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DIRECCIÓN DE NUEVAS CREACIONES
PRESENTACIÓN DE OPOSICIÓN

1. Oposición a solicitud de:

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

Número de expediente: 11-179-622

Solicitante: ABBOTT BIOTHERAPEUTICS CORP.

Apoderado: GERMAN CASTILLO GRAU.

Título de la Nueva Creación: ANTICUERPOS ANTI-VEGE Y SUS USOS.

Gaceta: 648 de 16 de julio de 2012, No. de publicación 962

2. Datos del Opositor

☐ Persona Natural

☒ Persona Jurídica

TECNOQUIMICAS S.A.

Nombre o denominación / Razón social completa

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

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

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

6. Fundamentos de la oposición

Causal: Incumplimiento de requisitos de patentabilidad (Título II, Capítulo I, Decisión 486 de 2000)

Justificación: VER ANEXO

Prórroga: SI ☒ NO ☐

7. Anexos

☒ Comprobante de pago de la tasa para la presentación de la solicitud No. 12 106278

☒ Poder, si fuera el caso con el que se acredita la representación.

☒ Pruebas de los fundamentos de la oposición

☒ Copias para traslado

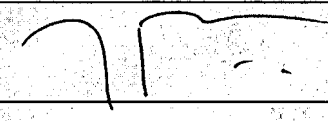
Fecha: 10-10-2012

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8. Firma

JOSÉ LUIS REYES VILLAMIZAR

Nombre y apellidos



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Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

E.

S.

D.

REF: Expediente 11-179-622

TÍTULO SOLICITADO: “ANTICUERPOS ANTI-VEGF Y SUS USOS”

PUBLICACIÓN: Gaceta 648 de 16 de julio de 2012, N° de publicación 962.

PRIORIDAD: US 61/218.005 17/06/2009

SOLICITANTE: ABBOTT BIOTHERAPEUTICS CORP.

APODERADO: GERMAN CASTILLO GRAU

TRÁMITE: PRESENTACIÓN DE OPOSICIÓN

JOSÉ LUIS REYES VILLAMIZAR, mayor de edad, identificado como aparece al pie de mi firma, abogado en ejercicio, titular de la tarjeta profesional número 44655 del Consejo Superior de la Judicatura, obrando en calidad de apoderado de la sociedad **TECNOQUÍMICAS S.A.**, en ejercicio de lo dispuesto por el artículo 42 de la Decisión 486 de 2000, muy respetuosamente me permito formular la siguiente

I. PETICIÓN

Sírvase negar el beneficio patentario a la solicitud contenida en el expediente **11-179-622**, por no cumplir con lo dispuesto en la Decisión 486 de 2000, con base en los fundamentos que se expondrán a continuación.

II. LEGÍTIMO INTERÉS

Asiste a **TECNOQUÍMICAS S.A.**, legítimo interés para presentar esta oposición, pues la eventual concesión de la solicitud restringiría injustificadamente el empleo de ciertas sustancias, derivados y procesos que a nuestro juicio carecen de nivel inventivo, en detrimento de sus legítimos intereses comerciales. De igual manera mis clientes están interesados, por su misma condición de industrias

farmacéuticas, en evitar la concesión de privilegios de patente que incumplan los requisitos materiales de patentabilidad establecidos en las normas vigentes, y que supongan un riesgo para la libre competencia.

III. FUNDAMENTOS DE LA OPOSICIÓN

La solicitud en curso, que se refiere a variantes del anticuerpo anti-VEGF **Bevacizumab** con inmunogenicidad reducida y/o afinidad mejorada hacia VEGF, en comparación con Bevacizumab o Ranibizumab, carece de los requisitos fundamentales para otorgársele el privilegio de patente de invención, particularmente en materia de Novedad y Nivel Inventivo si se tiene en cuenta la materia revelada entre otros, en documentos como:

- La publicación WO 03/099999 “Generation and selection of protein library in silico” de 04/12/2003 que describe de forma general la selección de librerías de proteína in silico.
- El documento de patente americano US 2002/0032315 “Anti-VEGF antibodies” de 14/03/2002 que se refiere a anticuerpos anti-VEGF.

Como primera instancia, es necesario mencionar que la presente solicitud se refiere a Bevacizumab y sus variantes, lo cual de entrada ya no presenta novedad o nivel inventivo, debido a que dichos anticuerpos ya se encuentran incluso disponibles comercialmente, y las variaciones en su secuencia son una técnica rutinaria en el estado del arte para modificar afinidad entre los fragmentos de unión al antígeno Fabs, e inmunogenicidad.

En la solicitud en trámite se mencionan tres CDRs de la cadena pesada denominadas aquí (en orden desde el terminal amino al carboxi) CDR-H1, CDR-H2 y CDR-H3, y tres CDRs de la cadena liviana, denominadas aquí (en orden desde el terminal amino al carboxi) CDR-L1, CDR-L2 y CDR-L3. Las secuencias de las CDRs de Bevacizumab se muestran en las Figuras 1A y 1B y su numeración se presenta en la Tabla 1 de la solicitud en trámite (para las CDRs de la cadena pesada) y la Tabla 2 (para las CDRs de la cadena liviana).

Adicional a lo anterior, la solicitud hace mención de un anticuerpo relacionado, Ranibizumab, que fue generado por fragmentos Fab madurados (maduración) derivados de Bevacizumab con una mayor afinidad hacia VEGF y adicionalmente

de menor tamaño (ver párrafo 0008 del documento WO 2010/148223 equivalente a la solicitud colombiana en trámite).

Ranibizumab tiene las secuencias de CDR-L1, CDR-L2, CDR-L3 y CDR-H2 idénticas a Bevacizumab, pero varía en sus secuencias de CDR-H1 y CDR-H3 con respecto a las de Bevacizumab. Las secuencias de las cadenas pesada y liviana de Ranibizumab se muestran en la Figura 1C de la solicitud en trámite y las CDRs se presentan en la Figura 1D (ver lista de figuras 36/39 en el documento WO 2010/148223 equivalente a la solicitud colombiana en trámite).

De esta forma, los anticuerpos de la solicitud en trámite generalmente tienen al menos una sustitución de aminoácido en al menos una CDR de la cadena pesada en comparación con Bevacizumab y Ranibizumab en la CDR-H2 que incluye la sustitución K64S lo que significa que como característica esencial solo se encuentra una sustitución determinada y muchas otras opcionales.

Ahora, para identificar lo reclamado por el solicitante, es necesario entender la reivindicación principal, así como su cobertura, veamos lo que describe textualmente:

1-. Un anticuerpo anti-VEGF o un fragmento de unión anti-VEGF de un anticuerpo, CARACTERIZADO porque comprende CDRs que tiene secuencias de amino ácidos correspondientes a SEQ ID NO: 3 (CDR-H1), SEQ ID NO: 4 (CDR-H2), SEQ ID NO: 5 (CDR-H3), SEQ ID NO: 6 (CDR-L1), SEQ ID NO: 7 (CDR-L2) y SEQ ID NO: 8 (CDR-L3), donde CDR-H2 incluye la sustitución K64S, y opcionalmente una o más mutaciones o combinaciones de mutaciones adicionales seleccionadas de una o más de las Tablas 6, 7, 8, 9, 10, 11, 12,-1 a 12-9 ó 13-16, donde las seis CDRs tienen en conjunto hasta 17 sustituciones de amino ácidos en comparación con las secuencias de las CDRs del anticuerpo Bevacizumab o el anticuerpo Ranibizumab.

Así, infringiendo los requisitos mínimos de patentabilidad, la solicitud reclama variantes de anticuerpos conocidos basándose en sustituciones, combinaciones, mutaciones, fragmentos de unión o anticuerpos que pueden obedecer a un sinnúmero de posibles compuestos o péptidos sin definición específica para la realización tanto de un buen análisis de anterioridades como para que un técnico medio en la materia pueda llevar a cabo la invención sin lugar a otras interpretaciones.

Adicionalmente, la materia reclamada es tan amplia que abarca cualquier anticuerpo con sustituciones no evaluadas y desarrollado por cualquier método

presente o futuro sin que éste se haya contemplado en los ejemplos y por lo tanto hace que lo revelado carezca de sustento.

En este orden de ideas, la Superintendencia de Industria y Comercio (en adelante SIC) ha sido clara en mencionar que las secuencias reclamadas deben ir en un capítulo aparte con el nombre “lista de secuencias” y en el formato indicado, lo que excluye la posibilidad de presentar sustituciones, combinaciones y posibilidades de productos biológicos no identificados plenamente por su secuencia en una estructura definida. Así, las reclamaciones no solo no cumplen con los requisitos de claridad, sustento y concisión sino que además no tienen un único concepto inventivo ya que no se enfocan en diferentes anticuerpos donde cada concepto se define a partir de una única sustitución que resuelva el problema técnico planteado. Sin los argumentos de un real efecto técnico, no se observan las diferencias que se producen entre las variadas combinaciones reclamadas.

