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TÍTULO SOLICITADO:

"MOLÉCULAS DE UNIÓN BIESPECÍFICAS PARA LA
TERAPIA ANTI-ANGIOGÉNESIS"

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TRÁMITE:

SUSTENTO 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.**, dentro del trámite de la referencia, en ejercicio de lo dispuesto por el artículo 42 inciso 2° de la Decisión 486 de 2000, por medio del presente escrito, procedo a sustentar y complementar la oposición presentada el 12 de Diciembre de 2012, en los siguientes términos:

I. RATIFICACIÓN

La solicitud en curso, que se refiere a una molécula de unión biespecífica que comprende un componente de unión a DII4 (siglas en inglés de *Delta Like Ligand 4*) y un componente de unión a VEGF (Factor de Crecimiento Vascular Endotelial), la cual como se demostró, carece de los requisitos de Novedad y Nivel Inventivo, de acuerdo a la materia técnica revelada por los documentos WO 2009/134776,

WO2008/060705 y WO2008/019144 tal y como se demostró en el respectivo escrito de oposición y del cual nos permitimos ratificar lo siguiente:

- El capítulo reivindicatorio presentado ante la SIC no cumple con los requisitos mínimos de claridad, principalmente en lo que concierne con los requisitos de concisión y soporte en la descripción ya que como se demostró, la reivindicación 1 no define el objeto reclamado adecuadamente por sus características estructurales sino por su función de unirse a Dll4 y VEGF.
- Se determinó que la definición del componente de unión a Dll4 no está dada por las características técnicas estructurales en cuanto a la secuencia de aminoácidos o por el certificado de depósito de material biológico necesarios en este caso para dar sustento a cualquier técnico medio para llevar a cabo el objeto reclamado.
- Las moléculas se encuentran definidas erróneamente por la velocidad de inhibición, lo cual obedece a resultados a alcanzar, demostrando así la falta de concisión y sustento de la solicitud.
- Las reivindicaciones de la solicitud en trámite no cumplen con los requisitos mínimos para la patentabilidad puesto que no mencionan las características técnicas para definir su objeto o categoría, ya que no hay demostración ni evaluación de la actividad o efecto reclamado, así mismo, las moléculas de ácido nucleico no han sido definidas por su secuencia de bases nitrogenadas ni la célula hospedadora; por su parte, la composición farmacéutica no presenta sino un compuesto como ingrediente activo, lo cual no define una composición.
- Se objeta además que la reivindicación 32 menciona como característica excluyente la composición que contiene *“al menos una molécula de unión a VEGF”* lo que de otra manera quiere decir que se incluyen composiciones que comprenden como agente activo, anticuerpos Bevacizumab y Ranibizumab, entre otros.
- La publicación internacional WO 2009/134776 “Dual variable domain immunoglobulins and uses thereof” de Ghayur Tariq, publicado el 5 de

noviembre de 2009 destruye por completo la novedad de la solicitud ya que describe, al igual que la solicitud, moléculas biespecíficas que comprenden componentes de unión a DLL4 así como componentes de unión a VEGF. El abstract por su parte, menciona como eje central del documento proteínas de unión multiespecíficas, métodos para realizarlas y su uso específico en diagnóstico, prevención y/o tratamiento de enfermedades inflamatorias crónicas y agudas, cáncer y otras enfermedades; las moléculas que se describen en dicho documento tienen una región Fc y son usadas para el tratamiento de cáncer (ver página 26, línea 18 a página 38, línea 9 y página 143, líneas 9 a 30).

Dicha anterioridad demuestra que la tecnología de anticuerpos biespecíficos como lo hace la solicitud en estudio, es conocida en el estado de la técnica ya que únicamente se pone en práctica el cambio de las secuencias afines que se obtienen de combinar dos anticuerpos que se unen a diferentes receptores o antígenos (ver página 7, línea 6) entre los cuales se nombran particularmente la combinación VEGF y DLL4.

Queda claro, entonces, que cualquier anticuerpo o molécula de unión a los compuestos DLL4 y VEGF ya han sido divulgados en el estado de la técnica y que cualquier definición particular de secuencias diferentes que se unen a los mismos obedecen únicamente a una caracterización secuencial de dichos anticuerpos.

- Además del documento del estado de la técnica mencionado arriba, la materia era evidente según las enseñanzas de los documentos citados a continuación ya que afectan el nivel inventivo del capítulo reivindicatorio de la solicitud en trámite:
 - La solicitud internacional WO2008/060705 de Yan Minhong "*Anti-DLL4 antibodies and methods using same*" publicado el 22 de mayo de 2008.
 - La solicitud internacional WO2008/019144 de Wiegand Stanley, "*Therapeutic methods for treating vascular eye disorders with DLL4 antagonists*" publicado el 14 de febrero de 2008.

- La publicación internacional WO 2008/060705 se establece como el estado de la técnica más cercano ya que describe un método para tratar cáncer que involucra la coadministración de anticuerpos anti-DLL4 y anti-VEGF (ver WO2008/060705 página 14).

Este documento a pesar de no hacer mención de los anticuerpos duales, ya presentaba la solución del mismo problema técnico de la solicitud en trámite, al incluir en el tratamiento los dos anticuerpos Anti-DLL4 y Anti-VEGF y se había formulado la solución de incluirlos en las mismas patologías de uso en la solicitud en estudio, incluso, esta anterioridad describe anticuerpos multivalentes como parte de la invención determinados por los dominios duales presentados por la solicitud en estudio y los demás documentos mencionados en este memorial (ver página 62)

- Por su parte, la publicación internacional WO2008/19144 (ver abstract), revela métodos para la realización de anticuerpos duales; por esta razón, cualquier técnico medio enfrentado al problema de obtener moléculas biespecíficas de unión para los tratamientos de cáncer se habría enfocado bajo las enseñanzas de cualquiera de los documentos citados a la realización de anticuerpos biespecíficos que se unen a DLL4 y VEGF con la misma probabilidad de éxito de la solicitud en trámite ya que dicha terapia enfocada a estos dos ligandos ya era conocida.

De esta manera, se ratifica que lo reclamado y sus descripciones no son materia patentable ya que no cumplen con lo dispuesto en cuanto a Novedad y Nivel Inventivo por la Decisión 486 de la Comunidad Andina de Naciones.

II. COMPLEMENTO DE INFORMACIÓN

Se han citado documentos de patente que anteceden lo divulgado por la presente solicitud, sin embargo existen adicionalmente publicaciones científicas que anteceden de igual forma la materia reclamada tales como el documento, veamos:

- Shen et al., *"Single variable domain antibody as a versatile building block for the construction of IgG-like bispecific antibodies"*: describe anticuerpos

biespecíficos y su uso en tratamientos antitumorales al ser dirigidos hacia células citotóxicas que expresan antígenos diana.

En dicho documento se sugiere además, el uso de los anticuerpos biespecíficos de forma simultánea para bloquear la función de dos blancos asociados al tumor para el tratamiento de cáncer como se observa en el siguiente aparte:

Abstract

Bispecific antibodies (BsAb) have been traditionally utilized to redirect cytotoxic effector cells and agents to kill tumor cells expressing the target antigens. Recently a new concept is emerging to develop BsAb that simultaneously block the functions of two tumor-associated targets, *eg.*, growth factor receptors, for enhanced antitumor efficacies. Broad clinical applications of BsAb have been, and still are, significantly hampered by the difficulty in producing the materials in sufficient quantity and quality by traditional approaches. Here we describe a recombinant approach for the production of an Fc domain-containing, IgG-like tetravalent BsAb, using a single variable domain (sVD) antibody as a versatile building block. In this method, a sVD of a defined specificity is genetically fused to either the N-terminus of the light chain or the C-terminus of the heavy chain of a functional IgG antibody of a different specificity. A model BsAb was constructed using a sVD to mouse platelet derived growth factor receptor alpha and a conventional IgG antibody to mouse platelet derived growth factor receptor beta. The BsAb were expressed in mammalian cells and purified to homogeneity by a one-step Protein A affinity chromatography. Further, the BsAb retained the antigen binding specificity and the receptor neutralizing activity of both of its parent antibodies. Importantly, the BsAb inhibited the activation of both its target receptors in tumor cells stimulated by both platelet derived growth factor AA and BB, whereas the parent monospecific antibody only inhibited the activation of a single receptor stimulated by its cognate ligand. This format of BsAb should be readily applicable to the production of other BsAb recognizing any pairs of antigens.

Al igual que en la solicitud, lo descrito por el documento citado se dirige incluso a las mismas moléculas blanco tales como el VEGF (páginas 66, segunda columna):

Carter, 2001). In another recent report, we identified a single variable domain (sVD) antibody directed against mouse platelet derived growth factor receptor alpha (mPDGFR α), and described the production of an Fc domain-containing BsAb molecule by direct fusion of the sVD antibody to the N-terminus of the light chain (LC) of a functional IgG antibody directed against mouse vascular endothelial growth factor receptor 2 (VEGFR2).

En dicho artículo se demuestra que las formas para la producción de anticuerpos biespecíficos retienen la especificidad para los dos sitios de unión y conservan la actividad neutralizante que presenta cada uno de sus anticuerpos parentales (ver discusión página 72):

specificities. The BsAb are expressed in mammalian cells and are capable of binding both of its target antigens. Further, the BsAb retained the activities of both parent antibodies in blocking receptor/ligand

interaction and in neutralizing ligand-induced biological activities. This approach should be universally applicable to the construction of BsAb from antibodies recognizing any pair of antigens.

