE LA JUNTA DIRECTIVA. M) PODRÁ EJERCER LA REPRESENTACIÓN LEGAL, SIN PERJUICIO DE LA MISMA FUNCIÓN ASIGNADA A LOS OTROS REPRESENTANTES LEGALES PARA OTORGAR PODERES A TERCEROS, NOMBRAR ABOGADOS Y PROCURADORES, INTERPONER RECLAMACIONES EN LA VÍA GUBERNATIVA, ANTE TRIBUNALES, JURISDICCIONES ORDINARIAS, LABORALES Y ESPECIALES, REPRESENTAR A LA SOCIEDAD ANTE ADMINISTRACIONES O HACIENDAS PÚBLICAS, PRIVADAS, NACIONALES, INTERNACIONALES Y AUTONÓMICAS, CONCURRIR A TODA CLASE DE SUBASTAS, CONCURSOS O SUMINISTROS OFICIALES O PARTICULARES, PUDIENDO POR TANTO REDACTAR, SUSCRIBIR, PRESENTAR Y, EN SU CASO, MEJORAR EN LICITACIÓN VERBAL O ESCRITA LAS OFERTAS O PROPOSICIONES PERTINENTES, ACEPTAR DONACIONES PARA LA SOCIEDAD Y ACEPTAR CON BENEFICIO DE INVENTARIO, LEGADOS, EJERCITAR ACCIONES O PONER EXCEPCIONES, PARA ACTUAR EN CALIDAD DE DEMANDANTE, DEMANDADO O TERCERO INTERVINIENTE. N) ORDENAR LOS PAGOS A REALIZAR POR LA SOCIEDAD PARA QUE EL TESORERO O QUIEN HAGA SUS VECES, EXPIDA LAS ÓRDENES DE PAGO USANDO EL MEDIO MÁS EXPEDITO PARA CONTAR CON LA CELERIDAD QUE LOS NEGOCIOS SOCIALES EXIGEN; TODO ELLO DENTRO DE LAS LIMITACIONES ESTABLECIDAS EN ESTOS ESTATUTOS.

REVISOR FISCAL. LA DESIGNACIÓN DEL REVISOR FISCAL Y SUS RESPECTIVOS SUPLENTE CORRESPONDE A LA ASAMBLEA GENERAL DE ACCIONISTAS Y SERÁ ELEGIDO POR LA MAYORÍA ABSOLUTA DE LOS ACCIONISTAS PRESENTES.

CERTIFICA:

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CERTIFICADO NACIONAL

CAMARA DE COMERCIO DE CALI

FECHA: 2010/10/12

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OPERACION: 09TYU1012006

PAGINA: 10

DOCUMENTO: ACTA No. 116 DEL 04 DE MARCH DE 2010

ORIGEN: ASAMBLEA GRAL ORDINARIA

INSCRIPCION: 11 DE MAY DE 2010 No. 5472 DEL LIBRO IX

FUE(RON) NOMBRADO(S):

PRESIDENTE DE LA JUNTA DIRECTIVA Y REPRESENTANTE LEGAL

ESTHER ISABEL VENTURA DE RENDON

C.C.38979831

VICEPRESIDENTE DE LA JUNTA DIRECTIVA Y SEGUNDO REPRESENTANTE LEGAL

JUAN MARIA RENDON GUTIERREZ

C.C.17125100

PRESIDENTE EJECUTIVO - TERCER REPRESENTANTE LEGAL

JUAN CAMILO PALACIO RAMIREZ

C.C.19455622

CUARTO REPRESENTANTE LEGAL

ROBERTO MAURICE VENTURA CRISPINO

C.C.9086003

QUINTO REPRESENTANTE LEGAL

JUAN PABLO REYES VILLAMIZAR

C.C.80419709

CERTIFICA:

DOCUMENTO: ACTA No. 116 DEL 04 DE MARCH DE 2010

ORIGEN: ASAMBLEA GRAL ORDINARIA

INSCRIPCION: 11 DE MAY DE 2010 No. 5471 DEL LIBRO IX

FUE(RON) NOMBRADO(S)

