Original Article

Expression, purification, and characterization of a diabody against the most important angiogenesis cell receptor: Vascular endothelial growth factor receptor 2

Mahdi Behdani[1], Sirous Zeinali[1], Morteza Karimipour[1], Hossein Khanahmad[1,2], Nader Asadzadeh[3], Kayhan Azadmanesh[4], Negar Seyed[1], Seyed Farzad Baniahmad[1], Mahdi Habibi Anbouhi[1]

[1]Department of Molecular Medicine, Pasteur Institute of Iran, [2]Department of Anatomical Sciences, Division of Genetics and Molecular Biology, Faculty of Medicine, Isfahan University of Medical Sciences, [3]Department of Animal Breeding and Genetics, Animal Science Research Institute of Iran, [4]Department of Virology, Pasteur Institute of Iran, Tehran, Iran

Abstract

Antibodies and their derivative fragments have long been used as tools in a variety of applications, in fundamental research work, biotechnology, diagnosis, and therapy. Camels produce single heavy-chain antibodies (VHH) in addition to usual antibodies. These minimal-sized binders are very robust and bind the antigen with high affinity in a monomeric state. Vascular endothelial growth factor recepror-2 (VEGFR2) is an important tumor-associated receptor that blockade of its signaling can lead to the inhibition of neovascularization and tumor metastasis. Here, we describe the construction, expression, and purification VEGFR2-specific Diabody. Two variable fragments of a same camel anti-VEGFR2 antibody were linked together by the upper hinge segment of antibody to make a diabody. We showed the ability of diabody to recognition of VEGFR2 on the cell surface by FACS. Diabodies can be produced in the low-cost prokaryotic expression system, so they are suitable molecules for diagnostic and therapeutic issues.

Key Words: Diabody, Nanobody, vascular endothelial growth factor recepror-2

Address for correspondence:
Dr. Hossein Khanahmad Shahreza, Department of Anatomical Sciences, Division of Genetics and Molecular Biology, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. Postal code: 8174673461. E-mail: hossein_khanahmad@yahoo.com
Received: 13.12.2011, **Accepted:** 18.02.2012

INTRODUCTION

Antibodies and their products have been used as versatile tools in many areas. Their applications in fundamental research work and also in diagnosis and therapy have been reported many times. For instance, utilization of antibodies as drug delivery vehicles, or in cancer therapy as triggers for immune response can be mentioned as some successful achievements.[1] High-yield production, solubility, stability, and small size are critical factors. Regarding this, many attempts to reduce the size of the conventional heterotetrameric IgG molecule (MW; 160 kDa), while retaining its antigen-binding properties, have been conducted. This resulted in a series of antibody fragment constructs, such as Fabs, Fvs, scFvs, dsFvs, and even single-

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.100126

How to cite this article: Behdani M, Zeinali S, Karimipour M, Khanahmad H, Asadzadeh N, Azadmanesh K, *et al.* Expression, purification, and characterization of a diabody against the most important angiogenesis cell receptor: Vascular endothelial growth factor receptor 2. Adv Biomed Res 2012;1:34.

domain VHs, which can be expressed in *Escherichia coli*, yeast or myeloma cells.[2]

Camelids generate antibodies that formed by two heavy chains, but no light chains. These immunoglobulins (MW; 95 kDa), referred to heavy-chain antibodies, constitute a major fraction of the functional antibodies in camels (up to 50% in camels). Refined structural changes in the variable domain of the naturally occurring heavy-chain antibodies (named as VHH or Nanobody) compensate for the absence of light chain variable domain.[3,4]

Vascular endothelial growth factor (VEGF) and its receptors; VEGFR-1, 2, 3, especially VEGFR2, play particularly an important role in angiogenesis under both physiological and pathological conditions.[5,6] VEGFR2 seems to be the major transducer of VEGF signals in endothelial cells that result in cell proliferation, migration, differentiation, tube formation, increasing vascular permeability, and maintenance of the vessels.[7] Thus, some therapies based on antibody could be one of the possible and also effective therapeutic strategies for inhibition of tumor growth and metastasis by blocking angiogenesis pathways in affected tissues through inhibition of VEGF or its receptor signaling system.[8]