Adicionalmente, el documento concluye que la aproximación de estas técnicas universales aplicadas a la construcción de anticuerpos biespecíficos reconocerían cualquier par de antígenos para los cuales hayan sido diseñados.

De esta forma no existe ninguna ventaja técnica diferente a la divulgada por el documento de Shen et al., que pueda ser demostrar por la solicitud, y más aún cuando en las reivindicaciones de la misma solo se utiliza una descripción superficial de los anticuerpos biespecíficos dados por su molécula de unión.

Por otra parte, entre la basta selección de documentos que afectan tanto la novedad como el nivel inventivo de la solicitud se puede citar adicionalmente la siguiente publicación internacional:

- WO 2008/101985 *"Amino acid sequences directed against vascular endothelial growth factor and polypeptides comprising the same for the treatment of conditions and diseases characterized by excessive and/or pathological angiogenesis or neovascularization"* de Merchiers et al., publicado el 28 de agosto de 2008, revela anticuerpos biespecíficos basados en dominios de cadena pesada VHH con la finalidad de ser usado en tratamiento contra el cáncer; también, describe anticuerpos biespecíficos anti-VEGF como se observa en el siguiente aparte:

The small size of the Nanobody also makes them ideally suited for the preparation of bispecific, or multispecific polypeptides. A bispecific anti-VEGF/anti-VEGFR Nanobody or a bispecific anti-VEGF/anti-tumor Nanobody, for example, will specifically target the tumor side, while the anti-VEGF activity in the circulation remains low with a reduced the risk of side effects. Bispecific Nanobodies binding to two different epitopes on VEGF (e.g. the VEGFR-1 binding site and the VEGFR-2 binding site) might be advantageous because of their higher potency.

La anterioridad mencionada revela anticuerpos biespecíficos de unión anti-VEGF dirigidos a moléculas para tratar enfermedades oculares tales como AMD. Los anticuerpos anti DII4 igualmente se dirigen hacia enfermedades oculares por lo que la selección de dichos blancos de unión resulta evidente (página 6, líneas 12 – 20):

Anti-VEGF strategies are also FDA approved for AMD patients, using a recombinant humanized anti-VEGF Fab (rhuFab VEGF, Ranibizumab or LucentisTM) (Chen et al. J. Mol. Biol. 1999, 293: 865-881; Ferrara et al. Retina 2006, 26: 859-870). rhuFab VEGF has been found to reduce angiogenesis and vascular leakage in a primate model of AMD (Krzystolik et al. Arch. Ophthalmol. 2002, 120: 338-346). Local delivery of VEGF inhibitors to the eye causes fewer side effects than systemic administration. High intraocular concentrations can be achieved by intravitreal injections. Repeated injections for the treatment of a chronic disease such as diabetic retinopathy is, however, not ideal because of the risk of endophthalmitis, vitreous hemorrhage, and retinal detachment.

Por lo anterior, los anticuerpos biespecíficos reclamados en la presente solicitud, no demuestran ningún efecto técnico sorprendente o inesperado frente al arte anterior. En conclusión, la materia reclamada en la presente solicitud carece de novedad y de un efecto técnico sorprendente que soporte el nivel inventivo de la misma.

III. PRUEBAS

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

- Shen et al., *"Single variable domain antibody as a versatile building block for the construction of IgG-like bispecific antibodies"*
- WO 2008/101985 *"Amino acid sequences directed against vascular endothelial growth factor and polypeptides comprising the same for the treatment of conditions and diseases characterized by excessive and/or pathological angiogenesis or neovascularization"* de Merchiers et al., publicado el 28 de agosto de 2008.

Señor Superintendente,



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

Research paper

Single variable domain antibody as a versatile building block for the construction of IgG-like bispecific antibodies

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Abstract

Bispecific antibodies (BsAb) have been traditionally utilized to redirect cytotoxic effector cells and agents to kill tumor cells expressing the target antigens. Recently a new concept is emerging to develop BsAb that simultaneously block the functions of two tumor-associated targets, *eg.*, growth factor receptors, for enhanced antitumor efficacies. Broad clinical applications of BsAb have been, and still are, significantly hampered by the difficulty in producing the materials in sufficient quantity and quality by traditional approaches. Here we describe a recombinant approach for the production of an Fc domain-containing, IgG-like tetravalent BsAb, using a single variable domain (sVD) antibody as a versatile building block. In this method, a sVD of a defined specificity is genetically fused to either the N-terminus of the light chain or the C-terminus of the heavy chain of a functional IgG antibody of a different specificity. A model BsAb was constructed using a sVD to mouse platelet derived growth factor receptor alpha and a conventional IgG antibody to mouse platelet derived growth factor receptor beta. The BsAb were expressed in mammalian cells and purified to homogeneity by a one-step Protein A affinity chromatography. Further, the BsAb retained the antigen binding specificity and the receptor neutralizing activity of both of its parent antibodies. Importantly, the BsAb inhibited the activation of both its target receptors in tumor cells stimulated by both platelet derived growth factor AA and BB, whereas the parent monospecific antibody only inhibited the activation of a single receptor stimulated by its cognate ligand. This format of BsAb should be readily applicable to the production of other BsAb recognizing any pairs of antigens.

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Keywords: Bispecific antibody; Single domain antibody; Dual-targeting; Antibody engineering; PDGFR α ; PDGFR β

Abbreviations: BsAb, Bispecific antibody; HRP, horse radish peroxidase; HC, heavy chain; LC, light chain; mPDGFR α , mouse PDGF receptor α ; mPDGFR β , mouse PDGF receptor β ; PBS, phosphate buffered saline; PDGF, platelet derived growth factor; RT, room temperature; sVD, single variable domain; V, variable; V_L, variable light chain; V_H, variable heavy light; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

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1. Introduction

Bispecific antibodies (BsAb) are immunoglobulin (Ig)-based molecules that can simultaneously bind to two different epitopes on either the same or distinct antigens (Kipriyanov and Le Gall, 2004; Kontermann, 2005; Marvin and Zhu, 2005). They have been traditionally utilized to redirect cytotoxic immune effector cells or agents for enhanced killing of tumor cells expressing the target antigens (Chang et al., 2002; Baeuerle et al., 2003; Lum and Davol, 2005). Recently, a novel concept is emerging to develop BsAb that target simultaneously two tumor-associated antigens (*e.g.*, growth factor receptors) for down-regulation of multiple cell proliferation/survival pathways in order to achieve enhanced antitumor activity (Lu et al., 2001, 2004a,b, 2005; Jimenez et al., 2005; Jendreyko et al., 2005; for review, see Marvin and Zhu, 2006). A major obstacle in broad development of BsAb has been the difficulty of producing materials in sufficient quality and quantity for both preclinical and clinical studies. Traditionally, the main method of production of BsAb was by co-expression of both the light chains (LC) and both the heavy chains (HC) of two parent antibodies of different specificities in a single cell through either the hybrid hybridoma technique (Milstein and Cuello, 1983) or DNA co-transfection. Unfortunately, assuming there is no pairing preference between any particular LC and HC, random association between the two LC and HC would lead to the production of 10 different IgG species. Consequently, the desired binding-competent BsAb are only a minor product (in theory, an eighth of the total), and purification from the other species is very difficult. Another traditional method for BsAb production is chemical conjugation of two antibodies (or their fragments) of different specificities (Brennan et al., 1985). The chemical modification process may inactivate the antibodies or promote aggregation. As purification from undesired products remains difficult, the resulting low yield and poor quality BsAb make this process, like the hybrid hybridoma approach, unsuitable for the large scale production.

In recent years, a variety of recombinant methods have been developed for the efficient production of BsAb, especially for BsAb antibody fragments (Carter et al., 1995; Todorovska et al., 2001). BsAb fragments may possess some advantages over the full length IgG-like molecules for certain clinical applications, such as for tumor radioimaging and targeting, owing to its better tissue penetration and faster clearance from the circulation. On the other hand, IgG-like BsAb may prove to be better choices over smaller BsAb fragments for other *in vivo* applications, specifically for oncology indications, by providing the Fc domain that not only confers long

serum half-life but also supports secondary immune function, such as antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity. Unlike the significant achievement in recombinant production of BsAb fragments, the successful design and production of full length IgG-like, *i.e.*, Fc domain-containing, BsAb have been generally limited (Marvin and Zhu, 2005; Carter, 2001). In another recent report, we identified a single variable domain (sVD) antibody directed against mouse platelet derived growth factor receptor alpha (mPDGFR α), and described the production of an Fc domain-containing BsAb molecule by direct fusion of the sVD antibody to the N-terminus of the light chain (LC) of a functional IgG antibody directed against mouse vascular endothelial growth factor receptor 2 (VEGFR2). The resulting BsAb retained the binding specificities and the receptor-neutralizing activities of both of the parent antibodies (Shen et al., 2006). In this study, utilizing the same anti-PDGFR α sVD antibody and an IgG antibody directed against mouse platelet derived growth factor receptor beta (mPDGFR β) as the building blocks, we constructed an anti-PDGFR α $\times$ anti-PDGFR β BsAb in two different formats; in one format, the sVD was fused to the N-terminus of the LC of the IgG antibody (as previously described, see Shen et al., 2006), while in the other format, the sVD was fused to the C-terminus of the heavy chain (HC) of the IgG antibody. We demonstrated that both formats led to the production of functional BsAb that retained the binding specificities and neutralizing activities of their parent antibodies.