Así, en el caso en el que se reconociera un efecto técnico sorprendente, dicho efecto obedecería al encontrado para un solo anticuerpo definido por su estructura determinada o un grupo plenamente definido y sustentado, y no en términos de combinaciones y/o permutaciones aleatorias en la secuencia; por otro lado, si se reconociera que no existe un efecto sorprendente de un compuesto exclusivo, no existe razón para pensar que los anticuerpos reclamados difieran del efecto obvio con relación a Bevacizumab o Ranibizumab, y por lo tanto, la solicitud carece de plano de novedad y nivel inventivo para su concesión.

Objeciones a la Novedad y Nivel Inventivo

La solicitud internacional WO 03/099999 describe diferentes anticuerpos, su estructura y la forma como se realizan cambios en los mismos en análisis computacionales (in silico) para predecir el plegamiento y función de los anticuerpos según su estructura y cambios en la secuencia. Esto con el fin de determinar cuáles cambios en los aminoácidos posibilitarían un sitio de unión al antígeno efectivo. (ver página 4, líneas 28 – 36, página 5 y 9).

Computer-implemented analysis and modeling of antibody combining site (or antigen binding site) are based on homology analysis comparing the target antibody sequence with those of antibodies with known structures or structural motifs in existing data bases (e.g. the Brookhaven Protein Data Bank). By using such homology-based modeling methods approximate three-dimensional structure of the target antibody is constructed. Early antibody modeling was based on the conjecture that CDR loops with identical length and different sequence may adopt similar conformations. Kabat and Wu (1972) Proc.

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Dicha solicitud internacional no solo menciona cómo resolver el problema planteado de forma general para cualquier antígeno, sino que va más allá y presenta los cambios en el anticuerpo específico anti VEGF de la solicitud (página 31, líneas 25 a 35 y ejemplo 1, página 141 y ejemplo 2). En dichos ejemplos se describen diferentes mutantes desarrollados a partir de regiones CDR de anticuerpo, parental anti-VEGF definido por las secuencias SEQ ID NO 1 y 2 idénticas a las SEQ ID NO 1 y 2 de la presente solicitud. El objetivo de la realización de este proceso involucrando mutaciones es incrementar la afinidad de los fragmentos de unión CDRs en el anticuerpo (ver página 142, línea 7 del ejemplo y página 5), que es la técnica rutinaria en el estado del arte

25 In another embodiment, the heavy chain variable region (V_H) of the monoclonal antibody against VEGF comprises an amino acid sequence of SEQ ID No: 126, and the light chain variable region (V_L) of the monoclonal antibody against VEGF comprises an amino acid sequence of SEQ ID No: 127.

30 In yet another embodiment, the heavy chain variable region (V_H) of the monoclonal antibody against VEGF comprises an amino acid sequence selected from group consisting of SEQ ID Nos: 126, 128, 129, 130, and 131, and the light chain variable region (V_L) of the monoclonal antibody against VEGF comprises an amino acid sequence of SEQ ID

35 No: 127.

The antibodies designed by using the methods of present

-31-

Por otro lado, la publicación americana US 2002/0032315 detalla la construcción de diferentes mutantes de anticuerpos anti-VEGF (ver ejemplo 3), construidos a partir de las regiones CDR de afinidad incrementada (ver párrafos 0288, 0304 y tabla 15).

EXAMPLE 3

[0268] In this example, CDR randomization, affinity maturation by monovalent Fab phage display, and cumulative combination of mutations were used to enhance the affinity of a humanized anti-VEGF antibody.

[0269] Construction of Humanized Antibody pY1101:

[0270] Phage-displayed antibody vector phMB4-19-1.6 (see FIGS. 8A-E) was used as a parent. In this construct, anti-VEGF is expressed as a Fab fragment with its heavy chain fused to the N-terminus of the truncated g3p. Both the light and heavy chains are under the control of phoA promoter with an upstream slll signal-sequence for secretion into the periplasm. Point mutations outside the CDR regions were made by site-directed mutagenesis to improve affinity

La reivindicación 9 de dicha publicación da las secuencias de las CDRs del anticuerpo anti-VEGF; dicho anticuerpo reclamado por la anterioridad divulga secuencias idénticas a aquellas SEQ 3-8 de la presente solicitud.

9. The humanized anti-VEGF antibody of claim 1 having a light chain variable domain comprising the following hypervariable region amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

No obstante la materia técnica en este caso es igual y el requisito de novedad debe tenerse como no superado por la solicitud, la interpretación en cuanto a la diferencia en las secuencias o estructura secuencial posible de cada uno de los objetos en la solicitud, aunque imposible de definir, pueden verse como diferente. En ese caso, la única diferencia (literal) con relación a la presente solicitud y los documentos citados es la definición de los anticuerpos por 6 CDRs que en conjunto pueden tener variaciones de aminoácidos entre sí.

Sin embargo, es necesario recalcar que el efecto técnico resultante de esta diferencia no involucra resultados sorprendentes, sino un evidente cambio en la afinidad de la unión antígeno-anticuerpo, predecible para cualquier técnico medio versado en la materia que realizara investigación rutinaria en el área técnica citada.

Para resolver el problema de proveer anticuerpos alternativos anti-VEGF mutados de forma inespecífica en regiones CDR, la solución propuesta por la solicitud en trámite obedece a cualquiera de los anticuerpos descritos tanto por las anterioridades como los definidos en la solicitud, haciendo que tal solución no

presente mérito inventivo sólo por las sustituciones mencionadas.

En conclusión, no existe nivel inventivo debido a que los documentos ya proponían mutaciones en las regiones CDR con el fin de mejorar la afinidad del anticuerpo.

Entonces, con el fin de obtener anticuerpos adicionales Anti-VEGF con afinidad aumentada, una persona versada habría tenido la motivación para seguir el camino propuesto por las anterioridades, obtener y seleccionar anticuerpos con CDRs modificadas, todo esto sin evidencia comprobada de todas sus ventajas. De hecho, los datos presentados en los ejemplos no muestran si el problema fue resuelto; lo que además indica que la resolución del problema teniendo como base todos los posibles anticuerpos reclamados, cae dentro del campo de la especulación ya que solo se obtuvieron anticuerpos alternativos, sin efectos sorprendentes.

Adicional a lo anterior, las reivindicaciones de la solicitud en trámite fallan en presentar correctamente la materia patentable al reclamar métodos de tratamiento que son excluidos de recibir el beneficio de patente (ver reivindicaciones 70 – 74), según lo contemplado en la Decisión 486 de la Comunidad Andina.

De esta forma se establece que la solicitud no cumple con los requisitos mínimos para obtener el beneficio de patente en lo que respecta a claridad, sustento, descripción suficiente, y menos aun los requisitos de Novedad además que no se asocia ningún salto cuantitativo en el estado de la técnica que indique que la solicitud cumple con el requisito de Nivel Inventivo.

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, (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 que el Despacho considere oficiosamente, en particular de las allegadas por el suscrito a los expedientes mencionados en este escrito, las siguientes:

- ❖ La publicación WO 03/099999, Portada, 4, 5, 9, 31, 141, 142,
- ❖ El documento de patente americano US 2002/0032315, portada, páginas 41 a 47.

VI. ANEXOS

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

1. Certificado de existencia y representación legal de TECNOQUÍMICAS S.A.
2. Poder para actuar.
3. Constancia de pago de las tasas de tramitación de oposición.
4. Constancia de pago para sustentar la solicitud de prórroga a que se refiere el artículo 42, inciso 2º de la Decisión 486.
5. Copia para correr traslado al solicitante.
6. El mencionado en el acápite de pruebas.

VII. 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. Tecnoquímicas S.A., las recibirá en la secretaría de su Despacho o en su sede localizada en la Calle 23 # 7-39, de la ciudad de Cali.

Señor Superintendente,



JOSÉ LUIS REYES VILLAMIZAR

C.C. 79'152.473 de Usaquén

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

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- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
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— without international search report and to be republished upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/099999 A2

(54) Title: GENERATION AND SELECTION OF PROTEIN LIBRARY IN SILICO

(57) Abstract: The present invention provides a methodology for efficiently generating and screening protein libraries for optimized proteins with desirable biological functions, such as improved binding affinity towards biologically and/or therapeutically important target molecules. The process is carried out computationally in a high throughput manner by mining the ever-expanding databases of protein sequences of all organisms, especially human. In one embodiment, a method for constructing a library of designed proteins, comprising the steps of: providing an amino acid sequence derived from a lead protein, the amino acid sequence being designated as a lead sequence; comparing the lead sequence with a plurality of tester protein sequences; and selecting from the plurality of tester protein sequences at least two peptide segments that have at least 15% sequence identity with the lead sequence, the selected peptide segments forming a hit library; and forming a library of designed proteins by substituting the lead sequence with the hit library. The library of designed proteins can be expressed in vitro or in vivo to produce a library of recombinant proteins that can be screened for novel or improved function(s) over the lead protein, such as an antibody against therapeutically important target.

presumably due to the accumulation of point mutations specifically in both heavy-and light-chain V region coding sequences and a selected expansion of high-affinity antibody-bearing B cell clones.