JUNTA DIRECTIVA

PRINCIPALES

PRIMER RENGLON

ESTHER ISABEL VENTURA DE RENDON

C.C.38979831

SEGUNDO RENGLON

JUAN MARIA RENDON GUTIERREZ

C.C.17125100

TERCER RENGLON

LUIS ALFONSO MORA TEJADA

C.C.4610260

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OPERACION: 09TYU1012006

PAGINA: 11

SUPLENTE

PRIMER RENGLON

JUAN MANUEL RENDON VENTURA

C.C.79939921

SEGUNDO RENGLON

ADRIANA RENDON DE ANTORCHA

C.C.66901857

TERCER RENGLON

CARLOS ANTONIO ESPINOSA PEREZ

C.C.16684716

CERTIFICA:

DOCUMENTO: ACTA No. 118 DEL 29 DE JUNE DE 2010

ORIGEN: ASAMBLEA DE ACCIONISTAS

INSCRIPCION: 12 DE AUGUST DE 2010 No. 9529 DEL LIBRO IX

FUE(RON) NOMBRADO(S):

REVISOR FISCAL FIRMA

DELOITTE & TOUCHE LTDA D & T LTDA

NIT.860005813-4

CERTIFICA:

DOCUMENTO: DOCUMENTO PRIVADO DEL 02 DE AUGUST DE 2010

ORIGEN: DELOITTE & TOUCHE LTDA D & T LTDA

INSCRIPCION: 12 DE AUGUST DE 2010 No. 9530 DEL LIBRO IX

FUE(RON) NOMBRADO(S):

REVISOR FISCAL PRINCIPAL

LEANDRO FELIPE PAZ AGELVIS

C.C.14700811

REVISOR FISCAL SUPLENTE

HEYMAN ANTONIO GIL BALCAZAR

C.C.16796791

CERTIFICA:

CAPITAL AUTORIZADO: \$20,000,000,000

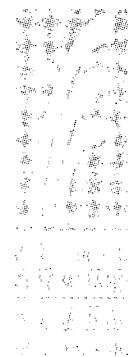
NUMERO DE ACCIONES: 2,000,000,000

VALOR NOMINAL: \$10

CAPITAL SUSCRITO: \$12,545,212,380

NUMERO DE ACCIONES: 1,254,521,238

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PAGINA: 12

VALOR NOMINAL: \$10

CAPITAL PAGADO: \$12,545,212,380

NUMERO DE ACCIONES: 1,254,521,238

VALOR NOMINAL: \$10

CERTIFICA:

DOCUMENTO: DOCUMENTO PRIVADO DEL 03 DE DICIEMBRE DE 2009

INSCRIPCIÓN: 28 DE DICIEMBRE DE 2009 BAJO EL NRO. 14869 DEL LIBRO IX

CONSTA LA SITUACIÓN DE CONTROL:

CONTROLANTE: DORAL INVESTMENTS INTERNATIONAL INC

DOMICILIO: ISLAS VIRGENES BRITÁNICAS

ACTIVIDAD: OTRAS ACTIVIDADES EMPRESARIALES

CONTROLADA: LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.

NIT: 890301463-8

DOMICILIO: CALI VALLE

NACIONALIDAD: COLOMBIANA

ACTIVIDAD: LA FABRICACIÓN, DISTRIBUCIÓN Y VENTA EN EL TERRITORIO DE LA REPÚBLICA DE COLOMBIA O EN EL EXTRANJERO DE CUALESQUIERA TIPOS DE VACUNAS, PRODUCTOS QUÍMICOS, FARMACÉUTICOS, ALIMENTOS FUNCIONALES, COSMÉTICOS Y FISIOLÓGICOS, REACTIVOS, ANTIBIÓTICOS PARA USO HUMANO Y VETERINARIO, PRODUCTOS DIETÉTICOS, COLORANTES DE PERFUMERÍA Y AFINES.

PRESUPUESTO DE CONTROL: POSEE PARTICIPACIÓN ACCIONARIA MAYOR AL 50% A PARTIR DEL 10 DE NOVIEMBRE DE 2009.