Our research group has previously characterized a high affinity VEGFR2-specific Nanobody, and *in vitro* studies demonstrated the ability of this Nanobody, termed 3VGR19, to bind VEGFR2 on the cell surface.[9] In this study, we show that the affinity of binding to antigen does not change by constructing the diabody. This study is an introduction to *in vivo* tests to evaluate the performance of this antibody.

MATERIALS AND METHODS

Diabody gene construction

The VEGFR2-specific Nanobody gene[9] was amplified from pHEN-4 plasmid, which contains VEGFR2-specific Nanobody (3VGR19) gene by using forward, A6E (5´-GAT GTG CAG CTG CAG GAG TCT GGR GGA GG-3´), and reverse 38 (5´-GGA CTA GTG CGG CCG CTG GAG ACG GTG ACC TGG GT-3´) primers and subcloned into the pHEN6C vector in PstI and BstEII restriction sites. For diabody construction, the 3VGR19 gene amplified again with BiNb-Sense (5´-GCC CAG CCG GCC ATG GCC CAG KTG CAG CTA CAG GAG TCN GGN GG-3´) and BiNb-Llama-IgG2C-hinge (5´-GCC TGA TTC CTG CAG CTG CAC CTG TGC CAT TGG AGC TTT GGG AGC TTT GGA GCT GGG GTC TTC GCT GTG GTG CGC TGA GGA GAC GGT GAC CTG GGT-3´) primers. The PCR product was purified and digested with PstI and NcoI and ligated with pHEN6C vector that contains the first Nanobody.[9] Furthermore, the linker sequence of hinge region of Llama IgG2 was added to the respected construct with BiNb-Llama-IgG2C-hinge primer. The resulting plasmid was confirmed by sequencing and named as pHEN6c-3VGR19-Dia.

Expression and purification

The recombinant plasmid pHEN6C-3VGR19-Dia was transformed in competent WK-6 E. coli cells and the cells were plated on Luria–Bertani (LB) agar plates supplemented with 1% glucose and 100 µg/mL ampicillin. After an overnight incubation, fresh colonies inoculated in 5 mL terrific broth (TB) medium with additional 20% glucose and 2 mM MgCl2, then scaled up bacterial culture from 5 mL to 5 L in shaker incubator at 37°C until the OD 600 reached to 0.6 and then induced with 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG). After induction, cells were allowed to grow and express for 12–16 h before harvesting the cell pellet. The cells were harvested by 8 min centrifugation at about 6500×*g*. After pelleting the cells, the periplasmic proteins were extracted by the osmotic shock.[10] This periplasmic extract was loaded on a His-Select column (Sigma-Aldrich). After washing with PBS, the bonded proteins were eluted with 500 mM imidazole and loaded on Sephadex S75 columns (Pharmacia Biotech) and concentrated on Vivaspin concentrators (Sartorius Stedim Biotech) with a molecular mass cut off of 5 kDa.

SDS-PAGE and western blotting

SDS–PAGE was performed in a 12% (w/v) NuPAGE™ in accordance with the method described by supplier (Invitrogen-USA). The purified protein samples mixed with the same volume of loading buffer were boiled at 100°C for 10 min and subsequently subjected to SDS–PAGE. The gel was stained with Coomassie Brilliant Blue G-250 and destained with the destain solution (2.5% methanol and 10% acetic acid) for 3–5 h. For western blotting, SDS–PAGE separated proteins were blotted onto a nitrocellulose membrane. After blocking with 2% skim milk in PBS, the separated proteins were detected with the anti-His tag mouse antibody (Sigma-Aldrich) and the goat antimouse IgG-HRP conjugate antibody (Sigma-Aldrich) using the color development (18 mg 4-chloro-1-naphtol, 18 µl H2O2, 6 ml methanol and 30 ml PBS) for detecting peroxidase. The purified 3VGR19 Nanobody was used as control. A broad range protein marker (Fermentas) was used as a molecular weight marker.

