Bispecific antibodies for topical delivery to inhibit abnormal choroidal angiogenesis

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Abzyme Therapeutics
A novel approach to generate antibodies

Abstract

The age-related macular degeneration (AMD) characterized by the formation of subretinal choroidal neovascularization is the main cause of blindness in the elderly. Vascular endothelial growth factor (VEGF) regulates angiogenesis and enhances vascular permeability that results in visual acuity deterioration. Blockade of VEGF action is currently the most effective strategy in preventing choroidal angiogenesis and reducing vascular permeability. The intravitreal injection of anti-VEGF drugs has been widely employed to reduce the disease progression and improve the visual outcomes of the affected patients. Unfortunately, injection requires the administration in hospital, poses a risk of severe infection, retinal detachment, endophthalmitis, intraocular inflammation, increase of intraocular pressure, and vitreous hemorrhage, as well as, has low patient compliance. In addition, only 60% of AMD cases are VEGF-positive that can be treated by anti-VEGF therapeutics.

Abzyme's ophthalmology program with 12 proprietary targets, including VEGF, is to develop a new antibody therapeutic for self-administrable, noninvasive, topical delivery to inhibit abnormal angiogenesis in the retina and choroid and inflammation. To overcome the retinal and choroidal barriers we develop antibodies with following attributes: (i) Small size antibodies with positive charges to enhance cell membrane penetration; (ii) Small size TfR-directed bispecific antibodies are designed to overcome the retinal and choroidal barriers via TfR-mediated transcytosis and transcorneal transport.

Abzyme's *ex-vivo* so-called ***S***elf-***D***iversifying ***A***ntibody ***L***ibrary or SDALib generation platform and bispecific Abz2 modular antibody engineering approach are utilized to produce antibodies with desired attributes. The SDALib not only shortens time for antibody discovery, but is also applicable for non-immunogenic or toxic antigens and avoids the use of animals. The Abz2 platform allows rapid reformatting existing antibodies into well-expressed and easy-to-purified bispecific antibodies.

Here we present data of rapid generation of positively-charged VEGF single domain VHH antibodies using SDALib platform and well-expressed and easy-to-purified bispecific VEGF/TfR antibodies with low affinity to TfR and high affinity to VEGF using Abz2 approach. The animal studies are being performed to determine the accumulation of monovalent anti-VEGF VHH and bispecific antibodies in the vitreous humor and retina in mouse model that receives topical eye drops.

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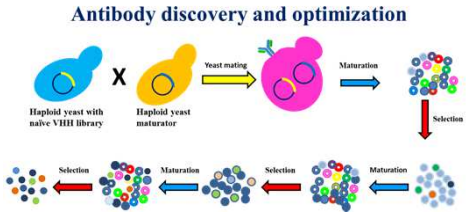
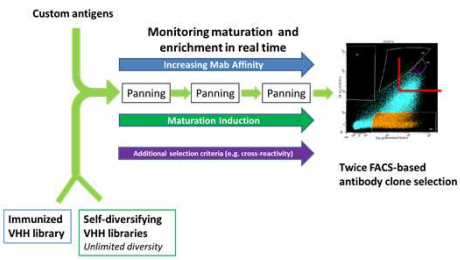
Experimental approach

Our state of the art rapid recombinant monoclonal antibody discovery technologies utilize yeast triple-mode system consisting of *in vivo* antibody maturation, cell surface presentation, and secretion. Our systems consist of antibody maturation by inducible *in vivo* mutation without the need to isolate and re-transform DNA into the cells, a surface expression of the antibodies for quick screening and selection of clones with desired attributes adaptable to different requirements (e.g. cross-reactivity of an antibody), and finally inducible secretion of the antibody for a detailed analysis of the selected antibody clone.

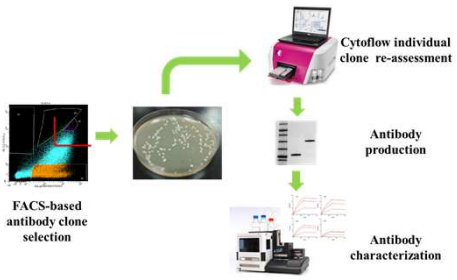
Our Abz2 modular approach includes two steps (i) development of modular single domain antibodies that remain functional once fused to the C-terminus of the existing antibodies and (ii) production of bispecific Mabs.