2. Materials and methods

2.1. Proteins and cells

Receptor proteins, mPDGFR α -Fc and mPDGFR β -Fc fusions, the ligands, the recombinant PDGF-AA and PDGF-BB, and the biotinylated anti-mPDGFR α and anti-PDGFR β antibodies were purchased from R&D Systems (Minneapolis, MN). Streptavidin–peroxidase conjugate was purchased from Jackson ImmunoResearch Laboratory. D122, a murine Lewis lung cell line, was obtained from American Type Culture Collection (ATCC, Manassas, VA). The identification and characterization of 1F2, the sVD antibody directed against mPDGFR α , have been previously described (Shen et al., 2006).

2.2. Identification of anti-mVEGFR2 antibody 1B3

The anti-mPDGFR β antibody, 1B3 IgG, was generated from a Fab fragment isolated from a Fab phage display library from Dyax Corp. (Hoet et al., 2005) by

immunopanning on mPDGFR β -Fc fusion protein immobilized on Maxisorp Star tubes, following a previously described procedure (Lu et al., 2002). 1B3 IgG binds specifically to mPDGFR β with a binding affinity of 0.04 nM as determined by BIAcore analysis. Further, the antibody blocks efficiently mPDGFR β /PDGF-BB interaction with an IC₅₀, the antibody concentration that inhibits 50% of binding of mPDGFR β to immobilized PDGF-BB in an ELISA-based assay, of approximately 1.2 nM (our unpublished results).

2.3. Construction of bispecific anti-mPDGFR α $\times$ anti-mPDGFR β antibodies based on the single V_H domain antibody 1F2

Two BsAb constructs were generated using the single V_H domain anti-mPDGFR α antibody, 1F2, and the full length IgG anti-mPDGFR β antibody, 1B3, as the building blocks (Fig. 1). In the first construct, 1F2 V_H domain was linked on its C-terminus via a 5 amino acid

linker, Ala-Ser-Thr-Lys-Gly, to the N-terminus of 1B3 LC. The resultant polypeptide, 1F2(V_H)-1B3(V_L-C_L), was then co-expressed with the 1B3 HC, 1B3(V_H-C_{H1}-C_{H2}-C_{H3}), to form the bispecific molecule, 1F2-1B3IgG. In the second construct, 1F2 V_H domain was linked on its N-terminus via a 6 amino acid linker, Asn-Ser-Pro-Pro-Ala-Ala, to the C-terminus of 1B3 HC. The resultant polypeptide, 1B3(V_H-C_{H1}-C_{H2}-C_{H3})-1F2(V_H), was then co-expressed with the 1B3 LC, 1B3(V_L-C_L), to form the bispecific molecule, 1B3IgG-1F2.

2.4. Expression and purification of BsAb

Both BsAb constructs were transiently expressed in COS-7 cells transfected with respective expression vector using Lipofectamine 2000 following the manufacturer's protocols (Invitrogen, Carlsbad, CA). The conditioned media were collected at 72 h after transfection, and pooled. Antibodies were purified from the supernatant of cell cultures by affinity chromatography affinity

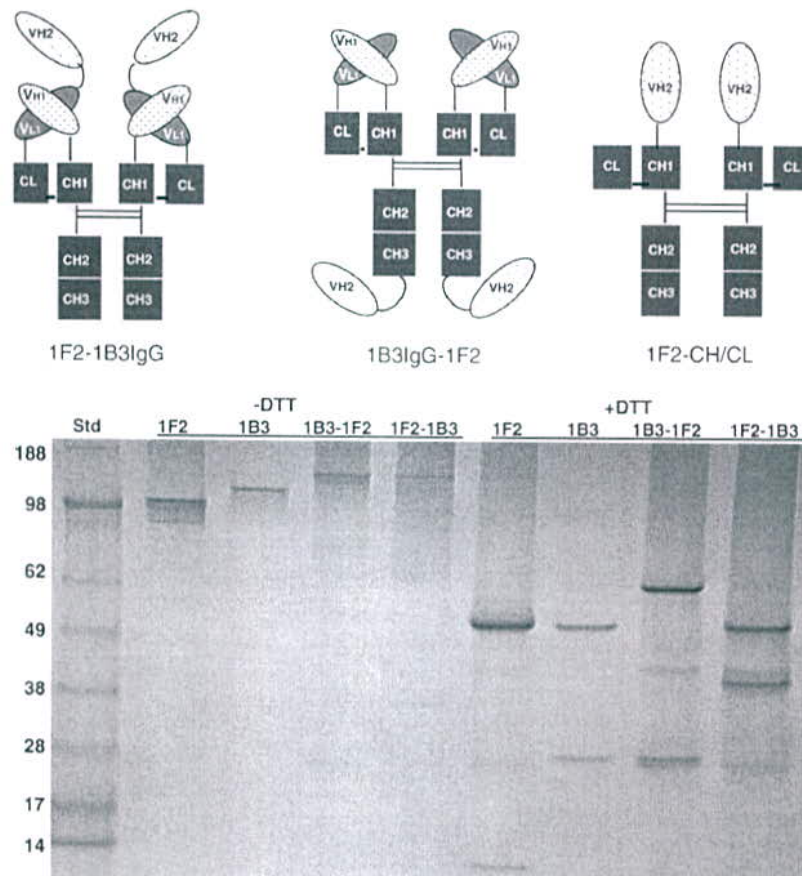


Fig. 1. (A) Schematic representation of the sVD/IgG fusion BsAb molecules, 1F2-1B3IgG and 1B3IgG-1F2, and monospecific, bivalent 1F2-C_{H1}/C_L. In BsAb format, 1F2 V_H was fused to either the N-terminus of 1B3 LC or the C-terminus of the 1B3 HC via a linker. Co-expression of the fusion polypeptide with either the normal 1B3 HC or the LC leads to the production of the bispecific molecules. (B) Expression and purification of the various antibody molecules in mammalian cells. The antibodies were transiently expressed in mammalian cells, purified by Protein A affinity chromatography, and analyzed by SDS-PAGE under both non-reducing (-DTT) and reducing (+DTT) conditions. Also shown on left are the positions of molecular weight standards in kDa.

chromatography using Protein-A columns following the manufacture's protocol (Amersham Biosciences). The purity and the molecular size of the purified proteins were analyzed by electrophoresis under both reducing and non-reducing conditions using a NuPAGE™ 4–12% Bis-Tris gel (Invitrogen) followed by visualization by staining with SimplyBlue™ SafeStain (Invitrogen). The antibody concentrations were determined by ELISA, using an anti-human Fc antibody as the capturing agent and an anti-human κ chain antibody–HRP conjugate as the detection agent.

2.5. Dual antigen binding of the BsAbs to mPDGFR α and mPDGFR β

Two assays were carried out to determine the dual antigen binding capability of the BsAbs. In the quantitative receptor binding assay, various amounts of purified antibodies were added to mPDGFR α or mPDGFR β -coated plates (1 μ g/ml), and incubated at RT for 1 h, after which the plates were washed 3 times with PBST. The plates were then incubated at RT for an additional 1 h with a rabbit anti-human κ chain antibody–HRP conjugate (Sigma). The plates were then washed 3 times, TMB peroxidase substrate (KPL, Gaithersburg, MD) was added, and the absorbance at 450 nm was read using a microplate reader (Molecular Device, Sunnyvale, CA). In second assay, the cross-linking assay, the BsAbs were tested for their capability in binding both of their target receptors simultaneously. Briefly, the BsAbs or their parent monospecific antibodies were first incubated with mPDGFR α or mPDGFR β in solution at RT for 1 h. The mixtures were transferred to a microtiter plate precoated with the second receptor, mPDGFR β or mPDGFR α (1 μ g/ml $\times$ 100 μ l/well overnight at 4 °C), respectively, and incubated at RT for 1 h, followed by incubation with a biotinylated polyclonal antibody to the first receptor (in solution), anti-mPDGFR α or anti-mPDGFR β , respectively, at RT for an additional hour. The plate was then incubated with a streptavidin–HRP conjugate (Jackson ImmunoResearch Laboratory), and the plate-bound BsAb-mPDGFR α or BsAb-mPDGFR β -complex was quantified by the addition of HRP substrate as described above.

2.6. Quantitative blocking assay

In the blocking assay, various amounts of antibodies were first mixed with a fixed amount of mPDGFR α (0.25 μ g/ml) or mPDGFR β (0.25 μ g/ml) and incubated at RT for 30 min. In another plate, PDGF-AA or PDGF-BB (0.1 μ g/ml) were captured by precoated polyclonal

anti-PDGF-AA antibody (0.5 μ g/ml) or anti-PDGF-BB antibody (0.5 μ g/ml). The receptor/antibody mixture was then transferred to the PDGF-AA or PDGF-BB-coated plate and incubated at RT for 1 h. After washing 3 times, the plates were incubated with a biotinylated anti-mPDGFR α or a biotinylated anti-mPDGFR β antibody (R&D Systems), followed by incubation with a streptavidin–HRP conjugate (Jackson ImmunoResearch Laboratory) for 1 h and developed as described above. IC₅₀, the antibody concentration that yielded 50% blockade of mPDGFR α or mPDGFR β from binding to its respective ligand, was determined.

