Alpbach – 14th March 2007

ESF ProteomeBinders Workshop on Affinity Proteomics:

Anticalins and other alternative scaffolds

Protein Scaffolds for the engineering of novel binding sites

The Lipocalin protein family: prominent members

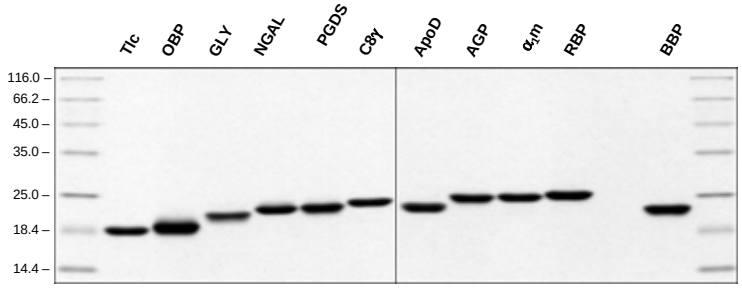
Lipocalin	Abbre-viation	No. of residues	Oligomeric state	Glyco-sylation	No. of SS-bonds	Physiological ligand(s)
α_1-Acid glycoprotein	AGP	183	monomer	+	2	? (numerous drugs)
Apolipoprotein D	ApoD*	169	mon./HDL-assoc.	+	2	Progesterone/Arachidonate
Apolipoprotein M	ApoM	188	HDL-anchored	+	3	?
Complement component 8 γ chain	C8γ*	182	heterodimer	–	1	?
Epididymal lipocalin / E-RABP	LCN6	142	monomer	+	0	Retinoic acid ?
Glycodelin	PP14	162	dimer	+	2	?
α_1-Microglobulin / protein HC	α_1m	183	fusion → mon./het.	+	1	Heme
Odorant-binding protein	OBP	157	monomer/dimer	–	1	Odorant compounds
Prostaglandin D synthase / β-Trace	PGDS*	169	monomer	+	1	Prostaglandin D/H_2
Retinol-binding protein	RBP*	183	monomer	–	3	Retinol (Vitamin A)
Siderocalin / **NGAL**	LCN2*	178	monomer/het.	+	1	Fe(III) siderophore
Tear lipocalin	LCN1/Tlc*	158	monomer/dimer	–	1	Retinoids/Rifampin/Lipids...
β-Lactoglobulin	Blg*	162	monomer/dimer	–	2	Fatty acids/Retinol
Aphrodisin	–	151	monomer	+	2	?
Major urinary protein	MUP*	166	dimer	–	1	2-(s-Butyl)thiazoline etc.
Bilin-binding protein	BBP*	173	dimer (monomer)	–	2	Biliverdin IX_γ
Lazarillo	LAZ	171	GPI-linked	+	1-2	?
Bacterial lipocalin	Blc*	159	lipoprotein	–	0	?

(human members of the family) ***Crystal structure known**

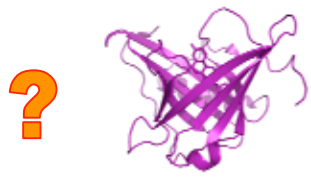
Prof. Dr. Arne Skerra – Lehrstuhl für Biologische Chemie

Standardized bacterial production of ten human lipocalins

1. Secretion of recombinant lipocalins into the periplasm of *E. coli* via the OmpA signal peptide
2. Purification from the periplasmic cell fraction via the ***Strep*-tag II** and gel filtration
3. Coomassie-stained SDS PAGE (15 %):

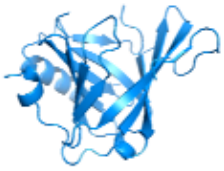


3D gallery of 10 human lipocalins: β-barrel fold with 4 variable loops



AGP

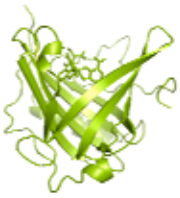
ApoD
[PDB: 2HZQ]



C8γ
[PDB: 1IW2]