Flowcytometry analysis

The VEGFR2 expressing cell 293KDR (Sibtech-USA) and the VEGFR2 negative cell HEK293 (Ncbi-Iran) were used for diabody evaluation in FACS. The cell lines were cultured in DMEM medium supplemented with 10% FBS. After washing three times with PBS-BSA 1%,

approximately 3×10^5 of cells diluted in a total volume of 100 µL. One microgram of VEGFR2-specific and control diabody (anti-scorpion diabody) was added, and cells incubated for 1 h on ice. Following three times washing with PBS-BSA1%, cells incubated with 1 µg mouse anti-His-tag antibody (Sigma-Aldrich) for 1 h on ice. Detection of bounded nanobodies performed by staining with 0.2 µg rat antimouse antibody PE-conjugated (BD Biosciences). Excess fluorescein-labeled antibody was removed by two times washing with PBS-BSA1% and cells analyzed on a BD FACS Canto II (BD Biosciences).

RESULTS

Expression and purification of VEGFR2-diabody

The VEGFR2-specific Nanobody sequence required for construction of diabody was amplified by PCR from previous characterized pHEN4-3VGR19 plasmid.[9] The fusion gene constructed in the pHEN6c vector, resulting in a three-fragment protein (3VGR19-Linker-3VGR19) as shown in Figure 1. The sequence of the construct and surrounding expression region was confirmed by DNA sequencing. The recombinant protein 3VGR19-Dia was expressed as His-tag fusions and purified by affinity chromatography and gel filtration. The gel-filtration profile of diabody showed a longer retention time as compared with the Nanobody [Figure 2]. One liter of the culture typically yielded about 5 mg of purified diabody.

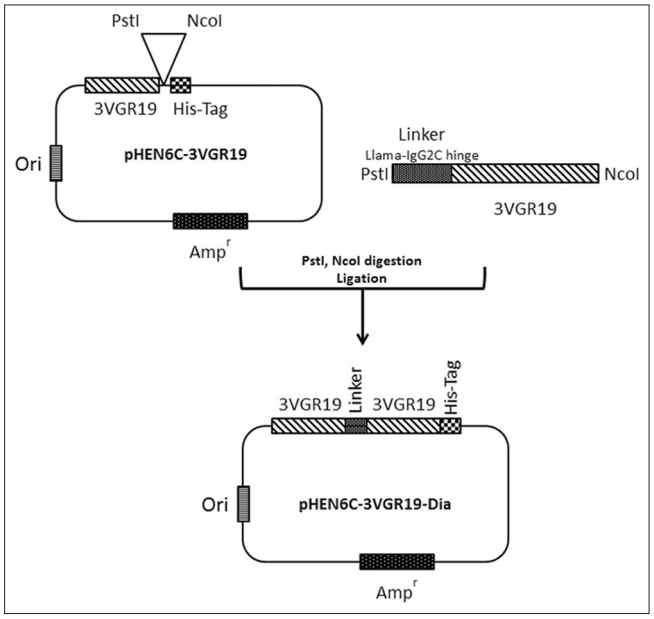

Figure 1: Construction of pHEN6C-3VGR19-Dia bacterial expression plasmid. The pHEN6C-3VGR19 is a plasmid with one copy of VEGFR2-specific Nanobody cloned between the BstEII and PstI sites. A PCR reaction was performed with the BiNb-Sense and BiNb-Llama-IgG2C-hinge primers and subcloned in the pHEN6C-3VGR19 vector, restricted with PstI and NcoI and ligated to each other. The final construct was named pHEN6c-3VGR19-Dia

SDS-PAGE and western blotting

After gel filtration chromatography, the purity of the protein was more than 95%. The purification was confirmed using SDS-PAGE. The VEGFR2 diabody is present as a single band of about 35 kDa [Figure 3a]. The western blot analysis was done with anti-His tag antibody and revealed that the purified proteins were transferred to the nitrocellulose membrane successfully and migrated at approximately 35 kDa [Figure 3b].