5 Structurally, various functions of an antibody are confined to discrete protein domains (regions). The sites that recognize and bind antigen consist of three hyper-variable or complementarity-determining regions (CDRs) that lie within the variable (V_H and V_L) regions at the N-terminal ends of the two H and two L chains. The constant domains are not involved directly in binding the antibody to an antigen, but are
10 involved in various effector functions, such as participation of the antibody in antibody-dependent cellular cytotoxicity.

The domains of natural light and heavy chains have the same general structures, and each domain comprises four framework regions, whose sequences are somewhat conserved, connected by three CDRs.
15 The four framework regions largely adopt a β -sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held in close proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site.

20 Generally all antibodies adopt a characteristic "immunoglobulin fold". Specifically, both the variable and constant domains of an antigen binding fragment (Fab, consisting of V_L and C_L of the light chain and V_H and C_H1 of the heavy chain) consist of two twisted antiparallel β -sheets which form a β - sandwich structure. The constant regions have
25 three- and four-stranded β -sheets arranged in a Greek key-like motif, while variable regions have a further two short β strands producing a five-stranded β -sheet.

The V_L and V_H domains interact via the five-stranded β sheets to form a nine-stranded β barrel of about 8.4Å radius, with the strands at
30 the domain interface inclined at approximately 50° to one another. The domain pairing brings the CDR loops into close proximity. The CDRs themselves form some 25% of the V_L/V_H domain interface.

The six CDRs, (CDR-L1, -L2 and -L3 for the light chain, and CDR-H1, -H2 and -H3 for the heavy chain), are supported on the β
35 barrel framework, forming the antigen binding site. While their

sequences are hypervariable in comparison with the rest of the immunoglobulin structure, some of the loops show a relatively high degree of both sequence and structural conservation. In particular, CDR-L2 and CDR-H1 are highly conserved in conformation.

5 Chothia and co-workers have shown that five of the six CDR loops (all except CDR-H3) adopt a discrete, limited number of main-chain conformations (termed canonical structures of the CDRs) by analysis of conserved key residues. Chothia and Lesk (1987) *J. Mol. Biol.* 196:901-917; Chothia et al. (1989) *Nature (London)* 342:877; and
10 Chothia et al. (1998) *J. Mol. Biol.* 278:457-479. The adopted structure depends on both the CDR length and the identity of certain key amino acid residues, both in the CDR and in the contacting framework, involved in its packing. The canonical conformations were determined by specific packing, hydrogen bonding interactions, and stereochemical
15 constraints of only these key residues which serve as structural determinants.

 Various methods have been developed for modeling the three dimensional structures of the antigen binding site of an antibody. Other than x-ray crystallography, nuclear magnetic resonance (NMR)
20 spectroscopy has been used in combination with computer model building to study the atomic details of antibody-ligand interactions. Dwek et al. (1975) *Eur. J. Biochem.* 53:25-39. Dwek and coworkers used spin-labeled hapten to deduce the combining site of the MoPC 315 myeloma protein for dinitrophenyl. Similar analysis has also been done
25 using anti-spin labeled monoclonal antibodies (Anglister et al. (1987) *Biochem.* 26: 6958-6064) and on the anti-2-phenyloxazolone Fv fragments (McManus and Riechmann (1991) *Biochem.* 30:5851-5857).

 Computer-implemented analysis and modeling of antibody combining site (or antigen binding site) are based on homology analysis
30 comparing the target antibody sequence with those of antibodies with known structures or structural motifs in existing data bases (e.g. the Brookhaven Protein Data Bank). By using such homology-based modeling methods approximate three-dimensional structure of the target antibody is constructed. Early antibody modeling was based on
35 the conjecture that CDR loops with identical length and different sequence may adopt similar conformations. Kabat and Wu (1972) *Proc.*

SUMMARY OF THE INVENTION

The present invention provides an innovative methodology for efficiently generating and screening protein libraries for optimized
5 proteins with desirable biological functions, such as improved binding affinity towards biologically and/or therapeutically important target molecules. The process is carried out computationally in a high throughput manner by mining the ever-expanding databases of protein sequences of all organisms, especially human. The evolutionary data of
10 proteins are utilized to expand both sequence and structure space of the protein libraries for functional screening in vitro or in vivo. By using the inventive methodology, an expanded and yet functionally biased library of proteins such as antibodies can be constructed based on computational evaluation of extremely diverse protein sequences and
15 functionally relevant structures in silico.

In one aspect of the invention, a method is provided for designing and selecting protein(s) with desirable function(s). The method is preferably implemented in a computer through in silico selection of protein sequences based on the amino acid sequence of a target
20 structural/functional motif or domain in a lead protein, herein after referred to as the "lead sequence". The lead sequence is employed to search databases of protein sequences. The choice of the database depends on the specific functional requirement of the designed motifs. For example, if the lead protein is an enzyme and the target motif
25 includes the active site of the enzyme, databases of proteins/peptides of a particular origin, organism, species or combinations thereof, may be queried using various search criteria to yield a hit list of sequences each of which can substitute the target motif in the lead protein. A similar approach may be used for designing other motifs or domains of the lead
30 protein. The designed sequences for each individual motif/domain may be combined to generate a library of designed proteins. In addition, to reduce immunogenicity of the designed proteins for human applications such as therapeutics or diagnosis, databases of proteins of human origin or humanized proteins are preferably searched to yield the hit list
35 of sequences, especially for motifs derived from sites of the lead protein that are not structurally or functionally critical. The library of designed

non-antibody polypeptide chains fused to the V_H and V_L chains, respectively. For example, these two non-antibody polypeptide may be derived from a heterodimeric receptors GABA_B R1 (GR1) and R2 (GR2), respectively.

5 Alternatively, the recombinant antibodies displayed on phage particles may be a single-chain antibody containing V_H and V_L linked by a peptide linker. The display of the single chain antibody on the surface of phage particles may be facilitated by a heterodimer formed between a fusion of the single chain antibody with GR1 and a fusion of phage pIII capsid protein with GR2.

10 The target antigen to be screened against includes small molecules and macromolecules such as proteins, peptides, nucleic acids and polycarbohydrates.

15 In yet another aspect of the present invention, a computer-readable medium is provided. The computer medium comprises logic for constructing a library of mutant antibodies based on a lead antibody, the logic comprising:

logic which

20 takes as an input an amino acid sequence that comprises at least 3 consecutive amino acid residues in a CDR of the lead antibody, the amino acid sequence being a lead sequence;

compares the lead sequence with a plurality of tester protein sequences;

25 selects from the plurality of tester protein sequences at least two peptide segments that have at least 15% sequence identity with lead sequence; and

generates as an output the selected peptide segments which form a hit library.

30 In yet another aspect of the present invention, monoclonal antibodies are provided that are capable of binding to human vascular endothelial growth factor (VEGF) with a binding affinity higher than 10^6 M⁻¹. The monoclonal antibody may be a fully assembled antibody, a Fab fragment, a Fv fragment or a single chain antibody (scFv).

functional screening is the ultimate test on the validity and accuracy of the in silico methods, some intrinsic limits related to scoring function and structure template in the computational screening can be tested experimentally.

5 Sixth, the use of simple structural correlation to partition long sequences allows the control of the library size so that it is experimentally manageable without a significant loss of diversity. It also makes it possible to design sequence libraries for a lead sequence with little structural information available.

10 Finally, the adaptability and parameterization of the scoring function permits refinement with each experimental cycle. The experimentally screened clones represent an actual positional variant in a profile that can be used as a feedback for refining the scoring function by refining the various scoring terms.

15 In summary, exploring the function space by combining direct experimental screening, within experimental limit, with indirect computational screening in sequence and structure space of a target protein is a powerful approach to protein engineering and design as we demonstrate here for antibodies.

20

EXAMPLE

Methods of the present invention were used for in silico construction of antibody libraries. The vascular endothelial growth factor (VEGF) is chosen as the antigen for the present proof-of-principle experiments in order to demonstrate the present invention in antibody design. A rich collection of sequence and structure information is available for VEGF and its receptor (Muller YA, Christinger HW, Keyt BA, de Vos AM (1997) Structure 5, 1325-1338; Wiesmann C, Fuh G, Christinger HW, Eigenbrot C, Wells JA, de Vos AM (1997) Cell 91, 695-704), a complex between VEGF and its humanized antibody (Muller YA, Christinger HW, Li B, Cunningham BC, Lowman HB, de Vos AM (1998) Structure 6, 1153-1167, and a complex between VEGF and its matured antibody (Chen Y, Wiesmann C, Fuh G, Li B, Christinger HW, McKay P, de Vos AM (1999) J Mol Biol 293, 865-881). These provide a good platform for testing the methods of the present invention. By using the

methods provided by the present invention, several digital libraries of anti-VEGF antibodies were designed in silico by utilizing incrementally enriched information from an antibody sequence, the structure of an antibody, the complex structure between an antibody and its antigen. Populations of the antibody libraries were screened in vitro for high affinity binding to VEGF via two independent novel phage display systems with antibody binding unit in single or double chains.