PP14

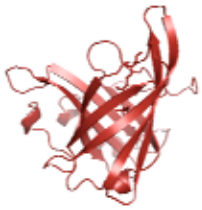
PGDS
[PDB: 2CZU]



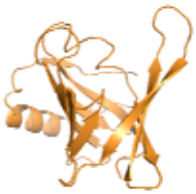
NGAL
[PDB: 1L6M]

OBP

α_1m

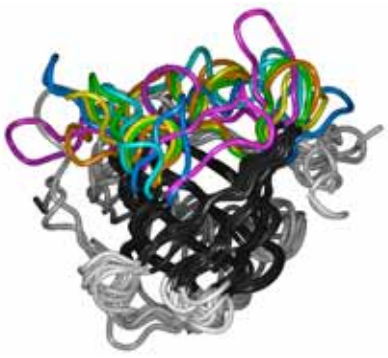


RBP
[PDB: 1RBP]

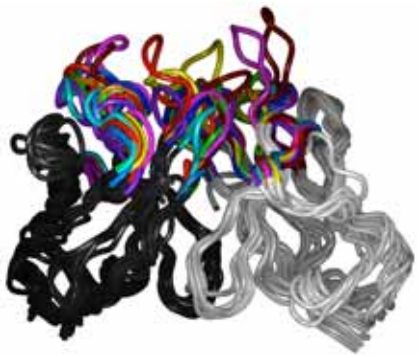


Tlc
[PDB: 1XKI]

