binding (Fab) and/or the single chain variable fragment (scFv), to single binding domains for proteins based on immunoglobulins with V_H domains, which in turn have been used to develop new immunotherapeutic and immunodiagnostic strategies. Mimetics of the Fab's to smaller domains is advantageous since that increases the stability and the possibility for accessing antigenic epitopes that are not recognized by conventional antibodies.[2]

There are three isotypes of immunoglobulins or antibodies from cartilaginous fishes, two of them with two standard heavy and light chains, designated as IgM and IgW (also called IgX or IgNARC) and one atypical isotope called IgNAR which is a homodimer of heavy chains not associated with light chains. The shark antigen receptor immunoglobulins (referred as IgNAR or NAR) have a single variable domain (sdAb fragments) and two fork hypervariable structures to include the entire repertoire with union specificity to recognize the antigens. IgNARs are high soluble and high thermostable small molecules (12 kDa) and with good tissue penetration *in vivo*, which makes IgNARs a good resource for antibody engineering and therapeutic antibodies.[3, 4]

The present invention relates to selection and isolation of IgNAR antibodies, in particular of its variable region V_HNAR, originated in the immunized shark *Heterodontus francisci* or *Orectolobus maculatus* with affinity for cytokines and ability to neutralize their activity. These antibodies are originated generally by the immunological system of cartilaginous fish (sharks, skates, rays, and chimeras). The molecular arrangement of the IgNAR antibodies consists of five constant regions and one variable region which in addition is very similar to the V_H found in camelids, which possibly represents an evolutionary convergence at molecular level.[1, 5]

Nuttall and collaborators obtained a non-immune shark antibody library, through phage display technology based on variable regions of IgNAR of the shark *Orectolobus maculatus*. These regions have the ability to recognize proteins like gingipain K protease from *Porphyromonas gingivalis*, the mitochondrial import receptor Tom70, the lysozyme and the Apical Membrane Antigen 1 (AMA1) of *Plasmodium falciparum*, among others. These regions have been cloned in *Escherichia coli* expression

US Patent 8,496,933, Paniagua-Solís et al., refers to the selection, isolation and production of a protein belonging to variable regions named V_HNAR or vNAR, originated from IgNAR-type immunoglobulins of elasmobranches with antigen receptor abilities. This vNAR was named V13 and it was selected by its capacity to bind specifically to the vascular endothelial growth factor (VEGF). It works by neutralizing the activity of VEGF, and it has been characterized by its sequence, selected and optimized, and which is the closest state of the art to the invention, incorporated herein by reference in its entirety.

The trials on anti-VEGF therapies have tried a variety of dosing strategies such as: when to start treatment, dosing frequency, and how these strategies can be followed in medical treatment, since secondary or side effects as hypertension, proteinuria, bleeding, damage to the healing of surgical wounds, even fatal complications such as arterial thrombosis, gastrointestinal perforation and reversible posterior focal leukoencephalopathy, route of administration, the invasiveness of the methods, the high dose, bioavailability, instability as well as high costs, long treatments, among others, lead to the need of research for new molecules that have better performance. Even with such alternatives, it is required to develop better drugs that inhibit the activity of VEGF for eye treatments in order to remove or reduce side effects.

The present invention describes novel clones and molecules named V19, V32R and the aforementioned V13, characterized by their three-dimensional structure, their sequences and affinities to VEGF and useful in treating eye conditions, particularly for the treatment of diabetic retinopathy, macular degeneration, neovascular glaucoma or ocular conditions related to angiogenesis.

SUMMARY OF THE INVENTION

The invention is defined in the independent claims. Some optional features of the invention are defined in the dependent claims. The present invention relates to the generation of shark-based therapeutic monoclonal antibodies known as IgNARs which are comprised of heavy chains of immunoglobulins. Specifically the present invention relates to the selection of the variable domains (vNARs) of these heavy

chains. In this case, they are characterized by their ability to recognize the cytokine known as vascular endothelial growth factor (VEGF). The vNARs are of interest in the field of biotechnology due to their biological and biophysical properties. The vNAR antibodies are highly resistant to environmental conditions and have a high capacity for topical therapeutic action. Together with the variable domains derived from camel immunoglobulin (known as V_HH), the vNARs are the smallest biological molecules capable of recognizing antigens. Due to these properties the vNARs outweigh the disadvantages and drawbacks of conventional therapy with monoclonal antibodies.

Moreover, the present invention relates to the selection, isolation and purification of proteins belonging to variable regions named V_HNAR or vNAR, originated from IgNAR-type immunoglobulins of elasmobranches with antigen receptor abilities. The clones from which they originate are named VEGFvNAR V32R and V19; and the antibodies are named V32R and V19 (also defined as either v19 or v32R).