Flowcytometry analysis with purified VEGFR2-specific diabody

The ability of VEGFR2 diabody on recognition of

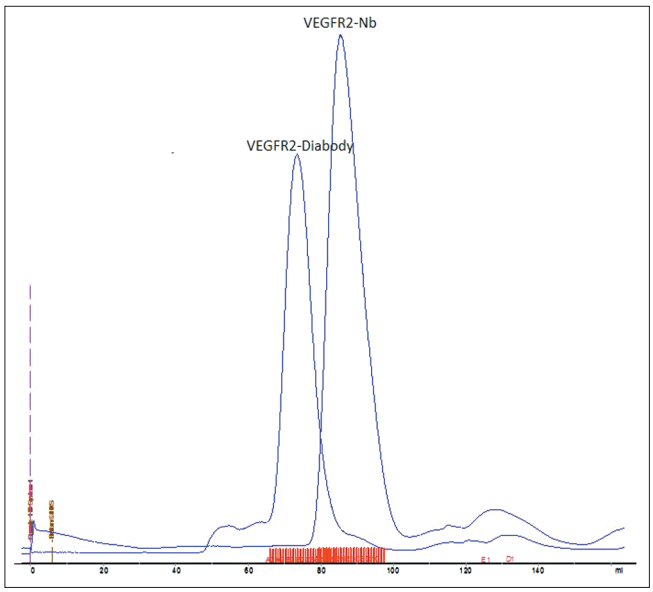

Figure 2: Size-exclusion chromatography profile of the IMAC-purified VEGFR2-Nanobody and VEGFR2-Diabody (as indicated), loaded onto a superdex S75 column in PBS buffer

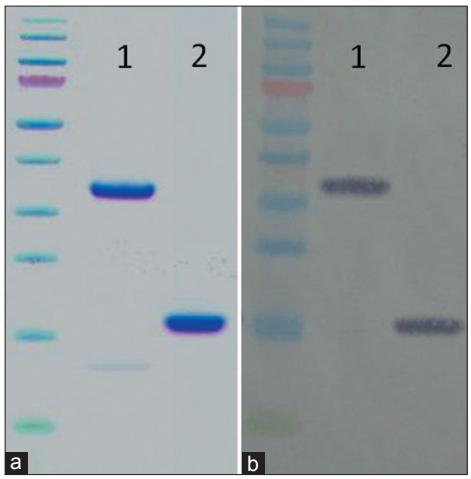

Figure 3: SDS-PAGE (a) and western-blotting (b) analysis of purified VEGFR2-Diabody (Lane 1) and 3VGR19 Nanobody (Lane 2). The MW of the marker lanes was from top to bottom 160,110, 90, 70, 55, 40, 35, 25, 15 and 10 kDa