CERTIFICA:

DOCUMENTO: DOCUMENTO PRIVADO DEL 30 DE ABRIL DE 2004

INSCRIPCIÓN: 03 DE MAYO DE 2004 No. 4859 DEL LIBRO IX

DOCUMENTO: DOCUMENTO PRIVADO DEL 29 DE ABRIL DE 2005

INSCRIPCIÓN: 06 DE MAYO DE 2005 No. 4983 DEL LIBRO IX

DOCUMENTO: DOCUMENTO PRIVADO DEL 15 DE JUNIO DE 2006

INSCRIPCIÓN: 21 DE JUNIO DE 2006 No. 7526 DEL LIBRO IX

DOCUMENTO: DOCUMENTO PRIVADO DEL 01 DE NOVIEMBRE DE 2006

INSCRIPCIÓN: 28 DE NOVIEMBRE DE 2006 No. 13380 DEL LIBRO IX

DOCUMENTO: DOCUMENTO PRIVADO DEL 19 DE DICIEMBRE DE 2006

INSCRIPCIÓN: 21 DE DICIEMBRE DE 2006 No. 14301 DEL LIBRO IX

DOCUMENTO: DOCUMENTO PRIVADO DEL 19 DE DICIEMBRE DE 2007

INSCRIPCIÓN: 21 DE DICIEMBRE DE 2007 No. 13604 DEL LIBRO IX

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PAGINA: 14

NACIONALIDAD:COLOMBIA

PRESUPUESTO DE CONTROL:NUMERAL 1, ARTÍCULO 27 DE LA LEY 222 DE 1995.

ACTIVIDAD: DISTRIBUCIÓN Y COMERCIALIZACIÓN DE PRODUCTOS FARMACÉUTICOS DE USO HUMANO.

SUBORDINADA

LAFRANCOL INTERNACIONAL S.A.S.

NIT. 815003912-2

DOMICILIO:PALMIRA VALLE

NACIONALIDAD:COLOMBIA

PRESUPUESTO DE CONTROL: NUMERAL 3, ARTICULO 27 DE LA LEY 222 DE 1995.

ACTIVIDAD: REALIZACIÓN DE ACTIVIDADES DESDE LA ZONA FRANCA, LAS CUALES CONSISTEN EN LA PRESTACIÓN DE TODO TIPO DE SERVICIOS ASÍ COMO DISTRIBUIR, VENDER, COMPRAR, IMPORTAR, EXPORTAR Y ALMACENAR TODO TIPO DE MATERIAS PRIMAS, PRODUCTOS TERMINADOS Y SEMITERMINADOS RELACIONADOS CON LA INDUSTRIA DE ALIMENTOS, COSMÉTICA Y FARMACÉUTICA.

SUBORDINADA

LAFRANCOL PERÚ S.A

DOMICILIO:LIMA

NACIONALIDAD:PERU

PRESUPUESTO DE CONTROL: NUMERAL 1, ARTÍCULO 27 DE LA LEY 222 DE 1995.

ACTIVIDAD: DISTRIBUCIÓN Y COMERCIALIZACIÓN DE PRODUCTOS FARMACÉUTICOS DE USO HUMANO.

SUBORDINADA

LABORATORIOS PAULY PHARMACEUTICAL S.A.S.

NIT. 800097402-6

DOMICILIO:CALI VALLE

NACIONALIDAD:COLOMBIA

PRESUPUESTO DE CONTROL:NUMERAL 1, ARTÍCULO 27 DE LA LEY 222 DE 1995.

ACTIVIDAD: DISTRIBUCIÓN Y COMERCIALIZACIÓN DE PRODUCTOS FARMACÉUTICOS DE USO HUMANO.

SUBORDINADA

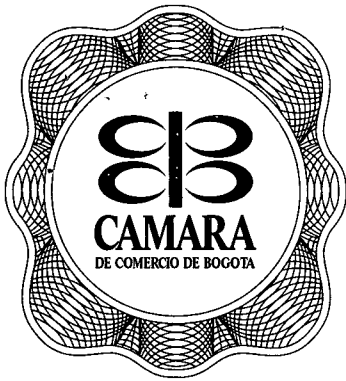
LAFRANCOL (USA) CORP

DOMICILIO:ESTADOS UNIDOS

NACIONALIDAD:ESTADOS UNIDOS

PRESUPUESTO DE CONTROL:NUMERAL 1, ARTICULO 27 DE LA LEY 222 DE 1995.