1. In silico Design of Anti-VEGF Antibody Libraries

10

VEGF is a key angiogenic factor in development and is involved in the growth of solid tumor by stimulating endothelial cells. A murine monoclonal antibody was found to block VEGF-dependent cell proliferation and slow the tumor growth in vivo (Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N (1993) Nature 362, 841-844). This murine antibody was humanized (Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M, Ferrara N (1997) Cancer Res. 57, 4593-4599; Baca M, Presta LG, O'Connor SJ, Wells JA (1997) J Biol Chem 272, 10678-10684) and affinity-matured by using phage-display and off-rate selection (Chen Y, Wiesmann C, Fuh G, Li B, Christinger HW, McKay P, de Vos AM (1999) J Mol Biol 293, 865-881). X-ray structure for the complex formed between VEGF and the parental antibody was reported (Muller YA, Chen Y, Christinger HW, Li B, Cunningham, BC, Lowman HB, de Vos AM (1998) Structure 6, 1153-1167.), as well as the one formed between VEGF and the matured antibody (Chen Y, Wiesmann C, Fuh G, Li B, Christinger HW, McKay P, de Vos AM, Lowman HB (1999) J. Mol Biol 293, 865-881).

Figure 9A shows the amino acid sequences of the variable regions of the humanized anti-VEGF antibody (therein after referred to as "parental anti-VEGF antibody") and the antibody affinity matured from the humanized anti-VEGF antibody (therein after referred to as "matured anti-VEGF antibody"). Each of the amino acid residues in the V_H CDRs that were observed to be in contact with the antigen is labeled as "c" underneath. **Figure 9B** is an alignment of the parental and matured anti-VEGF antibody in the V_H CDRs. The framework and CDRs are designated according to the Kabat criteria (Kabat EA, Redi-



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(54) **ANTI-VEGF ANTIBODIES**

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(57) **ABSTRACT**

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Humanized and variant anti-VEGF antibodies and various uses therefor are disclosed. The anti-VEGF antibodies have strong binding affinities for VEGF; inhibit VEGF-induced proliferation of endothelial cells in vitro; and inhibit tumor growth in vivo.

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from extending the diversity beyond the target human and parent murine framework sequences.

TABLE 6

Sequences Selected from the Humanized A4.6.1 Phagemid Fab Library													
Variant	Residue substitutions												
	V _L						V _H						
	4	71	24	37	67	69	71	73	75	76	78	93	94
murine A4.6.1	M	Y	A	V	F	F	L	T	A	S	A	A	K
hu2.0 (CDR-graft)	M	<u>F</u>	A	V	F	<u>I</u>	<u>R</u>	<u>N</u>	<u>K</u>	<u>N</u>	<u>L</u>	A	<u>R</u>
Phage-selected clones:													
hu2.1(2)	—	Y	—	I	—	—	—	—	—	—	V	—	K
hu2.2(2)	L	Y	—	I	—	—	—	—	—	—	V	—	K
hu2.6(1)	L	—	—	I	T	—	L	T	A	S	V	—	K
hu2.7(1)	L	—	—	I	—	—	—	—	—	—	V	—	K
hu2.10 (10)	—	Y	—	I	—	—	L	T	A	S	V	—	K

Differences between hu2.0 and murine A4.6.1 antibodies are underlined. The number of identical clones identifies for each phage-selected sequence is indicated in parentheses. Dashes in the sequences of phage-selected clones indicate selection of the human V_LKI-V_HIII framework sequence (i.e. as in hu2.0).

[0263] There were four other unique amino acid sequences among the remaining ten clones analyzed: hu2.1, hu2.2, hu2.6 and hu2.7. All of these clones, in addition to hu2.10, contained identical framework substitutions at positions V_H 37 (Ile), 78 (Val) and 94 (Lys), but retained the human V_HIII consensus sequence at positions 24 and 93. Four clones had lost the light chain coding sequence and did not bind VEGF when tested in a phage ELISA assay (Cunningham et al. *EMBO J.* 13:2508-251 (1994)). Such artifacts can often be minimized by reducing the number of sorting cycles or by propagating libraries on solid media.

[0264] Expression and Binding Affinity of Humanized A4.6.1 Variants:

[0265] Phage-selected variants hu2.1, hu2.2, hu2.6, hu2.7 and hu2.10 were expressed in *E. coli* using shake flasks and Fab fragments were purified from periplasmic extracts by protein G affinity chromatography. Recovered yields of Fab for these five clones ranged from 0.2 (hu2.6) to 1.7 mg/L (hu2.1). The affinity of each of these variants for antigen (VEGF) was measured by surface plasmon resonance on a BIAcore instrument (Table 7). Analysis of this binding data revealed that the consensus clone hu2.10 possessed the highest affinity for VEGF out of the five variants tested. Thus the Fab phagemid library was selectively enriched for the tightest binding clone. The calculated K_D for hu2.10 was 55 nM, at least 125-fold tighter than for hu2.0 which contains no framework changes (K_D>7 μM). The other four selected variants all exhibited weaker binding to VEGF, ranging down to a K_D of 360 nM for the weakest (hu2.7). Interestingly, the K_D for hu2.6, 67 nM, was only marginally weaker than that of hu2. 10 and yet only one copy of this clone was found among 20 clones sequenced. This may have due to a lower level of expression and display, as was the case when expressing the soluble Fab of this variant. However, despite the lower expression rate, this variant is useful as a humanized antibody.

TABLE 7

VEGF Binding Affinity of Humanized A4.6.1 Fab Variants				
Variant	k _{on} M ⁻¹ s ⁻¹ /10 ⁴	k _{off} 10 ⁴ s ⁻¹	K _D nM	K _D (A4.6.1) K _D (mut)
A4.6.1 chimera	5.4	0.85	1.6	>4000
hu2.0	ND	ND	>7000**	
Phage selected clones:				
hu2.1	0.70	18	260	170
hu2.2	0.47	16	340	210
hu2.6	0.67	4.5	67	40
hu2.7	0.67	24	360	230
hu2.10	0.63	3.5	55	35
*hu2.10V	2.0	1.8	9.3	5.8

*hu2.10V = hu2.10 with mutation V_LLeu→Val
Estimated errors in the BIAcore binding measurements are +/±25%.
**Too weak to measure; estimate of lower bound

[0266] Additional Improvement of Humanized Variant hu2.1:

[0267] Despite the large improvement in antigen affinity over the initial humanized variant, binding of hu2.10 to VEGF was still 35-fold weaker than a chimeric Fab fragment containing the murine A4.6.1 V_L and V_H domains. This considerable difference suggested that further optimization of the humanized framework might be possible through additional mutations. Of the Vernier residues identified by Foote & Winter *J. Mol. Biol.* 224:487-499 (1992), only residues V_L 46, V_H 2 and V_H 48 differed in the A4.6.1 versus human V_LKI-V_HIII framework (FIGS. 5A and 5B) but were not randomized in our phagemid library. A molecular model of the humanized A4.6.1 Fv fragment showed that VL 46 sits at the V_L-V_H interface and could influence the conformation of CDR-H3. Furthermore, this amino acid is almost always leucine in most V_LK frameworks (Kabat et al., supra), but is valine in A4.6.1. Accordingly, a Leu→Val substitution was made at this position in the background of hu2.10. Analysis of binding kinetics for this new variant, hu2.10V, indicated a further 6-fold improvement in the K_D for VEGF binding, demonstrating the importance of valine at position V_L 46 in antibody A4.6.1. The K_D for hu2.10V (9.3 nM) was thus within 6-fold that of the chimera. In contrast to V_L 46, no improvement in the binding affinity of hu2.10 was observed for replacement of either V_H 2 or V_H 48 with the corresponding residue from murine A4.6.1.

EXAMPLE 3

[0268] In this example, CDR randomization, affinity maturation by monovalent Fab phage display, and cumulative combination of mutations were used to enhance the affinity of a humanized anti-VEGF antibody.