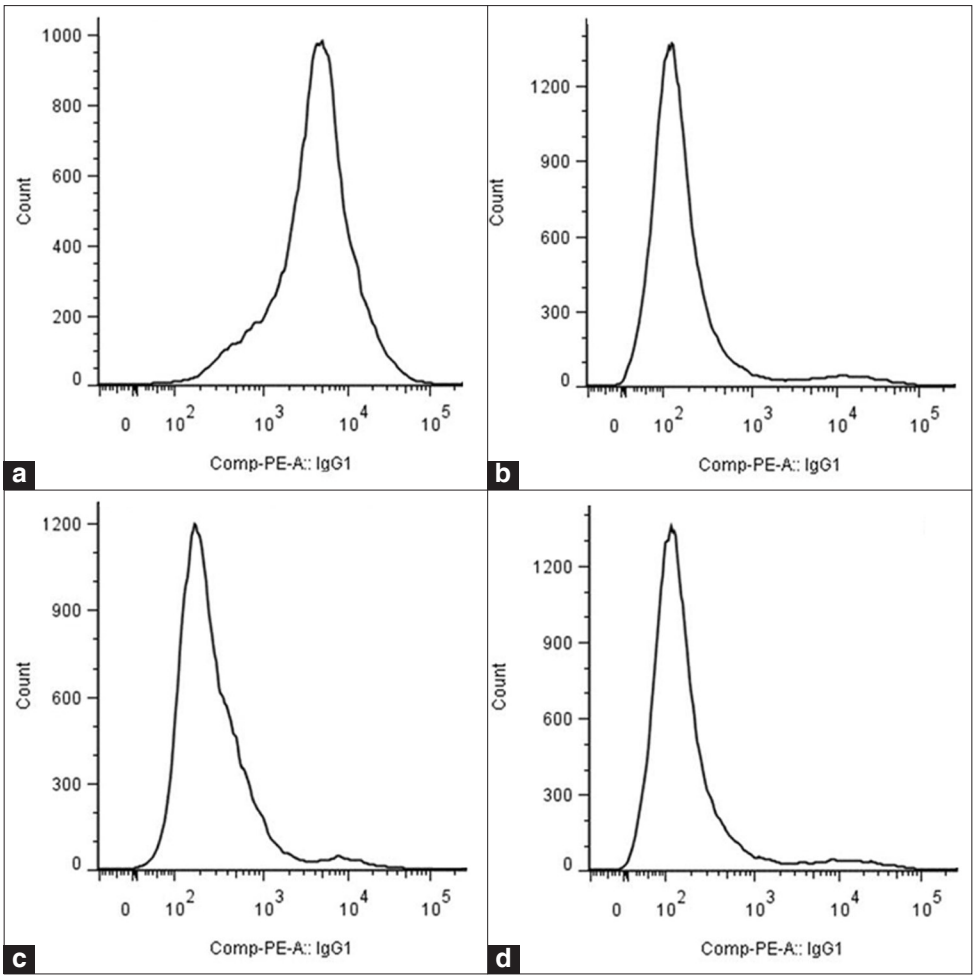

Figure 4: Flowcytometry analysis of VEGFR2-specific and control Diabody. a, b show the staining of 293KDR (a) and HEK293 (b) with VEGFR2-specific Diabody. c, d show the staining of 293KDR (c) and HEK293 (d) with control diabody (Anti-scorpion diabody)

receptor on the cell surface analyzed by flowcytometry. FACS analysis was performed with (a) 293KDR cell which is a stably transfected cell line expressing about 2.5×10^6 VEGFR2 per cell[11] and (b) HEK293, VEGFR2 negative cell.[9] Anti-scorpion venom diabody was used as control. In the 293KDR cell line, VEGFR2-diabody showed strong binding signals, as compared with signals obtained with the HEK293 cell line and the control diabody [Figure 4].