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CERTIFICADO NACIONAL

CAMARA DE COMERCIO DE CALI

FECHA: 2010/10/12

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ACTIVIDAD: DISTRIBUCIÓN Y COMERCIALIZACIÓN DE PRODUCTOS FARMACÉUTICOS DE USO HUMANO.

SUBORDINADA

GAMAR SOCIEDAD S A S

NIT. 890312050-7

DOMICILIO: CALI VALLE

NACIONALIDAD: COLOMBIA

PRESUPUESTO DE CONTROL: NUMERAL 1, ARTICULO 27 DE LA LEY 222 DE 1995.

ACTIVIDAD: TODAS LAS ACTIVIDADES RELACIONADAS CON LAS ARTES GRÁFICAS.

SUBORDINADA

LAFRANCOL GUATEMALA S.A.

NIT. 4088707

DOMICILIO: GUATEMALA

NACIONALIDAD: GUATEMALA

PRESUPUESTO DE CONTROL: NUMERAL 1, ARTÍCULO 27 DE LA LEY 222 DE 1995.

ACTIVIDAD: PRODUCCIÓN, FABRICACIÓN, EXPORTACIÓN Y DISTRIBUCIÓN DE PRODUCTOS FARMACÉUTICOS Y/O ALIMENTICIOS Y NATURALES DE CONSUMO HUMANO Y LA COMERCIALIZACIÓN DE TODA CLASE DE MERCANCÍAS POR CUENTA PROPIA O EN NOMBRE DE TERCEROS.

SUBORDINADA

DISTRIBUCIONES UQUIFA S A S

NIT. 800182021-7

DOMICILIO: CALI VALLE

NACIONALIDAD: COLOMBIA

PRESUPUESTO DE CONTROL: NUMERAL 1, ARTÍCULO 27 DE LA LEY 222 DE 1995.

ACTIVIDAD: DISTRIBUCIÓN Y COMERCIALIZACIÓN DE PRODUCTOS FARMACÉUTICOS Y ALIMENTOS FUNCIONALES DE CONSUMO HUMANO.

SUBORDINADA

LABORATORIOS NATURMEDIK S A S

NIT. 800226363-1

DOMICILIO: CALI VALLE

NACIONALIDAD: COLOMBIA

PRESUPUESTO DE CONTROL: NUMERAL 1, ARTÍCULO 27 DE LA LEY 222 DE 1995.

ACTIVIDAD: DISTRIBUCIÓN Y COMERCIALIZACIÓN DE PRODUCTOS FARMACÉUTICOS DE USO HUMANO.

*** CONTINUA ***

CERTIFICADO NACIONAL

CAMARA DE COMERCIO DE CALI

FECHA: 2010/10/12

HORA: 08:41:09

OPERACION: 09TYU1012006

PAGINA: 16

SUBORDINADA

LAFRANCOL DOMINICANA S.A.

DOMICILIO:SANTO DOMINGO REPÚBLICA DOMINICANA

NACIONALIDAD:REPÚBLICA DOMINICANA

PRESUPUESTO DE CONTROL:NUMERAL 1, ARTÍCULO 27 DE LA LEY 222.

ACTIVIDAD:FABRICACIÓN DE PRODUCTOS FARMACÉUTICOS.

SUBORDINADA

LABORATORIO FRANCO-COLOMBIANO DEL ECUADOR S.A.

DOMICILIO: GUAYAQUIL, REPÚBLICA DEL ECUADOR

NACIONALIDAD: ECUATORIANA

PRESUPUESTO DE CONTROL: NUMERAL 1, ARTICULO 27 DE LA LEY 222 DE 1995. LA FECHA DE LA PRIMERA TRANSACCION COMERCIAL FUE EN JUNIO DE 2009 Y LA CAPITALIZACION SE HIZO EN OCTUBRE DE 2009.