By changing the growth condition, surface expression of the antibodies is induced and the random mutation of the antibody encoding genes is enhanced to achieve an antibody maturation. Target specific clones are isolated by incubation of the cells with labeled antigen and magnetic beads followed by FACS. The cell selection conditions during the panning and sorting steps can be modified to achieve additional specifications of the antibody (e.g. exclusion or inclusion of cross-reactivity).

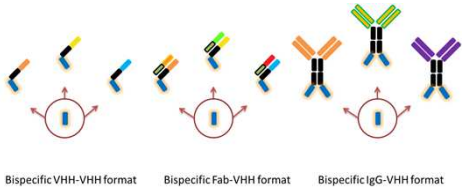


Sorted clones are isolated as single clones and further analyzed in the appropriate assays and for large production of the chosen antibodies.



Abzyme's modular antibody platform rapidly reformats existing antibody into bispecific antibody

We create bispecific antibodies by combining an active antibody with an active llama VHH at the C-terminus of the antibody. To increase the probability of a reactive VHH in the bispecific antibody, we isolate antigen specific llama VHH from a C-terminal expression library. The llama VHH fragment can be easily shuffled to other antibodies converting them into a bispecific antibody.



Results

We have rapidly isolated a suite of 12 anti-VEGF VHH antibodies. Most of them are reactive with human, cynomolgus, and murine VEGFs. All VHHs are grouped in three classes. Except clones 5 and 8, all bind to VEGF at epitopes different from Ranibizumab/Lucentis. Clones 5 and 8 binding to the Lucentis epitope do not react with murine VEGF-A. Previously Yu *et al* (1) have shown that Lucentis does not interact with mVEGF-A.

Alignment of VEGF VHH CDR3 regions

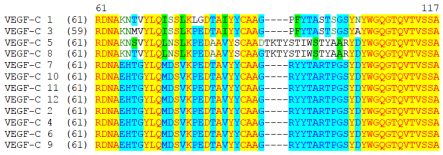


Table 1: Anti-VEGF VHH clone attributes

VHH clone	Family	Predicted MW (kDa)	Lucentis epitope	pI	Cross reactivity with mVEGF
VEGF1	1	12.9	No	9.47	+
VEGF2	3	13.7	No	8.2	+
VEGF3	1	14.4	No	9.69	+
VEGF4	3	13.5	No	7.95	+
VEGF5	2	14.1	Yes	8.58	No
VEGF6	3	13.5	No	7.95	+
VEGF7	3	13.7	No	7.95	+
VEGF8	2	14.0	Yes	9.36	No
VEGF9	3	13.5	No	7.95	+
VEGF10	3	13.7	No	7.95	+
VEGF11	3	13.7	No	8.98	+
VEGF12	3	13.7	No	8.98	+

Binding of VEGF 5 and VEGF 8 to VEGF is inhibited by Lucentis

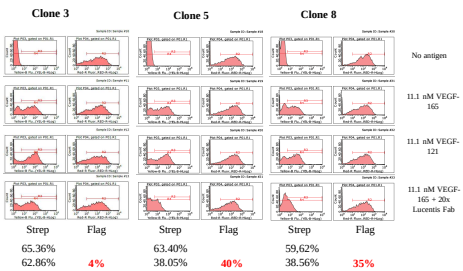
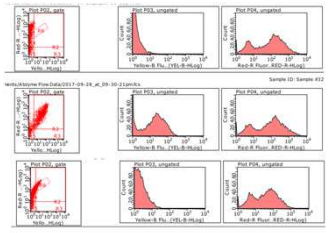


Fig. 1: Expressed VHHs were stained with anti-Flag dylight649 and the presence of biotinylated target protein was detected with PE conjugated Streptavidin.

De Cogan *et al.* had described that positively charged proteins can cross membranes by themselves (2). Therefore, we determined the pI of the VEGF VHHs. All are positively charged with pI ranges from 7.95 to 9.69.