Lipocalins & Antibodies: structural variability in the loop region

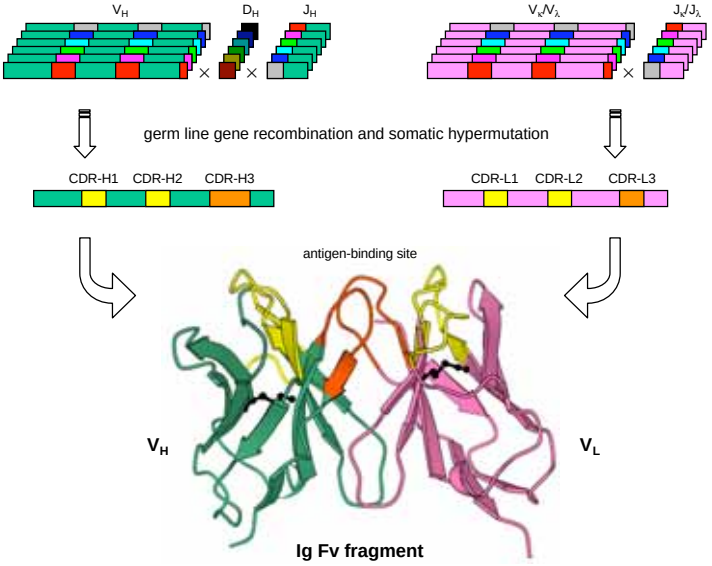


6 "prototypic" lipocalin structures:
RBP BBP MUP EPA BLG BDA

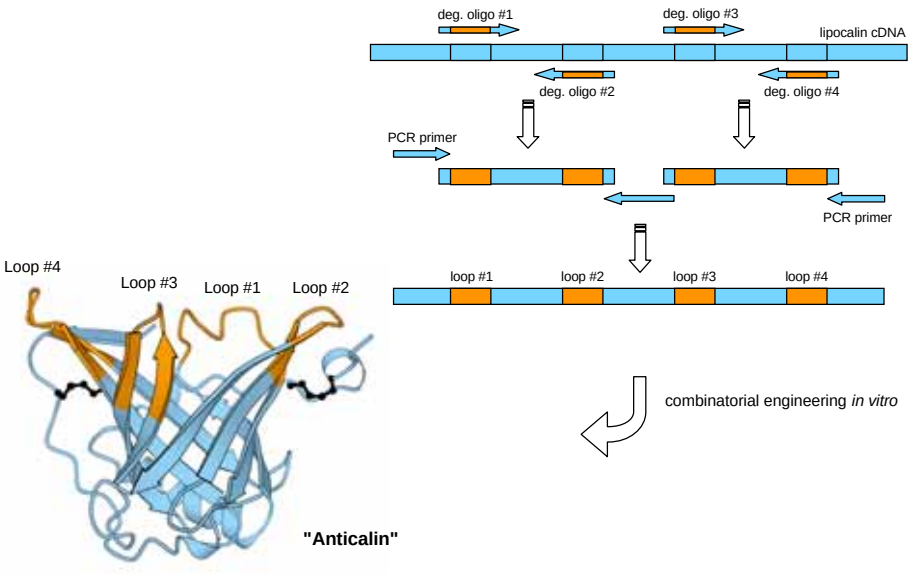


10 Fv fragments of different antibodies

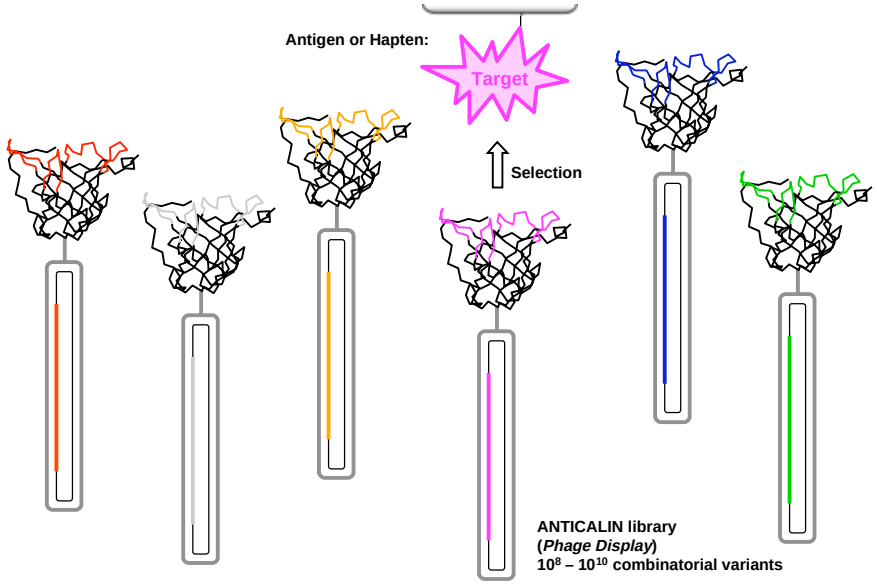
Generation of diversity in the immune system



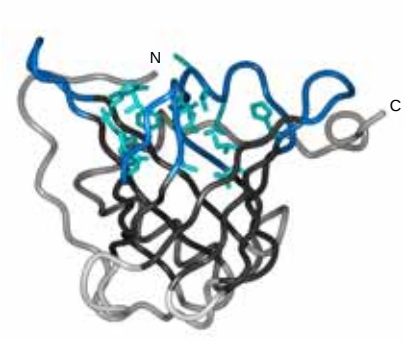
ANTICALIN®s: engineered lipocalins with novel binding properties



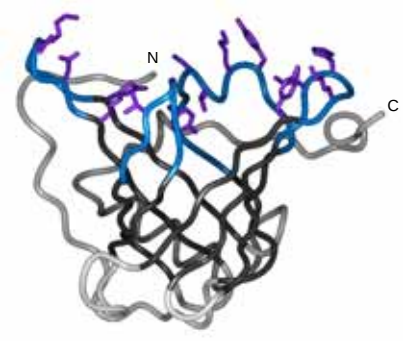
Selection from a lipocalin library with randomized binding sites



BBP random libraries designed against hapten or antigen targets



anti-hapten



anti-protein

Targets and applications for ANTICALINS

Established targets for ANTICALINS:

a) Haptens:
- fluorescein
- digoxigenin
- etc.