[0269] Construction of Humanized Antibody pY1101:

[0270] Phage-displayed antibody vector pHMB4-19-1.6 (see FIGS. 8A-E) was used as a parent. In this construct, anti-VEGF is expressed as a Fab fragment with its heavy chain fused to the N-terminus of the truncated g3p. Both the light and heavy chains are under the control of phoA promoter with an upstream stII signal-sequence for secretion into the periplasm. Point mutations outside the CDR regions were made by site-directed mutagenesis to improve affinity

for VEGF with oligonucleotides HL-242, HL-243, HL-245, HL-246, HL-254, HL-256, and HL-257 as shown in Table 8 below:

mixture of A, G, C, and T while S is an equal mixture of G and C) for saturation mutagenesis. VL1 and VH3 were chosen as potential candidates for affinity enhancement

TABLE 8

Oligos for Directed Mutations		
Oligo Number	Substitution/Region Comments	Sequence
HL-242 VL	M4L	5'-GATATCCAGTTGACCCAGTCCCCG-3' (SEQ ID NO:29)
HL-243 VL	L46V	5'-GCTCCGAAAGTACTGATTTAC-3' (SEQ ID NO:30)
HL-245 VH	CDR-7	5'-CGTCGTTTCACTTTTCTGCAGACACCTCCAGCAACACAGTATACCTGCAGATG-3' (SEQ ID NO:31)
HL-246 VH	R98K	5'-CTATTACTGTGCAAAGTACCCCCAC-3' (SEQ ID NO:32)
HL-254 VL	Y71F	5'-GGGACGGATTTCACTCTGACCATC-3' (SEQ ID NO:33)
HL-256 VH	137V	5'-GGTATGAACTGGGTCCGTCAGGCCCC-3' (SEQ ID NO:34)
HL-257 VH	CDR-7 A72L S76K N77S	5'-CGTCGTTTCACTTTTCTTTAGACACCTCCAAAAGCACAGCATACCTGCAGATGAA C-3' (SEQ ID NO:35)

[0271] The resulting variant was termed Y0101 (FIGS. 9A and 9B).

[0272] Construction of the First Generation of Antibody-Phage Libraries:

[0273] To prevent contamination by wild-type sequence, templates with the TAA stop codon at the targeted sites for randomization were prepared and used for constructing libraries by site-directed mutagenesis with oligonucleotides using the degenerate NNS codon (where N is an equal

(FIGS. 9A and B). Within the CDRs, two libraries were constructed from the pY0101 template. VL1 was mutated using stop-template oligonucleotides HL-248 and HL-249 (Table 9) and library oligonucleotides HL-258 and HL-259 (Table 10). Similarly, three libraries were constructed for VH3 using stop template oligonucleotides HL-250, HL-251, and HL-252 (Table 9), and library oligonucleotides HL-260, HL-261, and HL-262 (Table 10). Library construction is summarized in Tables 9 and 10 below.

TABLE 9

Template Oligos for Mutagenesis		
Oligo Number	Region Comments	Sequence
HL-248 VL1		5'-GGGTCACCATCACCTGCCTAAGCATAATAATAAAGCAACTATTTAAACTGG-3' (SEQ ID NO:36)
HL-249 VL1		5'-GCGCAAGTCAGGATATTTAATAATAATAAATGGTATCAACAGAAACCAGG-3' (SEQ ID NO:37)
HL-250 VH3		5'-GTCTATTACTGTGCAAAGTAATAACACTAATAAGGGAGCAGCCACTGG-3' (SEQ ID NO:38)
HL-251 VH3		5'-GGTACCCCACTATTATTAATAATAAATGGTATTTGACGCTCTGGGG-3' (SEQ ID NO:39)
HL-252 VH3		5'-CACTATTATGGGAGCAGCCACTAATAATAAAGTCTGGGTCAAGGAACCTTG-3' (SEQ ID NO:40)
HL-263 VH1		5'-TCCTGTGCAGCTTCTGGCTAATAATTCTAATAAAGGTATGAAGTGGTCCG-3' (SEQ ID NO:41)
HL-264 VH2		5'-GAATGGGTTGGATGGATTAACTAATAAAGGTTAACCGACCTATGCTGCGG-3' (SEQ ID NO:42)
YC-80 VH3		5'-CTGTGCAAAGTACCCGTAATATTAATAATAAAGTGGTATTTTCGAC-3' (SEQ ID NO:43)
YC-100 CDR7		5'-CGTTTCACTTTTCTTAAGACTAATCCAAATAAACAGCATACCTGCAG-3' (SEQ ID NO:44)
YC-102 VH2		5'-GAATGGGTTGGATGGATTAAATAATAAAGGTGAACCGACCTATG-3' (SEQ ID NO:45)

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[0274]

TABLE 10

Random Oligos for Library Construction		
Oligo Number	Region Comment	Sequence
HL-258	VL1	5'-GGGTCACCATCACCTGCNNSGCANNNNNSNNNSAGCAACTATTTAAACTGG-3' (SEQ ID NO:46)
HL-259	VL1	5'-GCGCAAGTCAGGATATTNNSNNNSNNNSNNSTGGTATCAACAGAAACCAGG-3' (SEQ ID NO:47)
HL-260	VH3	5'-GTCTATTACTGTGCAAAGNNSNNSCACNNSNNSGGGAGCAGCCACTGG-3' (SEQ ID NO:48)
HL-261	VH3	5'-GGTACCCCACTATTATNNSNNNSNNNSNNSTGGTATTTCGACGCTGCGG-3' (SEQ ID NO:49)
HL-262	VH3	5'-CACTATTATGGGAGCAGCCACNNSNNNSNNNSGTCTGGGGTCAAGGAACCCTG-3' (SEQ ID NO:50)
HL-265	VH1	5'-TCCTGTGCAGCTTCTGGCNSNNSTTCNNSNNNSGGTATGAACTGGGTCCG-3' (SEQ ID NO:51)
HL-266	VH2	5'-GAATGGGTTGGATGGATTAAACNNSNNNSGGTNNSCCGACCTATGCTGCGG-3' (SEQ ID NO:52)
YC-81	VH3	5'-CTGTGCAAAGTACCCGNNSTATNNSNNNSNNSCACTGGTATTTCGAC-3' (SEQ ID NO:53)
YC-101	CDR7	5'-CGTTTCACTTTTCTNNSGACNNSTCCAAANNSACAGCATACCTGCAG-3' (SEQ ID NO:54)
YC-103	VH2	5'-GAATGGGTTGGATGGATTNNSNNNSNNNSGGTGAACCGACCTATG-3' (SEQ ID NO:55)

[0275] The products of random mutagenesis reactions were electroporated into XL1-Blue *E.coli* cells (Stratagene) and amplified by growing 15-16 h with M13KO7 helper phage. The complexity of each library, ranging from 2x10⁷ to 1.5x10⁸, was estimated based upon plating of the initial transformation onto carbenicillin plates.

[0276] Initial Affinity Selections:

[0277] For each round of selection, approximately 10⁹-10¹⁰ phage were screened for binding to plates (Nunc Maxisorp 96-well) coated with 2 µg/mL VEGF (recombinant; residue 9-109 version) in 50 mM carbonate buffer, pH 9.6 and blocked with 5% instant milk in 50 mM carbonate buffer, pH 9.6. After 1-2 hour binding at room temperature, in the presence of 0.5% bovine serum albumin and 0.05% TWEEN 20TM in PBS, the phage solution was removed, and the plate was washed ten times with PBS/TWEENTM (0.05% TWEEN 20TM in PBS buffer). Typically, to select for enhanced affinity variants with slower dissociation rates, the plates were incubated with PBS/TWEENTM buffer for a period of time which lengthened progressively for each round of selection (from 0 minute for the first round, to 3 h for the ninth round of selection). After the PBS/TWEENTM buffer was removed, the remained phages were eluted with 0.1 M HCl and immediately neutralized with ½ volume of 1 M Tris, pH 8.0. The eluted phages were propagated by infecting XL1-Blue *E.coli* cells (Stratagene) for the next selection cycle.

[0278] Sequencing data revealed that both VL1 libraries, even after the eighth/ninth round of sorting, remained diverse, tolerating various type of residues at the sites of randomization. In contrast, the VH3 libraries retained only wild type residues or had very conservative substitutions. This suggested that the VL1 was more exposed to solvent and lay outside the binding interface. In contrast, VH3 did not show dramatically different sidechain substitutions, and therefore might be more intimately involved in antigen binding.

[0279] Phage-ELISA Assay of Binding Affinities:

[0280] From each of these libraries, representative clones (those represented by abundant sequences) were assayed for their affinities relative to that of parent clone pY0101 in a phage-ELISA assay. In such an assay, phages were first serially diluted to determine a fractional saturation titer which was then held constant and used to incubate with varying concentrations of VEGF (starting at 200 nM to 0 nM) in solution. The mixture was then transferred onto plate precoated with VEGF (2 µg/mL) and blocked with 5% instant milk, and allowed to equilibrate for 1 hour at room temperature. Thereafter, the phage solution was removed and the remaining bound phages were detected with a solution of rabbit anti-phage antibody mixed with goat anti-rabbit conjugate of horse radish peroxidase. After an hour incubation at room temperature, the plate was developed with a chromogenic substrate, o-phenylenediamine (Sigma). The reaction was stopped with addition of ½ volume of 2.5 M H₂SO₄. Optical density at 492 nm was measured on a spectrophotometric plate reader.