ACTIVIDAD: DEDICARSE A LA FABRICACIÓN, PRODUCCIÓN, EMPAQUE, COMERCIALIZACIÓN, DISTRIBUCIÓN, COMPRAVENTA, IMPORTACIÓN, EXPORTACIÓN Y/O REPRESENTACIÓN DE TODO TIPO DE PRODUCTOS FARMACÉUTICOS, HIGIÉNICOS, COSMÉTICOS, ALIMENTICIOS, DIETÉTICOS Y BAJOS EN CALORÍAS, VETERINARIOS, APARATOS E INSTRUMENTOS QUIRÚRGICOS, MÉDICOS, DENTALES Y VETERINARIOS. PODRÁ ADQUIRIR ACCIONES, PARTICIPACIONES O CUOTAS SOCIALES DE CUALQUIER CLASE DE COMPAÑÍA, SEA NACIONAL O EXTRANJERA, PUDIENDO INTERVENIR EN LA FUNDACIÓN O AUMENTO DE CAPITAL DE OTRAS SOCIEDADES QUE TENGAN OBJETIVOS SOCIALES RELACIONADOS CON EL SUYO. PODRÁ CONSTRUIR, ADQUIRIR EN PROPIEDAD, ARRENDAMIENTO O EN ASOCIACIÓN TODA CLASE DE PLANTAS INDUSTRIALES PARA PRODUCCIÓN, EMPAQUE, PROCESAMIENTO O CUALQUIER OTRA FORMA PARA LA COMERCIALIZACIÓN EN EL MERCADO INTERNO Y EXTERNO DE PRODUCTOS FARMACÉUTICOS YA SEA PARA EL CONSUMO HUMANO, VEGETAL O ANIMAL.

SUBORDINADA

FOCUS PHARMACEUTICAL S.A.

NIT: 900.104.555-8

DOMICILIO: BOGOTÁ D.C.

NACIONALIDAD: COLOMBIANA

PRESUPUESTO DE CONTROL: NUMERAL 1, ARTICULO 27 DE LA LEY 222 DE 1995. LA FECHA DE LA PRIMERA TRANSACCION COMERCIAL FUE EN ENERO DE 2010 Y LA CAPITALIZACION SE HIZO EN ENERO DE 2010.

ACTIVIDAD: EL OBJETO PRINCIPAL DE LA SOCIEDAD ES EL DESARROLLO, FABRICACIÓN O PRODUCCIÓN, DIRECTAMENTE O A TRAVÉS DE TERCEROS EN CUALQUIER MODALIDAD

*** CONTINUA ***



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CERTIFICADO NACIONAL

CAMARA DE COMERCIO DE CALI

FECHA: 2010/10/12

HORA: 08:41:09

OPERACION: 09TYU1012006

PAGINA: 17

NEGOCIAL, LA COMERCIALIZACIÓN Y DISTRIBUCIÓN, NACIONAL O INTERNACIONAL, DE TODA CLASE DE ARTÍCULOS DE GÉNERO FARMACÉUTICO, ESPECÍFICAMENTE DE MEDICAMENTOS ESA ACTIVIDAD PRINCIPAL CONLLEVA LA HABILITACIÓN PARA DESARROLLAR TODA OTRA CONEXA O NECESARIA, INCLUYENDO DE MANERA ESPECIAL PERO NO LIMITATIVA, LA INVESTIGACIÓN FARMACÉUTICA, EL DESARROLLO DE PRODUCTOS MÉDICOS Y FARMACÉUTICOS, EQUIPOS MÉDICOS, TRATAMIENTOS ALTERNATIVOS Y EN GENERAL, TODO AQUELLO VINCULADO A LA PROTECCIÓN DE LA SALUD HUMANA.

PODRÁ EFECTUAR LA INVERSIÓN EN BIENES INMUEBLES, URBANOS O RURALES Y LA ADQUISICIÓN, ADMINISTRACIÓN, ARRENDAMIENTO, GRAVAMEN Y ENAJENACIÓN DE LOS MISMOS.

LA INVERSIÓN DE FONDOS PROPIOS, EN BIENES INMUEBLES, BONOS, VALORES BURSÁTILES Y PARTES DE INTERÉS EN SOCIEDADES COMERCIALES, ASÍ COMO LA NEGOCIACIÓN DE TODA CLASE DE DERECHOS DE CRÉDITO.