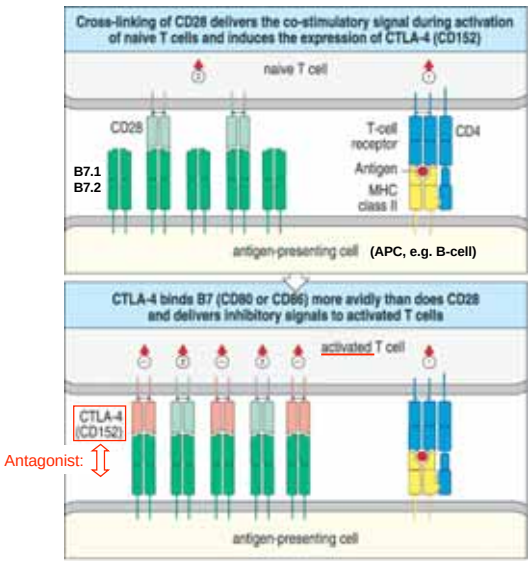
b) Peptides
- His_6-tag
- HBV epitope
- Aβ peptide

c) Protein antigens (medically relevant), e.g.:
- CD4
- CD33
- CTLA-4
- Cytokine receptor
- VEGF

Established applications for ANTICALINS:
- typical "immunochemical" assays
  - Western blot
  - ELISA
  - BIAcore
  - Flow-cytometric staining of cells
  - Histochemistry
- therapeutic applications ...

ANTICALIN libraries based on human scaffolds:
- NGAL (Siderocalin)
- Tear lipocalin
- Apolipoprotein D

Releasing the *immune brake* by an ANTICALIN as CTLA-4 antagonist

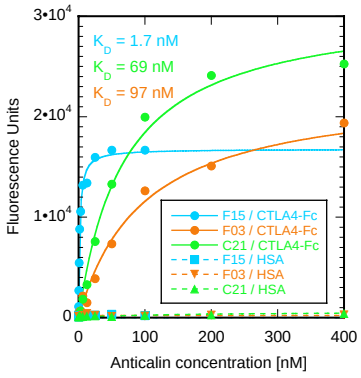


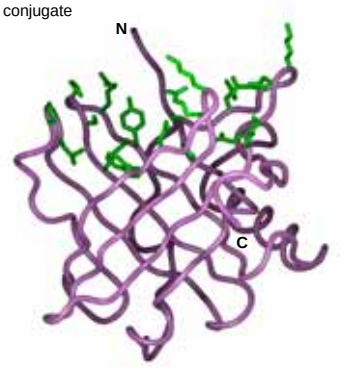
⇒ Clinical proof of concept for the immunotherapy of solid tumors (metastatic melanoma) with two human(ized) antibodies:
- *MDX-010* (Medarex): Phase III
- *CP670* (Pfizer): Phase I/II

from Janeway et al. "Immunobiology"

Selection of ANTICALINs with high affinity towards CTLA-4

- Selection of from a hNGAL library (20 randomized positions, complexity $2.0 \cdot 10^{10}$) via phage display followed by screening and identification of individual ANTICALINS via ELISA-HTS
- Target: **CTLA4-IgG1** (immunoadhesin of the extracellular T-cell co-receptor **CD152**)
  a) directly coated, 5 cycles of panning → ANTICALIN "F15"
  b) capturing via murine αhuFc antibody, 5 cycles of panning → ANTICALIN "C21" + "F03"
- ELISA: 1. capturing (5 µg/mL murine αhuFc antibody) of huCTLA4-Ig
  2. application of soluble, purified ANTICALIN (via *Strep*-tag II and gel filtration)
  3. detection via N-terminal T7-tag with mAb/HRP conjugate