2.7. Antibody affinity determination

The binding kinetics of various antibodies to their target receptors were measured using a BIAcore 3000 biosensor (BIAcore, Inc., Uppsala, Sweden). Briefly, mPDGFR α or mPDGFR β was immobilized onto a sensor chip and soluble antibodies were injected at concentrations ranging from 1.5 nM to 100 nM. Sensorgrams were obtained at each concentration and were evaluated using the program, BIA Evaluation 2.0. The affinity constant, K_d, was calculated from the ratio of dissociation rate (k_{off})/association rate (k_{on}).

2.8. In vitro stability study

The *in vitro* stability of 1F2-1B3IgG and 1B3IgG-1F2 was determined by incubation of the BsAbs and their parent antibodies, 1F2-C_H/C_L and 1B3 IgG, in 10% mouse serum/PBS at 37 °C for up to 7 days. Aliquots of untreated (day 0) and treated antibodies were taken at predefined intervals and were stored at –80 °C. Binding activity of the various samples against both mPDGFR α and mPDGFR β was determined by the direct binding ELISA described above.

2.9. Inhibition of ligand-stimulated receptor activation by the BsAbs

D122 cells, which express both mPDGFR α and mPDGFR β , were plated onto 6 cm dishes and grown to 70–80% confluence, after which the cells were washed twice in PBS and cultured overnight in serum free medium. The cells were first incubated with various antibodies at 37 °C for 30 min, followed by stimulation with PDGF-AA, PDGF-BB or a combination of PDGF-AA and PDGF-BB at 37 °C for 15 min. The cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Na₃VO₄, 1 μ g/ml

leupeptin, 1 $\mu\text{g/ml}$ pepstatin, and 1 $\mu\text{g/ml}$ aprotinin) for 1 h, followed by centrifugation of the lysate at 12,000 rpm for 10 min at 4 °C. The receptors were immunoprecipitated from the cell lysate supernatant by an anti-mPDGFR α antibody (R&D), an anti-mPDGFR β antibody (R&D), or a combination of both the antibodies, followed by the addition of 20 μl of ProA/G-sepharose beads (Santa Cruz Biotech). The precipitated receptor proteins were resolved on a NuPAGE™ 4–12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane. Phospho-mPDGFR α and phospho-mPDGFR β were detected on the blot using an anti-phospho-tyrosine antibody–HRP conjugate (Santa Cruz Biotech). Total receptor proteins loaded on the gel were assayed with antibodies to mPDGFR α or mPDGFR β (Santa Cruz Biotech).

3. Results

3.1. Construction and expression of sVD antibody-based BsAb

Two BsAb constructs, 1F2-1B3IgG and 1B3IgG-1F2, were generated using the gene sequences encoding the single V_H domain antibody 1F2 and the conventional IgG antibody, 1B3 (Fig. 1A). In the first format,

1F2 V_H domain was fused to the N-terminus of 1B3 V_L via a 5 amino acid linker to form 1F2(V_H)-1B3(LC), which was then co-expressed with the intact HC of 1B3. In the other format, 1F2 V_H domain was fused to the C-terminus of 1B3 HC via a 6 amino acid linker, to form 1B3(HC)-1F2(V_H). The resultant polypeptide was then co-expressed with the 1B3 LC. A previously described bivalent form of 1F2, 1F2-C_H/C_L, was used as a control in this study (Shen et al., 2006).

All the antibody molecules were transiently expressed in COS-7 cells and purified from the supernatant of cell cultures by affinity chromatography using Protein-A columns. The expression levels of the various antibodies were determined by ELISA. 1B3 IgG was the best expressed molecule with a level of approximately 13.9 ± 3.6 $\mu\text{g/ml}$ supernatant (mean $\pm$ SD of 3 to 4 independent experiments), followed by the bivalent monospecific 1F2-C_H/C_L at 6.0 ± 2.5 $\mu\text{g/ml}$. 1B3IgG-1F2 showed an expression level of 3.0 ± 2.8 $\mu\text{g/ml}$, whereas the expression of 1F2-1B3IgG was significantly lower at 0.5 ± 0.7 $\mu\text{g/ml}$.

The purified antibodies were analyzed by SDS-PAGE under both non-reducing (–DTT) and reducing conditions (+DTT) (Fig. 1B). When assayed under non-reducing conditions, both BsAb showed a single protein band of approximately 175 kD, whereas the divalent

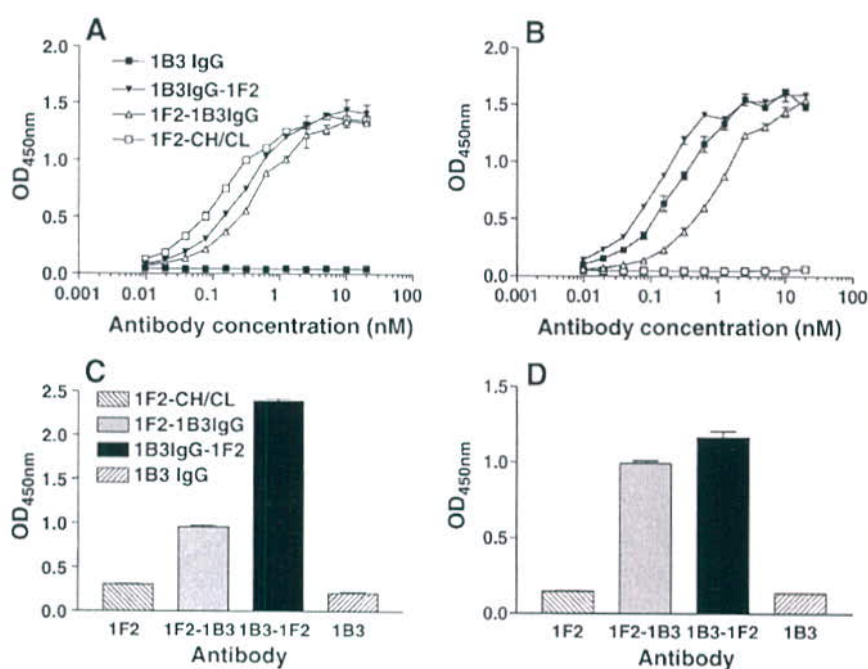


Fig. 2. Dual binding activity of the BsAb molecules. In the direct binding assay (A and B), various amounts of antibodies were first incubated in a 96-well plate precoated with mPDGFR α (A) or mPDGFR β (B) protein, followed by incubation with an anti-human κ antibody HRP conjugate. In the cross-linking assay (C and D), the BsAb or the monospecific antibodies were first incubated with mPDGFR α (C) or mPDGFR β (D) in solution and then transferred to a microtiter plate coated with the second receptor, mPDGFR β (C) or mPDGFR α (D), followed by incubation with a biotin-labeled polyclonal antibody to the first receptor (the receptor in solution), mPDGFR α or mPDGFR β , respectively. The plate was developed after further incubation with a streptavidin–HRP conjugate. Data shown represent the mean $\pm$ SD of triplicate determinations.

Table 1
Binding kinetics of the BsAb molecules to immobilized mPDGFR α and mPDGFR β

Antibody	mPDGFR α			mPDGFR β		
	kon ($\times 10^5$ 1/Ms)	koff ($\times 10^{-5}$ 1/s)	Kd ($\times 10^{-11}$ M)	kon ($\times 10^5$ 1/Ms)	koff ($\times 10^{-5}$ 1/s)	Kd ($\times 10^{-11}$ M)
1F2-C _H /C _L	4.3 $\pm$ 4.8 ^a	4.5 $\pm$ 1.1	3.7 $\pm$ 1.7	ND ^b	ND	ND
1B3IgG-1F2	8.1 $\pm$ 4.9	4.4 $\pm$ 3.5	8.0 $\pm$ 4.9	10.0 $\pm$ 5.0	7.0 $\pm$ 3.6	6.6 $\pm$ 1.1
1F2-1B3IgG	4.4 $\pm$ 0.91	6.7 $\pm$ 4.3	17.5 $\pm$ 1.3	6.7 $\pm$ 0.92	8.3 $\pm$ 6.9	11.5 $\pm$ 8.3
1B3 IgG	ND	ND	ND	5.9 $\pm$ 1.5	3.4 $\pm$ 0.25	6.1 $\pm$ 1.3

^a All numbers were determined by BIAcore analysis and represent the mean $\pm$ SE from triplicate determinations.

^b ND, not detectable.

monospecific 1F2-C_H/C_L demonstrated a single band at $\sim$ 125 kD, compared to that of 150 kD of 1B3 IgG, the conventional bivalent monospecific IgG (Fig. 1B). The two polypeptides of the antibodies were resolved under reducing conditions. While 1B3 IgG yielded two bands of 50 kD (the HC) and 25 kD (the LC), 1F2-C_H/C_L gave rise to two species of 50 kD (the HC) and 12.5 kD (the C_L domain). As expected, 1F2-1B3IgG showed two bands of 50 kD (the 1B3 HC) and 37.5 kD [the 1F2 (V_H)-1B3 LC fusion], whereas 1B3IgG-1F2 yielded two bands of 62.5 kD [the 1B3 HC-1F2(V_H) fusion] and 25 kD (the 1B3 LC).