[0281] Although all of the selected clones from these five libraries showed either weaker or similar affinities than that of wild type pY0101 in phage-ELISA assay, one particular variant (pY0192) from library HL-258 displayed an apparent advantage (about 10 fold) in the level of expression or phage display relative to pY0101. This clone contained mutations S24R, S26N, Q27E, D28Q, and 129L in the VL region (FIG. 9A). In addition, this variant was found to have a spurious mutation, M34I, in VH. This variant showed no significant difference in binding affinity to VEGF as compared with the pY0101 variant. To improve the level of Fab-display on phage, and the signal-to-noise ratio for phage-ELISA assays, the corresponding substitutions in pY0192 at VL1 were incorporated into the template background for constructing both CDR Ala-mutants and the second generation of anti-VEGF libraries.

[0282] Ala-Scanning the CDRs of Anti-VEGF:

[0283] To determine the energetics contributed by each of the amino acids in the CDR regions and thus better select

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target residues for randomization, the CDR regions were screened by substituting alanine for each residue. Each Ala mutant was constructed using site-directed mutagenesis with a synthetic oligonucleotide encoding for the specific alanine substitution. Where Ala was the wild-type residue, Ser was substituted to test the effect of a sidechain substitution. Phage clones having a single Ala mutation were purified and assayed in phage-ELISA as described above. Results of the Ala-scan demonstrated that Ala-substitution at various positions can have an effect, ranging from 2 to >150 fold reductions, on antigen binding affinity compared to pY0192. In addition, it confirmed a previous observation that VH3, but not VL1, was involved in antigen binding. Results of the CDR Ala-scan are summarized in Table 11 below.

TABLE 11

Relative VEGF Affinities of Ala-Scan Fab Variants			
Residue VL	IC50(mut) IC50 (wt)	Residue VH	IC50(mut) IC50 (wt)
R24A	1	G26A	2
A25S	1	Y27A	34
N26A	1	T28A	1
E27A	1	F29A	16
Q28A	1	T30A	1
L29A	1	N31A	>150
S30A	2	Y32A	>150
N31A	2	G33A	6
Y32A	2	I34A	6
L33A	2	N35A	66
N34A	4		
		W50A	>150
F50A	1	I51A	4
T51A	1	N52A	>150
S52A	1	T53A	9
S53A	1	Y54A	9
L54A	1	T55A	4
I55A	1	G56A	1
S56A	1	E57A	2
		P58A	1
Q89A	4	T59A	3
Q90A	3	Y60A	2
Y91A	14	A61S	1
S92A	1	A62S	1
T93A	1	D63A	1
V94A	2	F64A	1
P95A	3	K65A	1
W96A	>150	R66A	1
T97A	1		
		Y99A	>150
		P100A	38
		H101A	4
		Y102A	4
		Y103A	5
		G104A	2
		S105A	1
		S106A	>150
		H107A	2
		W108A	>150
		Y109A	19
		F110A	25
		D111A	2

[0284] All variants are in the background of pY0192 ("wt"; see FIGS. 9A-B). IC50's were determined in a competitive phage-ELISA assay.

[0285] The largest effects of Ala substitutions are seen in CDRs H1, H2, and H3, including Y27A (34-fold reduction in affinity), N31A, Y32A, W50A, N52A, Y99A, S106A and W108A (each >150-fold reduction); N35A (66-fold reduction), P100A (38-fold reduction) and F110A (25-fold reduction).

In contrast, only one VL substitution had a large impact on binding affinity, W96A (>150-fold reduction). These results point to the three VH CDRs as the main energetic determinants of Fab binding to VEGF, with some contribution from VL3.

[0286] Design of Second-Generation CDR Mutation Libraries:

[0287] Two additional libraries which randomized existing residues in anti-VEGF version Y0192 were designed based upon inspection of the crystal structure. In VH2, residues 52-55 were randomized because they lie within the binding interface with VEGF. An additional region of the Fab, termed "CDR7" (see FIG. 10B), was also targeted for randomization because several residues in this loop, while not contacting VEGF, do have contacts with the VH loops of the antibody. These represented potential sites for affinity improvement through secondary effects upon the interface residues. Residues L72, T74, and S77 were randomized in this CDR7 library.

[0288] Also based upon the crystal structure, one of the original CDR libraries was reconstructed to re-test the potential for affinity maturation in the VH1 CDR. Residues 27, 28, and 30-32 were randomized using the new Y0192 background.

[0289] Second-Generation Selections of Anti-VEGF Libraries:

[0290] Based on Ala-scan results as well as the crystal structure of the antigen-antibody (F(ab)-12) complex, a total of seventeen libraries were constructed using the pY0192 template and stop-template oligonucleotides (which code for a stop codon at the sites targeted for randomization) YC-80, YC-100, YC-102, HL-263, and HL-264 (Table 9 above). The corresponding randomization oligonucleotides (which employ NNS at the sites targeted for randomization) were YC81, YC-101, YC-103, HL-265, and HL-266 (Table 10 above). The resulting transformants yielded libraries with complexities ranging from 6x10⁷ to 5x10⁸ which suggests that the libraries were comprehensive in covering all possible variants. Phage libraries were sorted for 7-8 rounds using conditions as described in Table 12 below.

TABLE 12

Conditions for Secondary Selections of Fab Variants			
Round of Selection	Incubation Time (hr)	Incubation Solution	Incubation Temp. (° C.)
1	0	0	room temp.
2	1	ELISA buffer	room temp.
3	2	1 μM VEGF/ELISA	room temp.
4	18	1 μM VEGF/ELISA	room temp.
5	37	1 μM VEGF/ELISA	room temp.
6	17 hr @ room temp./ 30 hr @ 37° C.	1 μM VEGF/ELISA	room temp./ 37° C.
7	63	1 μM VEGF/ELISA	37° C.
8	121	1 μM VEGF/ELISA	37° C.

[0291] ELISA buffer contained 0.5% bovine serum albumin and 0.05% TWEEN 20™ in PBS. VEGF was included in the incubation buffer to minimize rebinding of phages to VEGF coated on the surface of the plate. Sorting of these libraries yielded phage enrichments over 7 to 8 rounds of selection.

[0292] Phage-ELISA Assays of Second Generation Clones:

[0293] After eight round of selections, ten to twenty clones from each library were isolated from carbenicillin containing plates harboring *E. coli* (XL1) colonies which had been infected with an eluted phage pool. Colonies were isolated and grown with helper phage to obtain single-stranded DNA for sequencing. CDR substitutions selected for more favorable binding to VEGF were deduced from the DNA sequences of phagemid clones. A sampling of selected clones is shown in Table 13 below.

TABLE 13

Protein Sequences of Anti-VEGF Variants from Second Generation Fab-Phage Libraries	
Variants from library YC-81	
Name	VH3 sequence (residues 99–111)
Y0238-1	YPYYRGTSHWYFD (SEQ ID NO: 56)
Y0238-2	YPYYINKSHWYFD (SEQ ID NO: 57)
Y0238-3	YPYYGTSHWYFD (SEQ ID NO: 58)
Y0238-4	YPYYNQSHWYFD (SEQ ID NO: 59)
Y0238-5	YPYYIAKSHWYFD (SEQ ID NO: 60)
Y0238-6	YPYYRDNShWYFD (SEQ ID NO: 61)
Y0238-7	YPYYWGTSHWYFD (SEQ ID NO: 62)
Y0238-8	YPYYRQNSHWYFD (SEQ ID NO: 63)
Y0238-9	YPYYRQSSHwyFD (SEQ ID NO: 64)
Y0238-10	YPYYRNTSHWYFD (SEQ ID NO: 65)
Y0238-11	YPYYKNTSHWYFD (SEQ ID NO: 66)
Y0238-12	YPYYIERSHWYFD (SEQ ID NO: 67)
Y0228-21	YPYYRNASHWYFD (SEQ ID NO: 68)
Y0228-22	YPYYTTRSHWYFD (SEQ ID NO: 69)
Y0228-23	YPYYEGSSHWYFD (SEQ ID NO: 70)
Y0228-24	YPYYRQRGHWYFD (SEQ ID NO: 71)
Y0228-26	YPYYTGRSHWYFD (SEQ ID NO: 72)
Y0228-27	YPYYTNTSHWYFD (SEQ ID NO: 73)
Y0228-28	YPYYRKGSHWYFD (SEQ ID NO: 74)
Y0228-29	YPYYTGSSHWYFD (SEQ ID NO: 75)
Y0228-30	YPYYRSGSHWYFD (SEQ ID NO: 76)
Y0229-20	YPYYTNRSHWYFD (SEQ ID NO: 77)
Y0229-21	YPYYRNSSHwyFD (SEQ ID NO: 78)
Y0229-22	YPYYKESSHWYFD (SEQ ID NO: 79)
Y0229-23	YPYYRDASHWYFD (SEQ ID NO: 80)
Y0229-24	YPYYRQKGHWYFD (SEQ ID NO: 81)
Y0229-25	YPYYKGGSHWYFD (SEQ ID NO: 82)
Y0229-26	YPYYYGASHWYFD (SEQ ID NO: 83)
Y0229-27	YPYYRGESHWYFD (SEQ ID NO: 84)
Y0229-28	YPYYRSTSHWYFD (SEQ ID NO: 85)

TABLE 13-continued

Variants from library HL-265	
Name	VH1 sequence (residue 26–35)
Y0243-1	GYDFTHYGMN (5/10 clones) (SEQ ID NO: 86)
Y0243-2	GYEFQHYGMN (SEQ ID NO: 87)
Y0243-3	GYEFTHYGMN (SEQ ID NO: 88)
Y0243-4	GYDFGHyGMN (SEQ ID NO: 89)
Y0243-5	GYDFSHYGMN (SEQ ID NO: 90)
Y0243-6	GYEFShYGMN (SEQ ID NO: 91)

Variants from library YC-101	
Name	VH “CDR7” sequence (residues 70–79)
Y0244-1	FSVDVSKSTA (SEQ ID NO: 92)
Y0244-2	FSLDKSKSTA (SEQ ID NO: 93)
Y0244-3	FSLDVWKSTA (SEQ ID NO: 94)
Y0244-4	FSIDKSKSTA (95)

[0294] The sequence of the randomized region only is shown as deduced from DNA sequencing.