DISCUSSION

Antibodies have different potential usages, especially as anticancer agents. However, they need still some improvements in many features to be completely applicable. Stability, affinity, specificity and their size along with pharmacokinetic properties are some of these challengeable and limiting problems.[12] One of the possible solutions for these limits, specifically their size can be solved by reducing the size of the conventional antibody to a Fab or scFv with cloning the corresponding gene fragments and expression in bacteria.[13] However, the unsatisfactory yield of functional, monomeric products in heterologous expression systems remains a barrier in the development of scFv derivatives for therapeutic purposes.[14] Alternatively, single-domain compounds with proper antigen binding specificity can be generated naive libraries of VH antibody fragments or synthetic libraries of various monomeric proteins serve as sources to retrieve antigen-specific molecules; unfortunately the low affinity of these kinds of antibodies is the major problem.[15] The discovery of functional heavy-chain antibodies in camelids generates a new opportunity to obtain soluble antigen-binding fragments of minimal size. These antibodies can be affinity-matured *in vivo* to yield molecules that interact via one variable domain (Nanobody) with the antigen with adequate affinity and specificity.[16] It shares a large sequence identical to human VH of family 3, but with four amino acid substitutions in framework 2 that render the surface more hydrophilic, thus explaining the soluble behavior and accompanying higher functional expression levels of nanobodies.[17] The beneficial advantage of these recombinant VHH proteins is their stability under different storage

conditions. It can be frozen and thawed without significant loss of biological activity indicating their greater stability compared with conventional antigen binders. These significant abilities have also been demonstrated by other scientists.[18,19] There is a limit in the efficiency of nanobodies because of the short serum half-life due to a rapid renal clearance. The mentioned problem can be eliminated with targeting the VHHs by some serum proteins such as albumin or immunoglobulin.[20-22] Resulted VHH has a half-life equal the half-life of the serum protein (albumin 2 days in mice and immunoglobulin 9 days). Another well-known alternative in this approach to increase serum half-life of proteins is the genetically addition of proteins together to generate dia, thria, tetrabody.

Previous studies in VEGFR2 antibodies showed these antibodies can inhibit the angiogenesis *in vitro* and *in vivo* models.[23-25] We also in our previous study selected a high affinity VEGFR2-specific Nanobody that was able to inhibit the tube formation.[9] In this study, we describe the successful construction, expression, and characterization of VEGR2-Diabody. The diabody molecule was extracted as soluble proteins from the periplasmic of *E. coli* in yields that are comparable with those of the monomeric VHHs. We, therefore expect that the high quantities require for treatment and diagnosis will be obtained easily. The recombinant diabody constructs obtained by linking two single-domain fragments can recognize the VEGFR2 on the cell surface of 293KDR cells as same as the VEGFR2-specific Nanobody.[9] Some reports of the construction of diabody have shown that diabody can act like the normal antibody for antigen recognition.[20,26]

As a conclusion, the easy production steps of Nanobody and diabody constructs based on camel single-domain antibody fragments makes them particularly attractive for use in therapeutic or diagnostic programs.