3.2. Dual specificities of sVD antibody-based BsAb

The antigen binding efficiency of the BsAb was determined on immobilized mPDGFR α and mPDGFR β . While 1F2-C_H/C_L and 1B3 IgG bound only to their respective targets, the BsAb reacted to both immobilized mPDGFR α and mPDGFR β (Fig. 2A and B). Between the two BsAb constructs, 1B3IgG-1F2 showed better binding activity to both its targets, and was almost as efficient as its monospecific divalent counterparts. The ED₅₀ values, *i.e.*, the antibody concentrations that yield 50% of maximum binding, to mPDGFR α were 0.14 nM, 0.25 nM and 0.44 nM for 1F2-C_H/C_L, 1B3IgG-1F2 and 1F2-1B3IgG, respectively, and to mPDGFR β were 0.26 nM, 0.13 nM and 1.05 nM, for 1B3 IgG, 1B3IgG-1F2 and 1F2-1B3IgG, respectively. On BIAcore analysis, the BsAb, 1F2-1B3IgG and 1B3IgG-1F2, bind to both mPDGFR α and mPDGFR β with sub-nanomolar affinities (Table 1). The affinity of 1B3IgG-1F2 is similar to that of the monospecific 1B3 IgG in binding to mPDGFR β , and is only about 2-fold lower than that of the monospecific 1F2-C_L/C_H in binding to mPDGFR α . On the other hand, the affinity of 1F2-1B3IgG is about 2-fold lower than that the monospecific 1B3 IgG in binding to mPDGFR β , and is $\sim$ 4-fold lower than the monospecific 1F2-C_L/C_H in binding to mPDGFR α .

A receptor cross-linking assay was carried out to investigate whether the BsAbs are capable of simultaneously binding to both of their target antigens. In this

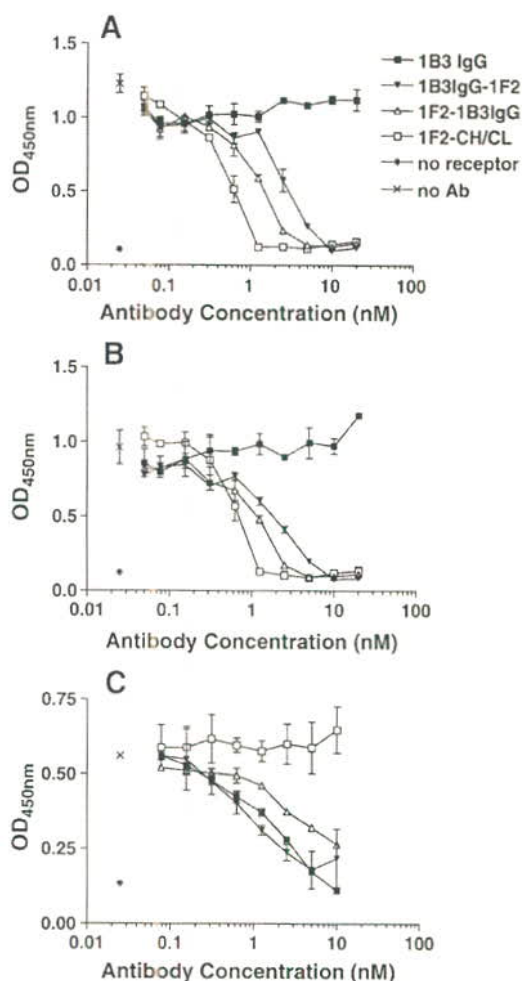


Fig. 3. Dual blocking assays of the BsAb. Various amounts of antibodies were incubated with a fixed amount of mPDGFR α (A and B) or mPDGFR β (C) proteins in solution for 30 min. The mixtures were then transferred to plates precoated with PDGF-AA (A) or PDGF-BB (B and C) and incubated for 1 h. The amounts of plate-bound receptors were then quantified by incubation with a biotinylated anti-mPDGFR α (A and B) or mPDGFR β (C) antibody, followed by a streptavidin-HRP conjugate. Data shown represent the mean $\pm$ SD of triplicate samples.

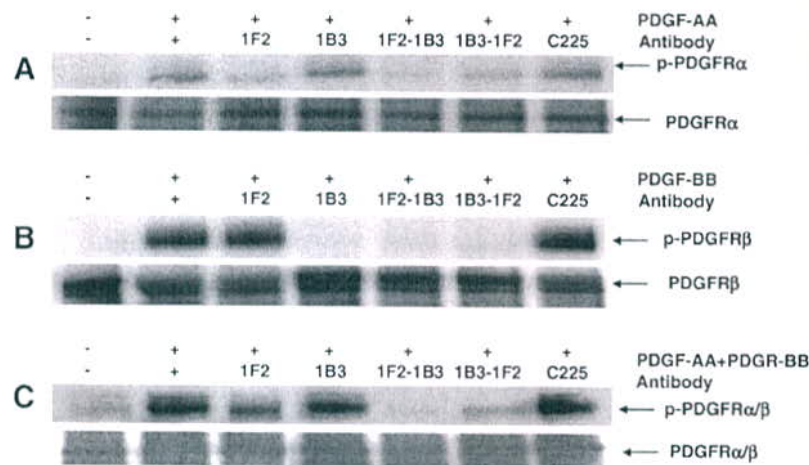


Fig. 4. Inhibition of PDGF-AA and PDGF-BB-stimulated receptor phosphorylation by the BsAb. D122 cells were first incubated with various antibodies at 37 °C for 30 min, followed by stimulation with PDGF-AA, PDGF-BB, or a combination of both ligands at 37 °C for 15 min. After cell lysis, the receptors were immunoprecipitated from the cell lysate supernatant by incubation with an anti-mPDGFR α , an anti-mPDGFR β , or a combination of both antibodies, followed by ProA/G-sepharose beads. The precipitated receptor proteins were resolved on a 4–12% Nupage Bis-Tris gel and transferred to a polyvinylidene difluoride membrane. Phospho-mPDGFR α and Phospho-mPDGFR β were detected on the blot using an anti-phospho-tyrosine antibody–HRP conjugate. Total receptor proteins loaded on the gel were assayed with antibodies to mPDGFR α and/or mPDGFR β .

assay, the BsAb was first allowed to bind to mPDGFR α or mPDGFR β receptor in solution, followed by incubation of complexes with the second receptor precoated on a microtiter plate, mPDGFR β or mPDGFR α , respectively, to test the capability of the BsAb in cross-linking the two receptors. As shown in Fig. 2C and D, both BsAb, but not the parent monospecific antibodies, efficiently cross-linked the two target receptors.

3.3. Inhibition of ligand/receptor interaction by the BsAb

Next we investigated whether the BsAb molecules are capable of blocking mPDGFR α and mPDGFR β from interacting with their ligands. While PDGF-BB binds both mPDGFR α and mPDGFR β , PDGF-AA only interacts with mPDGFR α . As shown in Fig. 3, the BsAb molecules blocked the interaction between PDGF-BB with both mPDGFR α and mPDGFR β , as well as between PDGF-AA and mPDGFR α . The IC_{50} for blocking mPDGFR α /PDGF-AA interaction (Fig. 3A) are ~ 2.8 nM and 1.3 nM for 1B3IgG-1F2 and 1F2-1B3IgG, respectively, compared to that of 0.5 nM for 1F2- C_H/C_L . On the other hand, the IC_{50} for blocking mPDGFR α /PDGF-BB (Fig. 3B) interaction are ~ 2.3 nM and 1.2 nM, for 1B3IgG-1F2 and 1F2-1B3IgG, respectively, compared to that of 0.62 nM for 1F2- C_H/C_L . Finally, the IC_{50} for blocking mPDGFR β /PDGF-BB (Fig. 3C) interaction are ~ 0.91 nM and 6.1 nM, for 1B3IgG-1F2 and 1F2-1B3IgG, respectively, compared to that of 1.1 nM for 1B3 IgG. As expected, 1B3 IgG had no

effects on mPDGFR α /PDGF-AA interaction, nor did 1F2- C_H/C_L have any effects on mPDGFR β /PDGF-BB interaction.

3.4. The BsAb inhibits ligand-induced activation of both mPDGFR α and mPDGFR β

The biological activity of the BsAb was investigated using D122 cells, a murine Lewis lung cell line that expresses both PDGFR α and PDGFR β receptors. D122 cells were first incubated with various antibodies, followed by stimulation with PDGF-AA, PDGF-BB or a combination of PDGF-AA and PDGF-BB. As shown in Fig. 4, the BsAb inhibited phosphorylation of both mPDGFR α and mPDGFR β that were stimulated by either only a single ligand or a combination of both ligands, whereas the monospecific parent antibodies only blocked the activation of a single receptor stimulated by its cognate ligand but had no effect on the activation of the other receptor. As a control, an antibody directed against epidermal growth factor receptor, C225, did not have any effect on ligand-stimulated activation of either mPDGFR α or mPDGFR β receptor.