CERTIFICA:

QUE A NOMBRE DE LA SOCIEDAD FIGURA MATRICULADO EN LA CAMARA DE COMERCIO BAJO EL NRO.5234- 2 ESTABLECIMIENTO DE COMERCIO: LABORATORIO FRANCO COLOMBIANO LAFRANCOL

UBICADO EN: ---K 1 46 84 DE CALI
RENOVO : POR EL AÑO 2010

CERTIFICA:

QUE A NOMBRE DE LA SOCIEDAD FIGURA MATRICULADO EN LA CAMARA DE COMERCIO BAJO EL NRO.369809- 2 ESTABLECIMIENTO DE COMERCIO: LABORATORIO FRANCO COLOMBIANO LAFRANCOL

UBICADO EN: --K 1 46A 70 DE CALI
FECHA MATRICULA : 29 DE ABRIL DE 1994
RENOVO : POR EL AÑO 2010

CERTIFICA:

QUE A NOMBRE DE LA SOCIEDAD FIGURA MATRICULADO EN LA CAMARA DE COMERCIO BAJO EL NRO.369810- 2 ESTABLECIMIENTO DE COMERCIO: LABORATORIO FRANCO COLOMBIANO LAFRANCOL

UBICADO EN: ---K 1A 46 A 32 DE CALI
FECHA MATRICULA : 29 DE ABRIL DE 1994
RENOVO : POR EL AÑO 2010

CERTIFICA:

QUE A NOMBRE DE LA SOCIEDAD FIGURA MATRICULADO EN LA CAMARA DE COMERCIO BAJO

*** CONTINUA ***

RECIBI
2010/10/12
08:41:09
09TYU1012006

CERTIFICADO NACIONAL

CAMARA DE COMERCIO DE CALI

FECHA: 2010/10/12

HORA: 08:41:09

OPERACION: 09TYU1012006

PAGINA: 18

EL NRO.734908- 2 ESTABLECIMIENTO DE COMERCIO: LABORATORIO FRANCO COLOMBIANO
LAFRANCOL

UBICADO EN: CRA. 1A NRO. 46A 28 DE CALI

FECHA MATRICULA : 28 DE MARCH DE 2008

RENOVO : POR EL AÑO 2010

CERTIFICA:

QUE A NOMBRE DE LA SOCIEDAD FIGURA MATRICULADO EN LA CAMARA DE COMERCIO BAJO
EL NRO.734911- 2 ESTABLECIMIENTO DE COMERCIO: LABORATORIO FRANCO COLOMBIANO
LAFRANCOL

UBICADO EN: CRA. 1B NRO. 46A 121 DE CALI

FECHA MATRICULA : 28 DE MARCH DE 2008

RENOVO : POR EL AÑO 2010

CERTIFICA:

QUE A NOMBRE DE LA SOCIEDAD FIGURA MATRICULADO EN LA CAMARA DE COMERCIO BAJO
EL NRO.734913- 2 ESTABLECIMIENTO DE COMERCIO: LABORATORIO FRANCO COLOMBIANO
LAFRANCOL

UBICADO EN: CRA. 3 NRO. 47 25 DE CALI

FECHA MATRICULA : 28 DE MARCH DE 2008

RENOVO : POR EL AÑO 2010

CERTIFICA:

QUE LA SOCIEDAD EFECTUO LA RENOVACION DE SU MATRICULA MERCANTIL EL 25 DE
MARCH DE 2010 .

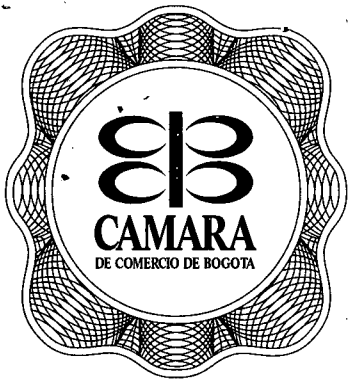
CERTIFICA:

QUE NO FIGURAN OTRAS INSCRIPCIONES QUE MODIFIQUEN TOTAL O PARCIALMENTE EL
PRESENTE CERTIFICADO.