The present invention describes new clones and molecules named V19, V32R and the aforementioned V13, characterized by their three-dimensional structure, their sequences and affinities to VEGF and useful in treating eye conditions, particularly for the treatment of diabetic retinopathy, macular degeneration, neovascular glaucoma or ocular conditions related to angiogenesis.

In order to demonstrate that the new clones are not a laboratory artifice and that they actually comprise differential antibodies that provide a surprising and unexpected technical advantage over the prior art, the characterization of the clone V13 has also been included herein – previously described in U.S. Patent 8,496,933 – and it has been subjected to the same isolation and purification protocols performed in the development of the invention in order to compare the results. Moreover, in order to improve yields, different tests and methods of expression and purification were carried out during the development of the invention in order to detect and get the best conditions for expression and purification of proteins, as well as the subsequent assessment on the performance of each of the obtained clones to bind and neutralize VEGF.

is observed that the higher the concentration of reference antibody, the tube formation decreases (**C-H**). **Ranibizumab (Genentech/Roche).*

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the generation of shark-based therapeutic monoclonal antibodies known as IgNAR (by the acronym of new antigen receptor) which are comprised of heavy chains of immunoglobulins. Specifically the present invention relates to the selection of the variable domains (vNAR) of these heavy chains. In this case, they are characterized by their ability to recognize the cytokine known as vascular endothelial growth factor (VEGF). The vNARs are of interest in the field of biotechnology due to their biological and biophysical properties. The vNAR products are highly resistant to environmental conditions and have a high capacity for topical therapeutic action. Together with the variable domains derived from camel immunoglobulin (known as V_HH); the vNARs are the smallest biological molecules capable of recognizing antigens. Due to these properties the vNARs outweigh the disadvantages and drawbacks of conventional therapy with monoclonal antibodies.

The present invention relates to the selection, isolation and purification of proteins belonging to variable regions named V_HNAR or vNAR, originated from IgNAR-type immunoglobulins of elasmobranches with antigen receptor abilities. The clones from which they originate are named VEGFvNAR V32R and V19; and the respective antibodies are named V32R and V19 (also defined as either v19 or v32R).

The present invention also relates to the development of vNAR-based biopharmaceuticals that block the vascular endothelial growth factor (VEGF) since they have a high and very specific affinity for VEGF. These generated molecules have been subjected to different isolation and purification protocols. They are characterized by their intrinsic properties such as their sequence and three-dimensional structure, as well as their affinity and ability to recognize its target molecule which results in a more efficient neutralizing capacity than of other related molecules.

CLAIMS

1. An isolated anti-VEGF vNAR protein selected from the group consisting of those having the amino acid sequence SEQ. ID. NO. 4 or SEQ. ID. NO.6 and having ability of recognition and enhanced affinity for its target molecule VEGF.

2. The anti-VEGF vNAR protein of claim 1, characterized by having the amino acid sequence of SEQ ID NO.4, and for being a monomer consisting of two groups of β sheets joined by a single disulfide bridge between cysteines 22 and 83 the first group corresponding to a variable region that recognizes and bind other proteins, and consisting of two β sheets interconnected by a loop formed between residues 85-100.

3. The anti-VEGF vNAR protein of claim 1, characterized by having the amino acid sequence of SEQ. ID. NO. 6, and for being a monomer consisting of two groups of β sheets joined by a single disulfide bridge between cysteines 24 and 85; the first group consisting of two β sheets interconnected by a loop formed between residues 90-110.

4. A DNA sequence comprising the nucleic acid of SEQ. ID. NO. 3 or 5 encoding the vNAR protein of claim 1 identified as SEQ. ID. NO. 3 or SEQ. ID. NO. 5 .

5. The isolated anti-VEGF vNAR protein selected from the group consisting of those having the amino acid sequence SEQ. ID. NO. 4 or SEQ. ID. NO.6 of claim 1, wherein:
    a) it originates from *Orectolobus maculatus* or *Heterodontus francisci; and*
    b) it binds to and neutralizes the activity of the vascular endothelial growth factor (VEGF) in the eye.

6. A pharmaceutical composition comprising the isolated anti-VEGF vNAR protein selected from the group consisting of those having the amino acid sequence SEQ. ID. NO. 4 or SEQ. ID. NO.6 of claim 1, and a pharmaceutically acceptable excipient.

7. The pharmaceutical composition of claim 6, wherein it is formulated for topical administration.

8. The pharmaceutical composition of claim 6, wherein the composition is formulated for topical ophthalmic administration.