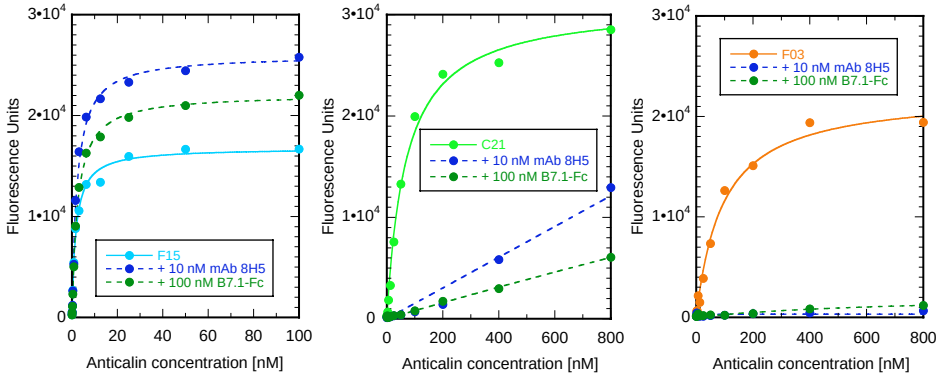




The **hNGAL** random library

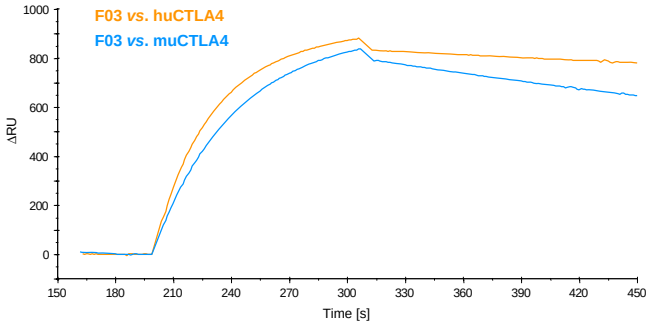
Epitope specificity of αCTLA-4 ANTICALINs

- ELISA with purified ANTICALINs as before, but after pre-incubation with competitors:
  (detection of bound ANTICALIN via N-terminal T7-tag with mAb/HRP conjugate)



The ANTICALIN "F03" cross-reacts with human and murine CTLA-4

- immobilization of ~2000 RU huCTLA4 or of ~2000 muCTLA4, both as huIgG1 Fc fusion proteins on a CM5 BIACORE chip coated with amine-coupled murine αhuFc antibody (14000 RU)
- application of the *Strep*-tag II-purified ANTICALIN *F03*, here at 5 µM concentration (20 µl/min)



Results:

- **huCTLA4**: k_{on} = 7.03 • 10^3 M^{-1} s^{-1} k_{off} = 3.3 • 10^{-4} s^{-1} **K_D = 47 nM**
- muCTLA4: k_{on} = 4.5 • 10^3 M^{-1} s^{-1} k_{off} = 8.9 • 10^{-4} s^{-1} K_D = 198 nM

Engineering of the αCTLA-4 ANTICALIN for higher affinity & stability

1. Engineering for improved target affinity:

- amplification of the central coding region of the anticalin "F03" via error-prone PCR
- phage display selection against huCTLA4-Ig (3 cycles), followed by filter sandwich colony screen (or ELISA HTS)

↳ "**J08**" (and "Q13")

↳ K_D = **7.9 nM** towards human CTLA-4 / 14.0 nM towards mouse CTLA-4

↳ T_m = 48.3 °C

2. Engineering for improved thermal stability:

- amplification of the central coding region of the anticalin "J08" via error-prone PCR
- phage display selection against huCTLA4-Ig at increased stringency, including preincubation of phagemids at elevated temperatures (T = 70 °C / 3 cycles), followed by ELISA HTS, in the presence of B7.1-Ig as competitor and/or including preincubation of protein extracts at 60 °C

↳ "**O10**"

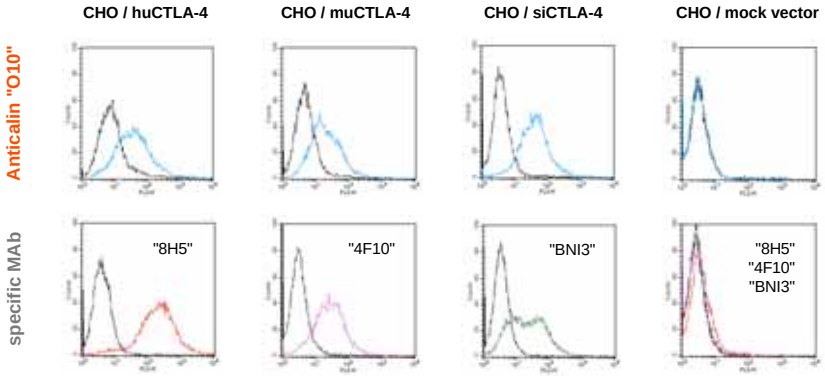
↳ K_D = **3.9 nM** towards human CTLA-4 / 15.3 nM towards murine CTLA-4