LOS ACTOS Y DOCUMENTOS REGISTRADOS QUEDAN EN FIRME CINCO (5) DIAS HABILES
DESPUES DE LA FECHA DE SU INSCRIPCION, SIEMPRE Y CUANDO DENTRO DE DICHO
TERMINO NO SEAN OBJETO DE RECURSOS EN LA VIA GUBERNATIVA.

DE CONFORMIDAD CON EL DECRETO 2150 DE 1.995 Y LA AUTORIZACION IMPARTIDA POR
LA SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO LA FIRMA MECANICA QUE APARECE A
CONTINUACION TIENE PLENA VALIDEZ PARA TODOS LOS EFECTOS LEGALES.

DADO EN CALI A LOS 12 DIAS DEL MES DE OCTOBER DEL AÑO 2010 HORA: 08:42:08



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24

CERTIFICADO NACIONAL

CAMARA DE COMERCIO DE CALI

FECHA: 2010/10/12

HORA: 08:41:09

OPERACION: 09TYU1012006

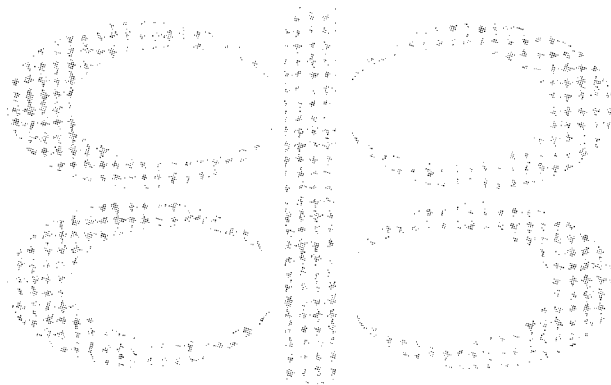
PAGINA: 19

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 900176099-2

RECIBO OFICIAL DE CAJA : 10 - 86,041
FECHA : OCTUBRE 13 DE 2010

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-047482- -00007-0000

Fecha: 2010-10-13 10:47:40 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 400 OPOSIOBSERVTER Folios: 52

**** CONSIGNACION ****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	Nº. PAGO	FECHA PGO	Vr. PAGO
REYES Y REYES ABOGADOS	CONSIGNACION	BANCO DE BOGOTA	062754397	403018534	13/10/2010	295,000.00

**** CONCEPTO ****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-04	PRESENTACION DE OBSERVACIONES	
12		MARCAS Y LEÑAS COMERCIALES	295,000.00
		TOTAL :	\$ 295,000.00

SON: DOSCIENTOS NOVENTA Y CINCO MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

DIVISIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 09 047482

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **618** DE

FECHA **19 DE JULIO 2010** EL TÉRMINO HÁBIL SEÑALADO POR LA LEY

EXPIRA EL **13 DE OCTUBRE DE 2010**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI X

NO

2020
Bogotá, D.C.,

Doctor:
Carlos R. Olarte
Apoderado

Oficio N°:  00327

ASUNTO:

Radicación PCT N°	09-47482
Trámite	011
Evento	378
Actuación	440
Folios	040

Con fundamento en lo previsto por el artículo 43 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica que la oposición presentada por el doctor José Luis Reyes Villamizar, en su calidad de apoderado de Lafrancol S.A., reúne los requisitos formales exigidos por la ley al momento de su presentación.

En cumplimiento de lo dispuesto en la citada norma, se le concede el término de sesenta (60) días hábiles siguientes a la fecha de notificación del presente oficio, para que haga valer sus argumentaciones y presente pruebas.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.


JOSÉ LUÍS SALAZAR LÓPEZ
Director de Nuevas Creaciones (E).

07 ENE. 2011

El anterior oficio fue notificado por fijación en lista de fecha, _____.

202088

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

SUPERINTENDENCIA

Radicación : 9-47482- -10-0 Fecha: 2011-06-01 08:45:19
Trámite : 11 PATENTEIYII
Evento : 379 FASENACIONALII
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.

Referencia RESOLUCION : 31052
Fecha : 31/05/2011
Expediente : 9-47482
Trámite : 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II
Evento : 379 FASE NACIONAL INCLUIDO CAPITULO II
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

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 : 31052
Fecha : 31/05/2011
Expediente No. : 09-47482

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.