[0295] When a number of clones were tested along with the parent clone pY0192 in phage-ELISA assay, none showed a distinctive improvement over the parental clone. This could be explained by the time-scale on which the assay was performed (<3 hours).

[0296] In order to quantify improvement in antigen binding over parent clone, several anti-VEGF variants’ DNA were transformed into *E. coli* strain 34B8, expressed as Fab, and purified by passing the periplasmic shockate through a protein G column (Pharmacia) as described in Example 2 above.

[0297] CDR Combination Variants:

[0298] To improve VEGF binding affinity further, mutations found by phage display were combined in different CDRs to create multiple-CDR mutants. In particular, the mutations identified in the most affinity-improved phage variants from VH1, VH2, and VH3 libraries were combined (Table 14) in order to test for additivity of their contributions to binding affinity.

TABLE 14

Combination CDR Anti-VEGF Variants			
Name	Parent clone	Mutagenesis oligo/ comments	Sequence
Y0313-1	Y0243-1	YC-115 (VH3: H101Y and S105T)	5'-GCAAAGTACCCGTACTATTATGGGAC GAGCCACTGGTATTTC-3' (SEQ ID NO:96)
Y0317	Y0313-1	YC-108 (revert VL15' back to wild type)	5'-GTCACCATCACCTGCAGCGCAAGTCA GGATATTAGCAACTATTTAAC-3' (SEQ ID NO:97)
Y0313-3	Y0238-3	YC-116 (VH3; T105S)	5'-CCGTACTATTATGGGAGCAGCCACTG GTATTTC-3' (SEQ ID NO:98)

[0299] Mutations from the indicated parental vectors were combined with those from the indicated oligonucleotide by site-directed mutagenesis to yield the combination variants listed.

[0300] Version Y0317 is equivalent to Y0313-1 except that the background mutation in VL1 was removed and its sequence reverted back to that in pY0101. The effects of mutating H101Y and S105T were tested by constructing a reversion mutant from Y0238-3.

[0301] BIAcore Analysis:

[0302] The VEGF-binding affinities of Fab fragments were calculated from association and dissociation rate constants measured using a BIAcore-2000™ surface plasmon resonance system (BIAcore, Inc., Piscataway, N.J.). A bio-sensor chip was activated for covalent coupling of VEGF using N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's (BIAcore, Inc., Piscataway, N.J.) instructions. VEGF was buffered exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 µg/mL. An aliquot (35 µL) was injected at a flow rate of 2 µL/min to achieve approximately 700-1400 response units (RU) of coupled protein. Finally, 1 M ethanolamine was injected as a blocking agent.

[0303] For kinetics measurements, two-fold serial dilutions of Fab were injected in PBS/TWEEN™ buffer (0.05% TWEEN 20™ in phosphate buffered saline) at 25° C. at a flow rate of 10 µL/min. On rates and off rates were calculated using standard protocols (Karlsson et al. *J. Immun. Methods* 145:229-240 (1991)). Equilibrium dissociation constants, Kd's from surface plasmon resonance (SPR) measurements were calculated as koff/kon. Data are shown in Table 15 below.

TABLE 15

Kinetics of Fab-VEGF binding from BIAcore™ measurements				
Variant	Kon (10 ⁴ /M/s)	koff (10 ⁻⁴ /s)	Kd (nM)	Kd (wt)/ Kd (mut)
Y0244-1	3.4	2.7	8	3.6
Y0244-4	5.2	1.7	3.3	0.9
Y0243-1	6.7	0.45	0.7	4.1
Y0238-3	1.7	≤0.04*	≤0.2*	≥14*
Y0238-7	1.5	≤0.06*	≤0.4*	≥7.3*
Y0238-10	1.6	0.09	0.6	4.8
Y0238-5	0.8	0.08	0.9	3.2
Y0238-1	2.6	0.09	0.4	7.3
Y0313-1	3.5	≤0.054*	≤0.15*	≥20*
Y0313-3	1.2	0.081	0.65	4.5

*The dissociation rate observed probably reflects an upper limit for the true dissociation rate in these experiments, since the off-rate is approaching the limit of detection by BIAcore.

[0304] The BIAcore™ data in Table 15 show that several variants had improved affinity over Y0192. For example, a CDRH1 variant, Y0243-1, showed 4.1 fold enhanced affinity, arising from mutations T281D and N31 H. Variant Y0238-3 showed at least a 14 fold improvement in binding affinity over Y0192. Both CDRH3 mutations contribute to the improved affinity of Y0238-3 because reversion of T105 to S (variant Y0313-3) reduces the affinity of Y0238-3 from 0.1 5nM to 0.65 nM (see Table 15). The greater affinity

enhancement relative to Y0192 was seen for Y0313-1, which contained CDRH3 mutations combined with CDRH1 mutations.

[0305] Cell-Based Assay of VEGF Inhibition:

[0306] Several versions of the A4.6.1 anti-VEGF antibody were tested for their ability to antagonize VEGF (recombinant; version 1-165) in induction of the growth of HuVECs (human umbilical vein endothelial cells). The 96-well plates were seeded with 1000 HuVECs per well and fasted in assay medium (F12:DMEM 50:50 supplemented with 1.5% dialyzed fetal bovine serum) for 24 h. The concentration of VEGF used for inducing the cells was determined by first titrating for the amount of VEGF that can stimulate 80% of maximal DNA synthesis. Fresh assay medium containing fixed amounts of VEGF (0.2 nM final concentration), and increasing concentrations of anti-VEGF Fab or Mab were then added. After 40 h of incubation, DNA synthesis was measured by incorporation of tritiated thymidine. Cells were pulsed with 0.5 µCi per well of [3H]-thymidine for 24 h and harvested for counting, using a TopCount gamma counter.

[0307] The results (FIG. 11) show that the full-length IgG form of F(ab)-12 was significantly more potent in inhibiting VEGF activity than the Fab form (here, Y0192 was used). However, both variants Y0238-3 and Y0313-1 showed even more potent inhibition of VEGF activity than either the Y0192 Fab or F(ab)-12 Mab. Comparing the Fab forms, variant Y0313-1 appeared >30-fold more potent than the wild-type Fab. It should be noted that the amount of VEGF (0.2 nM) used in this assay is potentially limiting for determination of an accurate IC50 for the mutant. For example, if the binding affinity (Kd) of the mutant is in fact <0.2 nM, the IC50 in this experiment will appear higher than under conditions of lower VEGF concentration. The result therefore supports the conclusion that the affinity-improved variant is at least 30-fold improved in affinity for VEGF, and that it effectively blocks VEGF activity in vitro. Since the variant Y0317 differs from Y0313-1 only in the reversion of the VL1 sequence to wild-type (FIG. 10A), it is predicted that Y0317 will have similar activity to Y0313-1.

[0308] Variant Y0317 (Fab) and humanized variant F(ab)-12 from Example 1 (full length and Fab) were compared for their ability to inhibit bovine capillary endothelial cell proliferation in response to a near maximally effective concentration of VEGF using the assay described in Example 1. As illustrated in FIG. 12, Y0317 was markedly more effective at inhibiting bovine capillary endothelial cell proliferation than the full length and Fab forms of F(ab)-12 in this assay. The Y0317 affinity matured Fab demonstrated an ED50 value in this assay which was at least about 20 fold lower than F(ab)-12 Fab.

What is claimed is:

1. A humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 1×10⁻⁸M.
2. A humanized anti-VEGF antibody which binds human VEGF with a K_d value of no more than about 5×10⁻⁹M.
3. A humanized anti-VEGF antibody which has an ED50 value of no more than about 5 nM for inhibiting VEGF-induced proliferation of endothelial cells in vitro.
4. A humanized anti-VEGF antibody which inhibits VEGF-induced angiogenesis in vivo.