↳ T_m = **65.5 °C**

αCTLA-4 ANTICALIN "O10" in flow cytofluorimetry (FACS analysis)

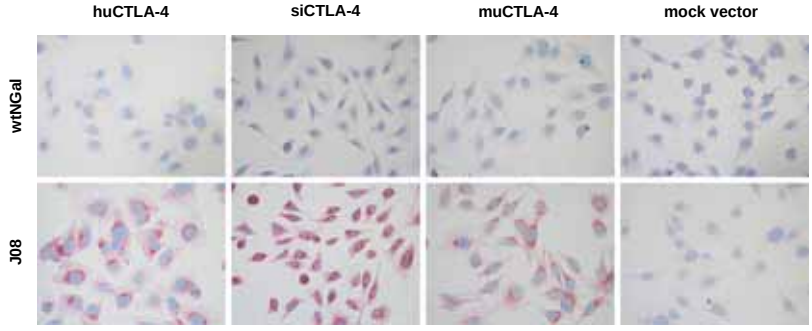
- CHO cells transfected with CTLA-4 cDNA from different species
- incubation of cells with purified ANTICALIN, followed by NGAL-specific mAb "HYB211-02"-Bio and detection via streptavidin-phycoerythrin (PE) conjugate
- alternatively, incubation with various species-specific αCTLA4 MAb (biotinylated or dye-labeled)

(black line: isotype control, i.e. IgG or wtNGAL)

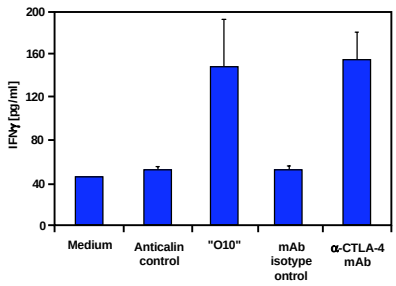


Histochemical staining of CTLA-4 positive cells with "J08"

- specific staining of CTLA4-transfected, acetone-fixed adherent CHO cells (cytospin preparation on frozen slides)
- detection of FITC-labelled ANTICALIN via anti-fluorescein mouse antibody and anti-mouse-AP conjugate, followed by FastRed staining and hematoxylin counterstaining



Enhanced T-cell activation *in vitro* by "O10" (PRS010) in MLR assay



Mixed Lymphocyte Reaction (MLR):

against allogeneic (MHC disparate) $CD80^+/CD86^+$ JY cells (human B-cells)

⇒ initial T-cell activation is attenuated by CTLA-4 unless blocked by cognate ANTICALIN or mAb

- co-culture of 10^5 washed human PHA T-cell blast cells (PBMC + 1 µg/ml PHA for 72 h) with 5,000 irradiated JY cells for 72 h
- 50 µg/ml of either CTLA-4 blocking antibodies or ANTICALINs are added at the start of the co-culturing period
- measurement of INFγ production after 48, 96 h (PHA blasts alone + test substance ≈ 20 pg/ml)

↳ **enhanced T-cell activity**
as revealed by
a) interferone production
b) cellular proliferation

⇒ potential for treatment of CTCL (*Cutaneous T Cell Lymphoma*) and immunotherapy of various solid tumors

Value of 'alternative scaffolds' for proteome research?