REFERENCES

1. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: Successes, limitations and hopes for the future. Br J Pharmacol 2009;157:220-33.
2. Weisser NE, Hall JC. Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. Biotechnol Adv 2009;27:502-20.
3. Rahbarizadeh F, Ahmadvand D, Sharifzadeh Z. Nanobody; An old concept and new vehicle for immunotargeting. Immunol Invest 2011;40:299-338.
4. Muyldermans S, Baral TN, Retamozzo VC, De Baetselier P, De Genst E, Kinne J, *et al*. Camelid immunoglobulins and Nanobody technology. Vet Immunol Immunopathol 2009;128:178-83.
5. Folkman J. Is angiogenesis an organizing principle in biology and medicine? J Pediatr Surg 2007;42:1-11.
6. Folkman J. Angiogenesis: An organizing principle for drug discovery? Nat Rev Drug Discov 2007;6:273-86.
7. Holmes K, Roberts OL, Thomas AM, Cross MJ. Vascular endothelial growth factor receptor-2: Structure, function, intracellular signalling and therapeutic inhibition. Cell Signal 2007;19:2003-12.
8. Ellis LM, Hicklin DJ. VEGF-targeted therapy: Mechanisms of anti-tumour activity. Nat Rev Cancer 2008;8:579-91.
9. Behdani M, Zeinali S, Khanahmad H, Karimipour M, Asadzadeh N, Azadmanesh K, *et al*. Generation and characterization of a functional Nanobody against the vascular endothelial growth factor receptor-2; angiogenesis cell receptor. Mol Immunol 2012;50:35-41.
10. Conrath KE, Lauwereys M, Galleni M, Matagne A, Frère JM, Kinne J, *et al*. Beta-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae. Antimicrob Agents Chemother 2001;45:2807-12.
11. Backer MV, Backer JM. Targeting endothelial cells overexpressing VEGFR-2: Selective toxicity of Shiga-like toxin-VEGF fusion proteins. Bioconjug Chem 2001;12:1066-73.
12. Cortez-Retamozo V, Backmann N, Senter PD, Wernery U, De Baetselier P, Muyldermans S, *et al*. Efficient cancer therapy with a Nanobody-based conjugate. Cancer Res 2004;64:2853-7.
13. Skerra A, Pluckthun A. Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. Science 1988;240:1038-41.
14. Fernandez LA. Prokaryotic expression of antibodies and affibodies. Curr Opin Biotechnol 2004;15:364-73.
15. Wang X, Campoli M, Ko E, Luo W, Ferrone S. Enhancement of scFv fragment reactivity with target antigens in binding assays following mixing with anti-tag monoclonal antibodies. J Immunol Methods 2004;294:23-35.
16. Saerens D, Ghassabeh GH, Muyldermans S. Single-domain antibodies as building blocks for novel therapeutics. Curr Opin Pharmacol 2008;8:600-8.
17. Vu KB, Ghahroudi MA, Wyns L, Muyldermans S. Comparison of llama VH sequences from conventional and heavy chain antibodies. Mol Immunol 1997;34:1121-31.
18. Bond CJ, Marsters JC, Sidhu SS. Contributions of CDR3 to V H H domain stability and the design of monobody scaffolds for naive antibody libraries. J Mol Biol 2003;332:643-55.
19. Dumoulin M, Conrath K, Van Meirhaeghe A, Meersman F, Heremans K, Frenken LG, *et al*. Single-domain antibody fragments with high conformational stability. Protein Sci 2002;11:500-15.
20. Coppieters K, Dreier T, Silence K, de Haard H, Lauwereys M, Casteels P, *et al*. Formatted anti-tumor necrosis factor alpha VHH proteins derived from camelids show superior potency and targeting to inflamed joints in a murine model of collagen-induced arthritis. Arthritis Rheum 2006;54:1856-66.
21. Roovers RC, Laeremans T, Huang L, De Taeye S, Verkleij AJ, Revets H, *et al*. Efficient inhibition of EGFR signaling and of tumour growth by antagonistic anti-EFGR Nanobodies. Cancer Immunol Immunother 2007;56:303-17.
22. Harmsen MM, Van Solt CB, Fijten HP, Van Setten MC. Prolonged *in vivo* residence times of llama single-domain antibody fragments in pigs by binding to porcine immunoglobulins. Vaccine 2005;23:4926-34.
23. Kendrew J, Eberlein C, Hedberg B, McDaid K, Smith NR, Weir HM, *et al*. An antibody targeted to VEGFR-2 Ig domains 4-7 inhibits VEGFR-2 activation and VEGFR-2-dependent angiogenesis without affecting ligand binding. Mol Cancer Ther 2011;10:770-83.
24. Lee SH. Tanibirumab (TTAC-0001): A fully human monoclonal antibody targets vascular endothelial growth factor receptor 2 (VEGFR-2). Arch Pharm Res 2011;34:1223-6.
25. Spratlin J. Ramucirumab (IMC-1121B): Monoclonal antibody inhibition of vascular endothelial growth factor receptor-2. Curr Oncol Rep 2011;13:97-102.
26. Els Conrath K, Lauwereys M, Wyns L, Muyldermans S. Camel single-domain antibodies as modular building units in bispecific and bivalent antibody constructs. J Biol Chem 2001;276:7346-50.

Source of Support: Nil, **Conflict of Interest:** None declared.