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5. The humanized anti-VEGF antibody of claim 4 wherein 5 mg/kg of the antibody inhibits at least about 50% of tumor growth in an A673 in vivo tumor model.

6. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYX₁FTX₂YGMN, wherein X₁ is T or D and X₂ is N or H; SEQ ID NO:128), CDRH2 (WINTYTGEPTYAADFQR; SEQ ID NO:2) and CDRH3 (YPX₁YYGX₂SHWYFDV, wherein X₁ is Y or H and X₂ is S or T; SEQ ID NO:129).

7. The humanized anti-VEGF antibody of claim 6 comprising the amino acid sequence of SEQ ID NO:7.

8. The humanized anti-VEGF antibody of claim 6 having a heavy chain variable domain comprising the following hypervariable region amino acid sequences: CDRH1 (GYTFTNYGMN; SEQ ID NO:1), CDRH2 (WINTYTGEPTYAADFQR; SEQ ID NO:2) and CDRH3 (YPHYGGSSHWYFDV; SEQ ID NO:3).

9. The humanized anti-VEGF antibody of claim 1 having a light chain variable domain comprising the following hypervariable region amino acid sequences: CDRL1 (SASQDISNYLN; SEQ ID NO:4), CDRL2 (FTSSLHS; SEQ ID NO:5) and CDRL3 (QQYSTVPWT; SEQ ID NO:6).

10. The humanized anti-VEGF antibody of claim 9 comprising the amino acid sequence of SEQ ID NO:8.

11. The humanized anti-VEGF antibody of claim 1 having a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:7 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO:8.

12. An anti-VEGF antibody light chain variable domain comprising the amino acid sequence: DIQX₁TQSPSSLSASVGDRVTITCSA SQDISNYLNWYQQKPGKAPKVLVYFTSSLHSGVPSRFS GSGSGTDFTLTSSLPEDFATYYCQQYSTVPWTF-GQGTKVEIKR (SEQ ID NO:124), wherein X₁ is M or L.

13. An anti-VEGF antibody heavy chain variable domain comprising the amino acid sequence: EVQLVESGGGLVQPG

GSLRLSCAASGYX₁FTX₂YGMNW VRQAPGK-
GLEWVGWINTYTGEPT YAADFQRRTF-
SLDTSKSTAY

LQMNSLR AEDTAVYYCAKYPX₃YYGX₄ SHWYFDVWGQGTILV TVSS (SEQ ID NO:125), wherein X₁ is T or D; X₂ is N or H; X₃ is Y or H and X₄ is S or T.

14. A variant of a parent anti-VEGF antibody, wherein said variant binds human VEGF and comprises an amino acid substitution in a hypervariable region of a heavy chain variable domain of said parent antibody.

15. The variant of claim 14 wherein said parent antibody is a human or humanized antibody.

16. The variant of claim 14 which binds human VEGF with a K_d value of no more than about 1×10⁻⁸M.

17. The variant of claim 14 which binds human VEGF with a K_d value of no more than about 5×10⁻⁹M.

18. The variant of claim 14 wherein the substitution is in CDRH1 of the heavy chain variable domain.

19. The variant of claim 14 wherein the substitution is in CDRH3 of the heavy chain variable domain.

20. The variant of claim 14 which has amino acid substitutions in both CDRH1 and CDRH3.

21. The variant of claim 14 which binds human VEGF with a K_d value less than that of said parent antibody.

22. The variant of claim 14 which has an ED50 value for inhibiting VEGF-induced proliferation of endothelial cells in vitro which is at least about 10 fold lower than that of said parent antibody.

23. The variant of claim 18 wherein the CDRH1 comprises the amino acid sequence: GYDFTHYGMN (SEQ ID NO:126)

24. The variant of claim 19 wherein the CDRH3 comprises the amino acid sequence: YPYYYGTSHWYFDV (SEQ ID NO:127).

25. The variant of claim 14 wherein the heavy chain variable domain comprises the amino acid sequence of SEQ ID NO:116.

26. The variant of claim 25 further comprising the light chain variable domain amino acid sequence of SEQ ID NO:124.

27. The variant of claim 26 comprising the light chain variable domain amino acid sequence of SEQ ID NO:115.

28. The humanized anti-VEGF antibody of claim 1 which is a full length antibody.

29. The humanized anti-VEGF antibody of claim 28 which is a human IgG.

30. The humanized anti-VEGF antibody of claim 1 which is an antibody fragment.

31. The antibody fragment of claim 30 which is a Fab.

32. A composition comprising the humanized anti-VEGF antibody of claim 1 and a pharmaceutically acceptable carrier.

33. A composition comprising the variant anti-VEGF antibody of claim 14 and a pharmaceutically acceptable carrier.

34. Isolated nucleic acid encoding the antibody of claim 1.

35. A vector comprising the nucleic acid of claim 34.

36. A host cell comprising the vector of claim 35.

37. A process of producing a humanized anti-VEGF antibody comprising culturing the host cell of claim 36 so that the nucleic acid is expressed.

38. The process of claim 37 further comprising recovering the humanized anti-VEGF antibody from the host cell culture.

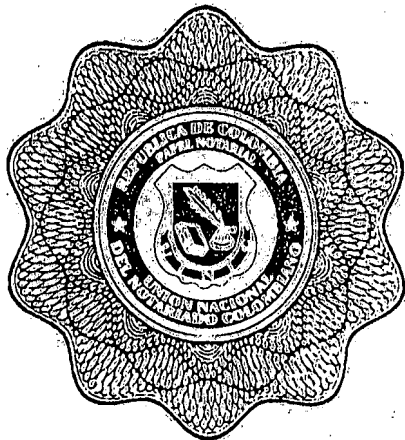
39. A method for inhibiting VEGF-induced angiogenesis in a mammal comprising administering a therapeutically effective amount of the humanized anti-VEGF antibody of claim 1 to the mammal.

40. The method of claim 39 wherein the mammal is a human.

41. The method of claim 39 wherein the mammal has a tumor.

42. The method of claim 39 wherein the mammal has a retinal disorder.

* * * * *



ESCRITURA NUMERO CUATRO MIL QUINIENTOS -
TREINTA Y NUEVE (No.4.539) -----
En la ciudad de Cali, Capital del Depar-
tamento del Valle del Cauca, República de
Colombia, a los VEINTITRES (23) días -
del mes de OCTUBRE del año DOS MIL UNO --

(2.001), ante mí, JORGE ENRIQUE CAICEDO ZAMORANO, Notario
T E R C E R O (3o.) del Círculo de Cali, - - - - -
- - - - -
- - - - -
compareció JUAN MANUEL BARBERI OSPINA mayor de edad, vecino de Cali,
identificado con la cédula de ciudadanía número 14.949.561 de Cali, y dijo: ----
PRIMERO.- Que obra en su carácter de Representante Legal de en nombre y
representación de TECNOQUIMICAS S.A. sociedad comercial organizada y existente
conforme a las leyes colombianas, con domicilio y sede principal en Cali constituida
por escritura pública número 2670 de la Notaría Segunda de Cali de fecha 12 de
junio de 1957, reformada en distintas oportunidades, según consta en el certificado
expedido por la Cámara de Comercio de Cali -----
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, identificado con C.C. # 79.152.473 de Bogotá, abogado en ejercicio, portador
de la T.P 44655 del C.S.de la J., domiciliado en Bogotá, D.C., República de Colombia
con dirección carrera 7 No. 88.23 oficina 205, de la misma ciudad, como su representante
con poder especial, amplio y suficiente, para obtener la protección de nuestra propiedad
industrial y contra la competencia desleal, a cuyo efecto lo autorizo, para que nos
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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.

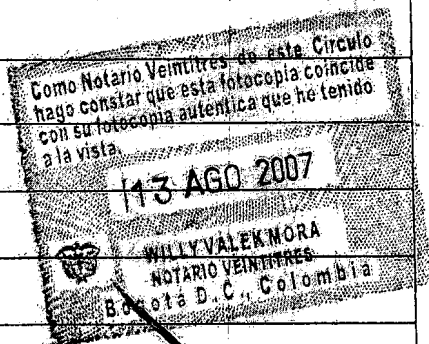
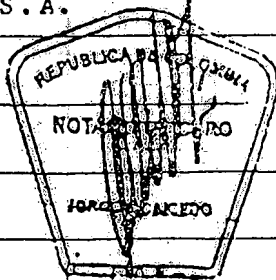
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Se agregan comprobantes. Derechos \$30.000.00 Decreto 1681 de Septiembre 16 de 1996. Reformado mediante Resolución No.5839 de Diciembre 27 del año 2.000 y Decreto 1326 de Julio 06 del año 2.001. Se corrió en la hoja número AA 3286877. ENMENDADO "OCTUBRE" VALE.

91 *Juan m. Barberi*

JUAN MANUEL BARBERI OSPINA

En rep. de TECNOQUIMICAS S.A.



El Notario Tercero de Cali

Dr. JORGE ENRIQUE CAICEDO ZAMORANO

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