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

Radicación :	9-47482- -9-0 Fecha: 2011-06-01 08:45:19
Trámite :	11 PATENTEIYII
Evento :	379 FASENACIONALII
Actuación :	432 COMUNICACTOADM
Destinatario :	CARLOS REYNALDO OLARTE GARCIA Folios 1

Señor (a) (es)
CARLOS REYNALDO OLARTE GARCIA
DIAGONAL 34 No. 5-28
BOGOTA D.C.

Referencia RESOLUCION : 31052
Fecha : 31/05/2011
Expediente : 9-47482
Trámite : 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II
Evento : 379 FASE NACIONAL INCLUIDO CAPITULO II
Actuación : 432 COMUNICACION ACTO ADMINISTRATIVO

Comedidamente 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

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCION

131052

Por la cual se declara abandonada una solicitud

Rad. PCT/ 09-47482

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 11 de mayo de 2009 con el N° 09-047482-00000-0000, el doctor Carlos Reinaldo Olarte García, en su calidad de apoderado de Irm Llc, presentó la solicitud de patente de invención titulada "ANTICUERPOS AGONISTAS DEL TRKB Y USOS DE LOS MISMOS", correspondiente a la solicitud internacional PCT/US 2007/083774 de fecha 06 de noviembre 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° 618 del 19 de julio de 2010.

TERCERO: Que mediante escrito radicado en esta Entidad el día 13 de octubre de 2010 con el N° 09-047482-00007-0000, encontrándose dentro del término establecido para el efecto, el doctor José Luis Reyes Villamizar, en su calidad de apoderado del Laboratorio Franco Colombiano Lafrancol S.A., presentó oposición fundamentada, la cual cumplió los requisitos formales previstos por la normativa andina. (Fls. 161 a 212).

CUARTO: Que mediante oficio N° 00327 del día 07 de enero de 2011 se corrió traslado de los documentos de oposición al solicitante, por el término de sesenta días para que se pronunciara al respecto, sin obtener respuesta alguna en tal sentido.

QUINTO: 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."*

SEXTO: Que contados seis meses desde la publicación en la Gaceta de la Propiedad Industrial, no se presentó petición de parte del interesado 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,

5431052

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCION

Por la cual se declara abandonada una solicitud

Rad. PCT/ 09-47482

RESUELVE:

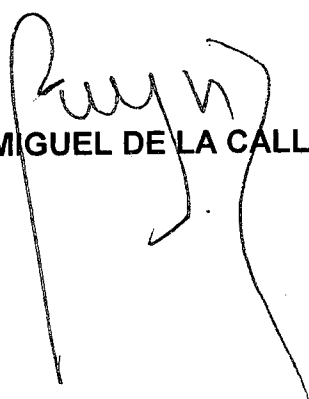
ARTÍCULO PRIMERO: Declarar abandonada la solicitud de patente de invención titulada "ANTICUERPOS AGONISTAS DEL TRKB Y USOS DE LOS MISMOS", que entró en fase nacional en virtud del Tratado de Cooperación en Materia de Patentes (PCT).

ARTÍCULO SEGUNDO: Notificar personalmente el contenido de la presente resolución al doctor Carlos Reinaldo Olarte García, en su calidad de apoderado de Irm Llc, y al doctor José Luis Reyes Villamizar, en su calidad de apoderado del Laboratorio Franco Colombiano Lafrancol S.A., sociedad opositora, 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 07 MAY 2009

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO,


JOSÉ MIGUEL DE LA CALLE RESTREPO

Doctor
CARLOS REINALDO OLARTE GARCÍA
Diagonal 34 N° 5 – 28.
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 31052 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 Blasimir Garmé
Identificado con 39853081

y T.P. No. _____
Con el fin de notificarme de la presente resolución
Recibí copia auténtica de la misma y se advirtió que
Como ella proceden los recursos de impugnación ante
La División de Signos Distintivos y el de Apelación

Para ante el Poder Judicial para la Propiedad Industrial
Dentro de los cinco (5) días hábiles siguientes a esta
Notificación.

Fecha 08 JUN. 2011

Maxo Poder

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NOTIFICADOR