3.5. In vitro stability of sVD antibody-based BsAb

In evaluating the stability of the BsAb and its parent molecules, the antibodies were incubated in 10% mouse serum in PBS at 37 °C for up to 7 days. Sample aliquots were taken at predefined intervals and examined for retention of binding activity to both target receptors. After

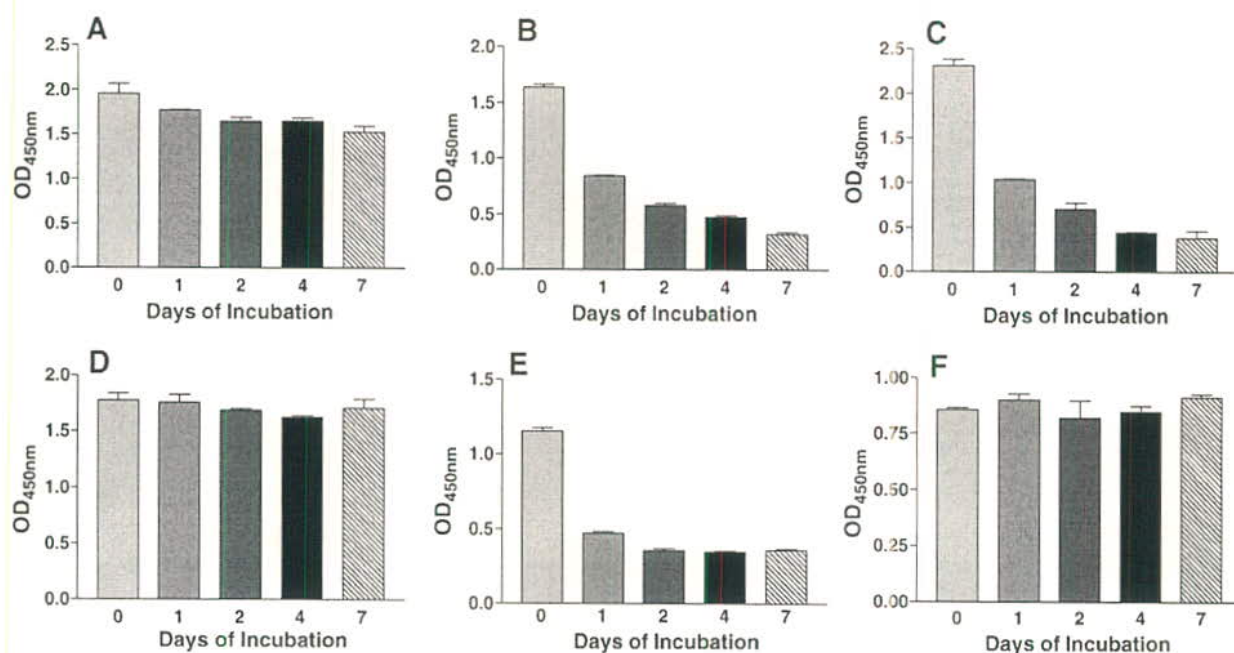


Fig. 5. Stability of the BsAb incubated at 37 °C in mouse serum. Various antibody preparations were added to 10% mouse serum (in PBS) and incubated at 37 °C. Aliquots of samples were removed at predefined intervals of incubation and assayed for efficiency for binding to both mPDGFRα (panels A to C) and mPDGFRβ (panels D to F) using an ELISA assay as described in the legend for Fig. 2. (A), 1F2-C_H/C_L; (D), 1B3 IgG; (B) and (E), 1F2-1B3IgG; (C) and (F), 1B3IgG-1F2. Data shown are those obtained at an antibody concentration of 2 nM, and represent the mean ± SD of triplicate determinations.

7-day incubation, the monospecific 1B3 IgG was still fully active to mPDGFRβ (Fig. 5D), whereas 1F2-C_H/C_L only retained approximately 78% of its original binding activity to mPDGFRα (Fig. 5A). 1B3IgG-1F2 BsAb retained full activity to mPDGFRβ (Fig. 5F), but showed progressive loss of binding to mPDGFRα during the prolonged incubation — after 24-h incubation, the BsAb lost ~56% mPDGFRα-binding activity, and at the end of study (7 days), only ~16% activity was retained (Fig. 5C). 1F2-1B3IgG BsAb showed significant loss in binding activity to both of its targets under the experimental conditions — after 24-h incubation, the BsAb lost ~49% and 59% binding activity to mPDGFRα and mPDGFRβ, respectively. After 7-day incubation, only ~20% (to mPDGFRα) and 31% (to mPDGFRβ) binding activity was retained (Fig. 5B and E).

4. Discussion

By using sVD as a versatile building block, we here describe a novel approach for the production of an Fc domain-containing, IgG-like, BsAb. In this method, the sVD was genetically fused to either the N-terminus or the C-terminus of an intact IgG antibody of different specificities. The BsAb are expressed in mammalian cells and are capable of binding both of its target antigens. Further, the BsAb retained the activities of both parent antibodies in blocking receptor/ligand

interaction and in neutralizing ligand-induced biological activities. This approach should be universally applicable to the construction of BsAb from antibodies recognizing any pair of antigens.

In the past several years, several approaches have been exploited for efficient production of IgG-like, Fc domain-containing BsAb molecules in mammalian cells (for reviews, see Carter, 2001; Marvin and Zhu, 2005). For example, production of homogeneous full length IgG-like BsAb has been achieved by the so-called “knobs-into-holes” engineering for efficient Ig C_{H3} domain heterodimerization plus the use of a common LC shared by two antibodies of different specificities (Ridgway et al., 1996; Atwell et al., 1997; Merchant et al., 1998). Alternatively, IgG-like BsAb have also been produced by linking two single chain Fv (scFv) of different specificities onto both the N-terminal of the C_L and the first constant heavy (C_{H1}) chains [the “(scFv)₄-IgG”] (Lu et al., 2004a; Zuo et al., 2000), or by fusing a scFv to the C-terminus of the HC of a full length IgG molecule of different specificities (Coloma and Morrison, 1997). A newly described IgG-like BsAb molecule, the “Di-diabody”, was efficiently expressed in mammalian cells (Lu et al., 2005). Though the Di-diabody demonstrated excellent dual-targeting activity *in vitro* and was as potent as the combination of the two parent antibodies in inhibiting tumor growth, it was significantly unstable *in vivo* most likely due to the lack of covalent linkage between the two polypeptide chains constituting

the bispecific molecule (our unpublished observation). The approach we described here, by genetically fusing a sVD antibody to either the N-terminus of the C-terminus of a full length IgG antibody of a different specificity, leads to the efficient production of a tetravalent (*ie.*, two binding sites to each target), Fc domain-containing BsAb. Like in the Di-diabody format (Lu et al., 2005), the intact natural Fc domain is expected to retain full capability in mediating immune effector functions including both antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity.

An apparent concern associated with the sVD–IgG or the IgG–sVD fusion BsAb format described here is that the direct fusion of a sVD to the N- or the C-terminus of an IgG may affect the overall folding as well as the antigen binding efficiency of both the parent molecules. It seemed that, for the pair of antibodies used in the study, 1F2 V_H and 1B3 IgG, the C-terminus fusion yielded better outcomes. 1B3IgG–1F2 showed a binding efficiency to both mPDGFR α and mPDGFR β that was very similar to its parent antibodies, whereas the binding of 1F2–1B3IgG to its targets was reduced by 2 to 4-fold (Fig. 2 and Table 1). This is in good consistency with that of our previous observations with the 1F2–2B4IgG BsAb (directed against mouse PDGFR α and mouse VEGFR2) (Shen et al., 2006). The effect of variable domain orientation on BsAb activity is, however, likely to be dependent upon the intrinsic properties of each individual antibody (Zhu et al., 1996; Kipriyanov et al., 2003; Lu et al., 2004a). In supporting this notion, we have observed that for a different sVD/IgG fusion BsAb, comprising of a sVD antibody to human VEGFR2 and a IgG antibody to human EGFR, the sVD–IgG N-terminus fusion led to a much better binding activity for the sVD while it had no effect on the activity of the IgG component, compared to its IgG–sVD C-terminus fusion counterpart (Lu, D., and Zhu, Z., unpublished observations). It has been suggested that individual antibodies may have different requirement for either free N- or free C-terminus of variable domains for maintaining antigen binding activity (Zhu et al., 1996; Kipriyanov et al., 2003; Lu et al., 2004a). Taken together with those of our previous observations (Shen et al., 2006; Lu et al., 2004a,b), these results underscore the importance of choosing optimal sVD/IgG orientations/arrangements for efficient production of active sVD-based BsAb.

In addition to antigen binding, product stability constitutes another important consideration in the development of antibody-based therapeutics. In our sVD/IgG fusion format one would expect that the BsAb have good stability since the sVD is genetically linked to

the IgG molecule. Thus it was rather surprising to note that both BsAb showed quick loss of mPDGFR α binding (sVD) activity when incubated in mouse serum at 37 °C. Two possible explanations on the loss of 1F2 activity include direct degradation of 1F2 sVD molecule and breakdown of 1F2 sVD from 1B3 IgG, leading to the loss of 1F2 from the BsAb molecule. The observation that the monospecific 1F2–C_H/C_L lost ~22% activity after the 7-day incubation is partly supportive to these hypotheses. It is interesting to note that, in contrast to the excellent stability shown by 1B3IgG–1F2 BsAb in binding to mPDGFR β (Fig. 5F), 1F2–1B3IgG lost ~60% mPDGFR β -binding activity at 24 h post-incubation (Fig. 5E). The loss of 1B3 activity in the latter case is most likely due to the negative influence imposed on the structural integrity and/or conformation of the IgG antibody during the breaking down of 1F2 sVD from the BsAb molecule (by either internal degradation or dissociation from 1B3 IgG). It is important to point out that, however, the instability issue observed with this particular BsAb molecule may not necessarily be generalized to other BsAb constructed in a similar manner. For example, for the anti-VEGFR2(sVD)–anti-EGFR(IgG) BsAb discussed above, the sVD–IgG N-terminus fusion BsAb (with the same linker as in 1F2–1B3IgG) retained ~84%, 58% and 36% of sVD activity at 1, 5 and 7 days post-incubation in mouse serum at 37 °C, respectively, while maintaining 100% of the IgG activity. Removal of the linker between the sVD and the IgG led to further improvement of BsAb stability — ~90%, 70% and 42% of sVD binding activity were retained at 1, 5 and 7 days post-incubation, respectively (Lu, D., and Zhu, Z., unpublished observations). It is, therefore, in addition to optimal sVD/IgG orientation/arrangement (as discussed above), the selection of sVD with good biochemical properties (*eg.*, good stability and expression), the choice of using (or not using) a linker between the sVD and IgG and the nature of the linker, should all be taken into consideration when designing and constructing a sVD-based BsAb molecule.