Functionally:

- **selection from synthetic libraries ⇒ control of many intrinsic and extrinsic parameters**
- **choice of selection techniques: phage display, ribosome display, colony screening, intracellular inhibition etc.**
- **monoclonality ⇒ reproducibility in binding behaviour and unlimited resources**
- **controllable specificity, e.g. by subtractive or competitive selection schemes**
- **binding activity may be enhanced as needed by *in vitro* affinity maturation**
- **higher robustness, smaller size, easier manufacture**
- **simple fusion or conjugation with reporter or affinity moieties**
- **recognition of native *vs*. denatured targets?**
- **preference of particular epitope shapes for different scaffolds?**

Survey of engineered protein scaffolds for molecular recognition

	Name	Template	Size	No. of loops	Crosslinks	Commercial exploitation
Ig-like domains	Nanobody	cameloid VHH	~ 130	3	2 S-S	www.ablynx.com
	dAb	human single V_H	~ 120	3	1 S-S	www.domantis.com
	iMab	Ig type fold	~ 110	3 – (4+4)	1 S-S	www.catchmabs.com
	Adnectin	^{10}Fn3 fibronectin III	94	2	–	www.adnexustx.com
loopy folds	Anticalin	lipocalin	160-180	4	0 – 2 S-S	www.pieris.biz
	Fluorobody?	"superfolder" GFP	238	≥ 1	fluorophore	–
	Microbody	knottin CBD/EETI-II	36/28	(β)*	2/3 S-S	www.nascacell.de
	Kunitz domain	BPTI/APPI	58	1 to 2	3 S-S	www.dyax.com
	aptamer	thioredoxin	108	1	1 S-S	–
2ndary struct.	Affiline	γB-crystallin/ubiquitin	198	(β)*	–	www.scilproteins.de
	Affibody	protein A	58	2•(α)*	–	www.affibody.com
	DARPin	ankyrin repeat	166	3•(α/β-turn)*	–	www.molecularpartners.ch
oligo -mer	Tetranectin	C-type lectin domain$_3$	ca. 155	3	3 S-S	www.boreanpharma.com
	Avimer	LDLR-A module$_n$	43	~ 4	3 + CA^{2+}	wwwext.amgen.com

(sound technology / scientific concept / patent application) (represented by consortium member)

Prof. Dr. Arne Skerra – Lehrstuhl für Biologische Chemie

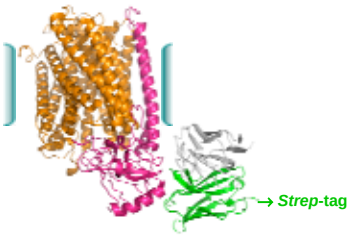
Value of alternative scaffolds for proteome research?

Practically:

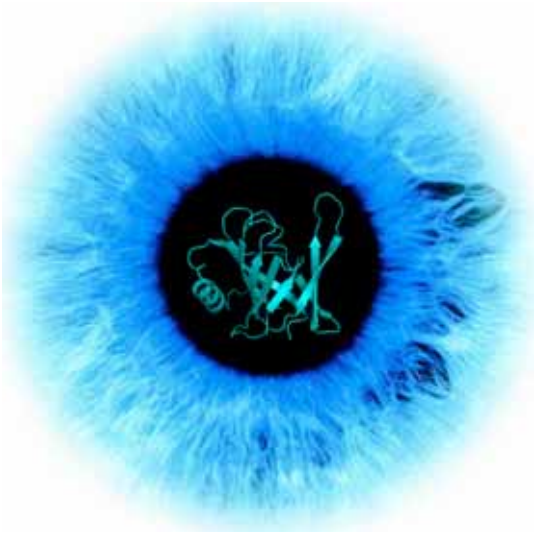
- **cost of generation (including affinity maturation) and validation**
- **availability of suitable "high throughput" facilities**
- **academic needs and capabilities *vs*. business requirements**

↳Short term future in specialized and/or commercially rewarding applications?

- **therapeutics [*intellectual property*]**
- ***in vivo* diagnostics [*plasma half life, receptor interactions*]**
- **highly functionalized reagents for unique applications or frequently assayed targets [*market volume*]**
- **crystallization tools?**



Contributors



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