9. The pharmaceutical composition of claim 6, wherein the composition is designed to be topically administered in a VEGF-related eye disease selected from: retinal neovascularization, choroidal neovascularization, corneal neovascularization, macular degeneration, age-related macular degeneration, retinal diseases, diabetic retinopathy, vitreous hemorrhage, retinal hemorrhage, choroiditis, retinal detachment, retinal drusen, neovascular glaucoma, choroid diseases, uveitis, myopia, eye diseases, fungal eye infections, telangiectasia, retinal artery occlusion, degenerative myopia, retinal vein occlusion, chorioretinitis, histoplasmosis, uveal diseases, rubella (german measles), ocular toxoplasmosis, epiretinal membrane, coloboma, choroid neoplasms, retinal degeneration, retinitis, retinal perforations, herpetic keratitis, retinopathy of prematurity, cystoid macular edema, papilledema, uveomeningoencephalitic syndrome, optic disk drusen, angioid streaks, retinitis pigmentosa, vision disorders, sympathetic ophthalmia, scar, ocular burns, recurrent ischemia, eye injuries, glaucoma, eye hemorrhage, scotoma, posterior uveitis, fungemia, retinal neoplasms, corneal diseases, pigmentary incontinence, hemoglobin c disease, fibrosis, opacity of the cornea, anterior uveitis, hyphema, sarcoidosis, aphakia, iatrogenic disease, panuveitis, eye cataract, postoperative complications, sickle cell anemia, retinal vasculitis, osteoma, cytomegalovirus retinitis, atrophy, phlebitis, keratoconus, sturge-weber syndrome, viral eye infections, eye abnormalities, substance-related disorders, penetrating eye injuries, diabetes

mellitus type 2, radiation injuries, sickle cell trait, pseudophakia, pigmented nevus, proliferative vitreoretinopathy, bleeding, diabetes mellitus type 1, nevus, optic nerve diseases, vascular diseases, candidiasis, chemical burns, microphthalmia.

10. The pharmaceutical composition of claim 9, wherein the VEGF-related eye disease is preferably selected from wet age-related macular degeneration, diabetic retinopathy, or neovascular glaucoma.

11. The use of an effective amount of the anti-VEGF vNAR protein selected from the group formed by consisting of those having the amino acid sequence SEQ. ID. NO. 4 or SEQ. ID. NO.6 of claim 1, for preparing a medicament administrable by the topical ophthalmic route for the treatment of a VEGF-related eye disease.

12. The use of claim 11, wherein the disease is also a pathology related to an angiogenic process, where excessive angiogenesis occurs when diseased cells produce abnormal amounts of VEGF.

13. The use of claim 12, wherein the angiogenic process is selected from the group consisting of age-related macular degeneration, diabetic retinopathy, and neovascular glaucoma.

14. The use of claim 13, wherein the angiogenic process is preferably diabetic retinopathy.

15. The use of claim 13, wherein the angiogenic process is preferably wet age-related macular degeneration.

16. The use of claim 13, wherein the angiogenic process is preferably neovascular glaucoma.

17. The use of at least one anti-VEGF vNAR protein selected from the group consisting of those having the amino acid sequence SEQ. ID. NO. 4 or SEQ. ID. NO.6 of claim 1, for the preparation of a medicament useful in the prevention or treatment of a VEGF-related ophthalmic condition selected from: retinal neovascularization, choroidal neovascularization, corneal neovascularization, macular degeneration, age-related macular degeneration, retinal diseases, diabetic retinopathy, vitreous hemorrhage, retinal hemorrhage, choroiditis, retinal detachment, retinal drusen, neovascular glaucoma, choroid diseases, uveitis, myopia, eye diseases, fungal eye infections, telangiectasia, retinal artery occlusion, degenerative myopia, retinal vein occlusion, chorioretinitis, histoplasmosis, uveal diseases, rubella (german measles), ocular toxoplasmosis, epiretinal membrane, coloboma, choroid neoplasms, retinal degeneration, retinitis, retinal perforations, herpetic keratitis, retinopathy of prematurity, cystoid macular edema, papilledema, uveomeningoencephalitic syndrome, optic disk drusen, angioid streaks, retinitis pigmentosa, vision disorders, sympathetic ophthalmia, scar, ocular burns, recurrent ischemia, eye injuries, glaucoma, eye hemorrhage, scotoma, posterior uveitis, fungemia, retinal neoplasms, corneal diseases, pigmentary incontinence, hemoglobin c disease, fibrosis, opacity of the cornea, anterior uveitis, hyphema, sarcoidosis, aphakia, iatrogenic disease, panuveitis, eye cataract, postoperative complications, sickle cell anemia, retinal vasculitis, osteoma, cytomegalovirus retinitis, atrophy, phlebitis, keratoconus, sturge-weber syndrome, viral eye infections, eye abnormalities, substance-related disorders, penetrating eye injuries, diabetes mellitus type 2, radiation injuries, sickle cell trait, pseudophakia, pigmented nevus, proliferative vitreoretinopathy, bleeding, diabetes mellitus type 1, nevus, optic nerve diseases, vascular diseases, candidiasis, chemical burns, microphthalmia.

18. The use of claim 17, wherein the ophthalmic condition is wet age-related macular degeneration, diabetic retinopathy, or neovascular glaucoma.