The prototype sVD antibody we utilized in this study, 1F2 V_H, was derived from a large Fab phage display library via immunopanning on immobilized mPDGFR α (Shen et al., 2006). Identification of sVD (V_L or V_H only) antibodies from both Fab and scFv phage display libraries has been, although infrequent, previously reported (Cai and Garen, 1996; our unpublished data). Recent progress in protein scaffold engineering has led to the production of a number of sVD antibody or sVD-like domain libraries, including those from the scaffolds of camelid V_HH, human V_H or V_L, lipocalins, shark V-

NAR, Protein A IgG-binding Z domain, and fibronectin type III domain (for reviews, see Holt et al., 2003; Binz and Pluckthun, 2005; Holliger and Hudson, 2005). With the availability of these libraries, sVD antibodies to various antigens can be readily identified. These sVD represent excellent building blocks for the construction of bi- or multispecific antibody molecules in both fragment and, especially, the IgG-like formats.

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(54) Title: AMINO ACID SEQUENCES DIRECTED AGAINST VASCULAR ENDOTHELIAL GROWTH FACTOR AND
POLYPEPTIDES COMPRISING THE SAME FOR THE TREATMENT OF CONDITIONS AND DISEASES CHARACTERIZED
BY EXCESSIVE AND/OR PATHOLOGICAL ANGIOGENESIS OR NEOVASCULARIZATION

(57) Abstract: The present invention relates to amino acid sequences that are directed against vascular endothelial growth factor
(VEGF), as well as to compounds or constructs, and in particular proteins and polypeptides, that comprise or essentially consist
of one or more such amino acid sequences. The amino acid sequences, compounds and constructs can be used for prophylactic,
therapeutic or diagnostic purposes, such as for the treatment of conditions and diseases characterized by excessive and/or pathological
angiogenesis or neovascularization.



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Amino acid sequences directed against vascular endothelial growth factor and polypeptides comprising the same for the treatment of conditions and diseases characterized by excessive and/or pathological angiogenesis or neovascularization

5 The present invention relates to amino acid sequences that are directed against (as defined herein) vascular endothelial growth factor (VEGF), as well as to compounds or constructs, and in particular proteins and polypeptides, that comprise or essentially consist of one or more such amino acid sequences (also referred to herein as "*amino acid sequences of the invention*", "*compounds of the invention*", and "*polypeptides of the invention*",
10 respectively).

 The invention also relates to nucleic acids encoding such amino acid sequences and polypeptides (also referred to herein as "*nucleic acids of the invention*" or "*nucleotide sequences of the invention*"); to methods for preparing such amino acid sequences and polypeptides; to host cells expressing or capable of expressing such amino acid sequences or
15 polypeptides; to compositions, and in particular to pharmaceutical compositions, that comprise such amino acid sequences, polypeptides, nucleic acids and/or host cells; and to uses of such amino acid sequences or polypeptides, nucleic acids, host cells and/or compositions, in particular for prophylactic, therapeutic or diagnostic purposes, such as the prophylactic, therapeutic or diagnostic purposes mentioned herein.

20 Other aspects, embodiments, advantages and applications of the invention will become clear from the further description herein.

 Angiogenesis is an important cellular event in which vascular endothelial cells proliferate, prune and reorganize to form new vessels from preexisting vascular network. The development of a vascular supply is essential for normal and pathological proliferative
25 processes (Folkman and Klagsbrun Science 1987, 235: 442-447). Delivery of oxygen and nutrients, as well as the removal of catabolic products, represent rate-limiting steps in the majority of growth processes occurring in multicellular organisms. In adults, angiogenesis is tightly controlled by an "angiogenic balance", i.e. a physiological balance between the stimulatory and inhibitory signals for blood vessel growth. In normal circumstances, the
30 formation of new blood vessels occurs during wound healing, organ regeneration, and in the female reproductive system during ovulation, menstruation, and formation of the placenta. It is also an important factor in several pathological processes such as tumor growth, rheumatoid arthritis, diabetic retinopathy, age-related macular degeneration, and psoriasis.

In view of the remarkable physiological and pathological importance of angiogenesis, much work has been dedicated to the elucidation of the factors capable of regulating this process. It is suggested that the angiogenesis process is regulated by a balance between pro and anti-angiogenic molecules, and is derailed in various diseases, especially cancer

(Carmeliet and Jain Nature 2000, 407: 249-257). A switch to the angiogenic phenotype depends on a local change in the balance between angiogenic stimulators and inhibitors.

One of the most important pro-angiogenic factors is vascular endothelial growth factor (VEGF), also termed VEGF-A or vascular permeability factor (VPF). VEGF belongs to a gene family that includes placenta growth factor (PlGF) (Maglione et al. Proc. Natl.

Acad. Sci USA 1991, 88: 9267-9271; Maglione et al. Oncogene 1993, 8: 925-931), VEGF-B (Olofsson et al. Proc. Natl. Acad. Sci USA 1996, 93: 2576-2581), VEGF-C (Joukov et al. EMBO J. 1996, 15: 1751-1758; Lee et al. Proc. Natl. Acad. Sci USA 1996, 93: 1988-1992), VEGF-D (Orlandini et al. Proc. Natl. Acad. Sci USA 1996, 93: 11675-11680, Achen et al. Proc. Natl. Acad. Sci USA 1998, 95: 548-553), VEGF-E (Hoeben et al. Pharmacol. Rev.

2004, 56: 549-580) and VEGF-F (Hoeben et al. Pharmacol. Rev. 2004, 56: 549-580). Human VEGF exists as at least six isoforms (VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206) that arise from alternative splicing of mRNA of a single gene (Ferrara and Davis-Smyth Endocr. Rev. 1997, 18: 1-22). VEGF165, the most abundant isoform, is a basic, heparin binding, dimeric glycoprotein with a molecular mass of 45,000 daltons.

Two VEGF tyrosine kinase receptors (VEGFR) have been identified that interact with VEGF, the fms-like tyrosine kinase Flt-1 (VEGFR-1 or Flt-1) and the kinase domain region, also referred to as fetal liver kinase (VEGFR-2, KDR or Flk-1) (Shibuya et al. Oncogene 1990, 5: 519-24; Matthews et al. Proc. Natl. Acad. Sci. USA 1991, 88: 9026-30; Terman et al. Oncogene 1991, 6: 1677-83; Terman et al. Biochem. Biophys. Res. Commun. 1992, 187: 1579-86; de Vries et al., Science 1992, 255: 989-91; Millauer et al. Cell 1993, 72: 835-46; Quinn et al. Proc. Natl. Acad. Sci. USA 1993, 90: 7533-7). VEGFR-1 has the highest affinity for VEGF, with a K_d of 10-20 pM (de Vries et al. Science 1992, 255: 989-91), and VEGFR-2 has a somewhat lower affinity for VEGF, with a K_d of 75-125 pM (Terman et al., Oncogene 1991, 6: 1677-83; Millauer et al. Cell 1993, 72: 835-46; Quinn et al. Proc. Natl. Acad. Sci. USA 1993, 90: 7533-7). A further detailed description of VEGF, the interaction of VEGF with its receptors and the function of VEGF in normal and pathological processes can

be found in Hoeben et al. (Pharmacol. Rev. 2004, 56: 549-580) and Ferrara (Endocrine Rev. 2004, 25: 581-611).

VEGF has been reported as a pivotal regulator of both normal and abnormal angiogenesis (Ferrara and Davis-Smyth Endocrine Rev. 1997, 18: 4-25; Ferrara J. Mol. Med. 1999, 77: 527-543). Compared to other growth factors that contribute to the processes of vascular formation, VEGF is unique in its high specificity for endothelial cells within the vascular system. VEGF is essential for embryonic vasculogenesis and angiogenesis (Carmeliet et al. Nature 1996, 380: 435-439; Ferrara et al. Nature 1996, 380: 439-442). Furthermore, VEGF is required for the cyclical blood vessel proliferation in the female reproductive tract and for bone growth and cartilage formation (Ferrara et al. Nature Med. 1998, 4: 336-340; Gerber et al. Nature Med. 1999, 5: 623-628).