Another proven method to cross the blood barrier method is the use of an anti-TfR antibody as a carrier molecule (3). To generate bispecific we isolated several modular anti-TfR VHHs which were reacting specific with TfR in the single clone cytoflow assay.



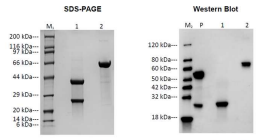
We subdivided the isolated TfR reactive antibody expressing clones into 6 families based on their sequence homologies. The pI of the different clones ranges from 8.98 up to 10.02. From each family we selected a few members to produce VHHs in *E. coli* and tested their reactivity in ELISA assays.

Clone	Ratio	Clone	Ratio	Clone	Ratio	Clone	Ratio	Clone	Ratio
1	**10.24**	10	**12.33**	56	**11.49**	53	**11.19**	52	**9.40**
68	**9.45**	22	**10.62**	51	**9.32**			13	**8.81**
70	**7.99**							8	**12.10**
								58	**5.12**

Table 2: ELISA results with VHHs for the different clone families. Based on the size of the family different number of clones were selected. The supernatants containing VHHs were tested against human TfR and BSA as control. Reactivity ratio was determined by dividing the signal of wells containing TfR by the signal of the wells containing BSA.

One of the anti-TfR VHH clones which is reactive with both human and murine TfR has been used to generate TfR x VEGF bispecific antibodies in the format VHH-Fab. Bispecific antibody was well-expressed and easy-to-purified from Hek293 cells.

SDS-PAGE gel and Western blotting of purified TfR x VEGF VHH-Fab bispecific antibody produced in HEK293 cells



Used abbreviations: M = Marker, P = control protein, Red = reducing conditions, NR = non-reducing

The bispecific antibody recognizes TfR of both species (human and mouse)

1st Antigen	Antibody	2nd antigen	Detection agent	Signal
VEGF	VEGF x TFR	Bio-hTFR	Strep-HRP	**2.85**
VEGF	VEGF x TFR	Bio-mTFR	Strep-HRP	**2.87**
VEGF	anti-VEGF	Bio-hTFR	Strep-HRP	0.12
VEGF	anti-VEGF	Bio-mTFR	Strep-HRP	0.13
BSA	VEGF x TFR	Bio-hTFR	Strep-HRP	0.27
BSA	anti-VEGF	Bio-TFR	Strep-HRP	0.21

VEGF is comparably recognized by both antibodies

Antigen	Antibody	Detection agent	Signal
VEGF	Bispecific	Anti-Fab HRP	**3.43**
VEGF	Monospecific anti-VEGF	Anti-Fab HRP	**3.15**
BSA	Bispecific	Anti-Fab HRP	**0.11**
BSA	Monospecific anti-VEGF	Anti-Fab HRP	**0.09**

Summary and outlook

We have successfully isolated a number of VHHs reacting highly specific with either VEGF-A or TfR in cytoflow and ELISA assays using Abzyme's platform. These VHHs will serve a foundation for bispecific antibody generation. VHH antibodies to other 11 eye disease targets are being generated.

We demonstrate that our modular antibody platform allows the quick generation of bispecific antibodies, easy exchange of one binding partner, and avoids many expression problems of other bispecific antibody formats.

Currently we are establishing the *in vitro* blood brain barrier model in the transwell system to test which of the TfR VHHs have the highest cell layer crossing capability mimicking the blood-brain barrier. For this test we are using hCMEC/D3 cells (4). Additionally, we will analyze in this model if the high pI of some of the anti-VEGF VHHs is sufficient to enable the VHHs to penetrate/cross cells (see De Cogan *et al.*).

Literature

1) Yu *et al*, "Interaction between bevacizumab and murine VEGF-A: a reassessment", Invest Ophthalmol Vis Sci. 2008. **49**:2
2) De Cogan *et al*, " Topical delivery of anti-VEGF drugs to the ocular posterior segment using cell-penetrating peptides", 2017, IOVS **58**:2578
3) Yu *et al*, "Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates", 2014, Science Trans Med **261**:1
4) B. Weksler *et al*, "The hCMEC/D3 cell line as a model of the human blood brain barrier", 2013, Fluids and Barriers of the CNS **10**:16

Support

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