In addition to being an angiogenic factor in angiogenesis and vasculogenesis, VEGF, as a pleiotropic growth factor, exhibits multiple biological effects in other physiological processes, such as endothelial cell survival, vessel permeability and vasodilation, monocyte chemotaxis and calcium influx (Ferrara and Davis-Smyth Endocrine Rev. 1997, 18: 4-25). Moreover, recent studies have reported mitogenic effects of VEGF on a few non-endothelial cell types, such as retinal pigment epithelial cells, pancreatic duct cells and Schwann cells (Guerrin et al. J. Cell Physiol. 1995, 164: 385-394; Oberge-Welsh et al. Mol. Cell. Endocrinol. 1997, 126: 125-132; Sondell et al. J. Neurosci. 1999, 19: 5731-5740).

VEGF is also implicated in the development of conditions or diseases that involve pathological angiogenesis. The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman et al. J Clin Invest 1993, 91: 153-159; Brown et al. Cancer Res. 1993, 53: 4727-4735; Brown et al. Human Pathol. 1995, 26: 86-91; Dvorak et al. Am J. Pathol. 1995, 146: 1029-1039; Mattern et al. Brit. J. Cancer. 1996, 73: 931-934).

The concentration of VEGF in eye fluids is highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (Aiello et al. N. Engl. J. Med. 1994, 331: 1480-1487).

Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez et al. Invest. Ophthalmol. Vis. Sci. 1996, 37: 855-868). Age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment.

VEGF up-regulation has also been observed in various inflammatory disorders (Dvorak J. Clin. Oncol. 2002, 20: 4368-4380). VEGF has been implicated in the pathogenesis of RA, an inflammatory disease in which angiogenesis plays a significant role (Koch et al. J. Immunol. 1994, 152: 4149-4156; Fava et al. J. Exp. Med. 1994, 180: 341-346). VEGF is strongly expressed by epidermal keratinocytes in wound healing and psoriasis, conditions that are characterized by increased microvascular permeability and angiogenesis (Detmar et al. J. Invest. Dermatol. 1995, 105: 44-50). VEGF up-regulation has also been observed in the development of brain edema. Diffuse, low-abundance, VEGF mRNA expression has been observed in the adult rat brain (Monacci et al. Am. J. Physiol. 1993, 264: C995-C1002).

The elucidation of VEGF and its role in angiogenesis and different processes has provided a potential new target of therapeutic intervention. The VEGF function has been inhibited by small molecules that block or prevent activation of VEGF receptor tyrosine kinases (Schlaeppli and Wood Cancer Metastasis Rev. 1999, 18: 473-481) and consequently interfere with the VEGF signal transduction pathway. Tumor cell-specific cytotoxic conjugates containing bacterial or plant toxins can inhibit the stimulating effect of VEGF on tumor angiogenesis. VEGF₁₆₅-DT385 conjugates (diphtheria toxin domains fused or chemically conjugated to VEGF₁₆₅), for example, efficiently inhibit tumor growth in vivo (Olson et al. Int. J. Cancer 1997, 73: 865-870). Tumor growth inhibition was also demonstrated with a retrovirus-delivered Flk-1 mutant (Millauer et al. Nature 1994, 367: 576-579) and soluble VEGF receptors (Kong et al. Hum. Gene Ther. 1998, 9: 823-833; Goldman et al. Proc. Natl. Acad. Sci. USA 1998, 95: 8795-8800; Gerber et al. Cancer Res. 2000, 60: 6253-6258; Kuo et al. Proc. Natl. Acad. Sci. USA 2001, 98: 4605-4610; Holash et al. Proc. Natl. Acad. Sci. USA 2002, 99: 11393-11398).

VEGF-neutralizing antibodies, such as A4.6.1 and MV833, have been developed to block VEGF from binding to its receptors and have shown preclinical antitumor activity (Kim et al. Nature 1993, 362: 841-844; Folkman Nat. Med. 1995, 1: 27-31; Presta et al. Cancer Res. 1997, 57: 4593-4599; Kanai et al. Int. J. Cancer 1998, 77: 933-936; Ferrara and Alitalo Nat. Med. 1999, 5: 1359-1364; 320, 340). Anti-VEGF antibody treatment generally converts fast-growing, angiogenesis-dependent, human tumor xenografts transplanted subcutaneously in nude or severe combined immune deficiency mice into small, avascularized microcolonies.

For a review of anti-VEGF approaches in clinical trials, see Campochiaro and Hackett (Oncogene 2003, 22: 6537-6548).

Most clinical experience has been obtained with A4.6.1, also called bevacizumab (Avastin®; Genentech, San Francisco, CA) (Bunn In: *Proceedings of the Annual Meeting of the American Society of Clinical Oncology* 2001, May 12-15, San Francisco, Vol. 20, pp 395-406, American Society of Clinical Oncology, Chestnut Hill, MA; Margolin et al. *J. Clin.*

5 Oncol. 2001, 19: 851-856). Avastin in combination with chemotherapy is FDA approved or in clinical trial for a large number of cancer indications. This product combination, however, is plagued by side-effects (hemorrhages, arterial thromboembolism, hypertension, gastrointestinal (GI) perforations, wound healing problems, proteinuria and congestive heart failure) which are primarily due to the fact that the anti-VEGF activity is not restricted to the
10 site of the tumor, but persists in circulation over a long period of time. This results in a shift of physiological to pathophysiological activity of the peripheral endothelial cells.

Anti-VEGF strategies are also FDA approved for AMD patients, using a recombinant humanized anti-VEGF Fab (rhuFab VEGF, Ranibizumab or Lucentis™) (Chen et al. *J. Mol. Biol.* 1999, 293: 865-881; Ferrara et al. *Retina* 2006, 26: 859-870). rhuFab VEGF has been
15 found to reduce angiogenesis and vascular leakage in a primate model of AMD (Krzystolik et al. *Arch. Ophthalmol.* 2002, 120: 338-346). Local delivery of VEGF inhibitors to the eye causes fewer side effects than systemic administration. High intraocular concentrations can be achieved by intravitreal injections. Repeated injections for the treatment of a chronic disease such as diabetic retinopathy is, however, not ideal because of the risk of
20 endophthalmitis, vitreous hemorrhage, and retinal detachment.

Nanobodies (as further defined herein) are more potent and more stable than conventional four-chain antibodies which leads to (1) lower dosage forms, less frequent dosage leading to less side effects; and (2) improved stability leading to a broader choice of administration routes, comprising oral or subcutaneous routes and slow-release formulations
25 in addition to the intravenous route. Slow-release formulation with stable anti-VEGF Nanobodies, for example, could be advantageous for treatment of AMD, avoiding the need of repeated injections and the side effects associated with it. In addition, their small size and short half-lives makes them specifically suited for treatment of AMD. The small size will facilitate the penetration of the Nanobodies deeper into the eye to reach the choroidal vessels.

30 Because of their small size, Nanobodies have the ability to cross membranes and penetrate into physiological compartments, tissues and organs not accessible to other, larger polypeptides and proteins. The small-sized Nanobodies have a shorter half-life and accumulate rapidly in the kidney and bladder where they stay for more than 48 hours. This

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makes them also ideally suited for the treatment of renal cell carcinoma and bladder cancer. Upon systemic administration of, for example, an anti-VEGF Nanobody, there would be a low anti-VEGF activity in the circulation with a reduced risk of side effects, while obtaining a high anti-VEGF activity at the side of the tumor and an effective treatment of the kidney or bladder carcinoma.

Because of their small size, Nanobodies can also selectively bind a specific epitope on VEGF (such as e.g. the VEGFR-2 binding site) while not (sterically) blocking other epitopes (such as e.g. the VEGFR-1 binding site). This may result in a selective inhibition of certain biological processes, while other biological processes are not inhibited, reducing the risk of side effects. It has indeed been shown that a monoclonal antibody (2C3) that blocks the binding of VEGF to VEGFR-2, but not to VEGFR-1, causes apoptosis of the endothelial cells of newly formed immature vessels, which are dependent on VEGF to maintain cell adhesion to a provisional extracellular matrix until periendothelial cells facilitate a more permanent mode of adhesion, while the integrity of mature vessels is not influenced by this antiangiogenic therapy (Brekken et al. Cancer Res. 2000, 60: 5117-5124).

The small size of the Nanobody also makes them ideally suited for the preparation of bispecific, or multispecific polypeptides. A bispecific anti-VEGF/anti-VEGFR Nanobody or a bispecific anti-VEGF/anti-tumor Nanobody, for example, will specifically target the tumor side, while the anti-VEGF activity in the circulation remains low with a reduced the risk of side effects. Bispecific Nanobodies binding to two different epitopes on VEGF (e.g. the VEGFR-1 binding site and the VEGFR-2 binding site) might be advantageous because of their higher potency.

The polypeptides and compositions of the present invention can generally be used to modulate, and in particular inhibit and/or prevent, binding of VEGF to VEGFR, and thus to modulate, and in particular inhibit or prevent, the signalling that is mediated by VEGF and/or VEGFR, to modulate the biological pathways in which VEGF and/or VEGFR are involved, and/or to modulate the biological mechanisms, responses and effects associated with such signalling or these pathways.

As such, the polypeptides and compositions of the present invention can be used for the prevention and treatment (as defined herein) of conditions and diseases characterized by excessive and/or pathological angiogenesis or neovascularization. Generally, "conditions and diseases characterized by excessive and/or pathological angiogenesis or neovascularization" can be defined as diseases and disorders that can be prevented and/or treated, respectively, by