Optimization of a VHH targeting Tau nucleation core and inhibiting Tau seeding

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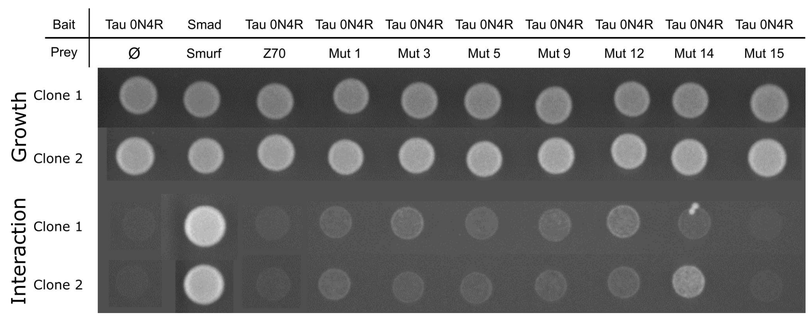
Tau is a neuronal protein linked to pathologies called tauopathies, including Alzheimer's disease. In Alzheimer's disease, tau aggregates into filaments leading to the observation of intraneuronal fibrillary tangles. VHHs (Variable domain of the heavy-chain only antibody) or nanobodies (Nbs), are antibody fragments of small size (<15kDa) easily produced in prokaryote recombinant systems. Naturally occurring antibodies only composed of the equivalent of the IgG heavy chain are found in Camelidae. VHHs correspond to the variable antigen-binding domain of these single chain antibodies.
We recently described VHH Z70, targeting Tau microtubule-binding domain composing the core of Tau fibrils and to reduce *in vitro* aggregation of Tau, Tau seeding in a cellular model and Tau pathology in a transgenic murine model. We are now looking into VHH Z70 optimization, which was carried out by random mutagenesis followed by yeast two-hybrid screening. We confirmed that optimized VHH kept the same epitope and had improved binding affinities. Interestingly, although these VHH had better affinities toward their epitope, *in vitro* aggregation and cellular seeding experiments revealed that their ability to inhibit Tau aggregation and seeding was not solely dependent on this aspect and proved difficult to predict. Indeed, our results demonstrate that VHH stability is another key to their efficacy. While our results show a good correlation between *in vitro* and intracellular activities of the VHH, both needs careful evaluation depending on the intended use, e.g. diagnostic tools or therapeutics.

Selecting Z70 mutants with better affinity

To generate optimized variants of VHH Z70, a strategy of limited random mutagenesis coupled with yeast two-hybrid screening was chosen for affinity optimization in intracellular conditions. Mutants of VHH Z70 with an improved affinity for Tau were selected on the His- medium by increasing the selection pressure using 3AT (3-amino-1,2,4-triazole), to reach conditions with limited to undetected interaction for VHH-Z70 with Tau (1 mM 3AT). 43 mutants were thus obtained and their sequence analyzed



Mutants contained 1 to 4 different point mutations resulting in amino acid substitutions and 33 different amino acid positions were found substituted at least once. Most substitutions occurred in the framework, with only 1 position in CDR1 (T32), 3 in CDR2 (E56, G58, S59) and 1 in CDR3 (P101). Interestingly, 3 positions were highly represented, G115 (23.6% of occurence, 21 occurrences), R47 (15.7%) and S23 (9%) whereas the others were randomly found between 1 and 4 times (< 5%).

a

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1         10        20        30        40        50        60
MAEVQLQASGGVFVQSGGSLRL*S*CAASG*ATSTFDG*MGWFRQAPGKE*R*EFVSAIS*YEQGSYT*YY
      G    Y R E     C S A   I   L     V  N KA      D VL
           H         I                           I       G E
                     N
        70        80        90        100       110       120
ADSVKGRFTISRDNSKNMVYLQMNSLRAEDTATYYCA*PAYEGDLYAFDS*YG*G*QGTQVTVSSAA
VV          VSPET AS      G     SC    S              NEEH
 N          K                       L
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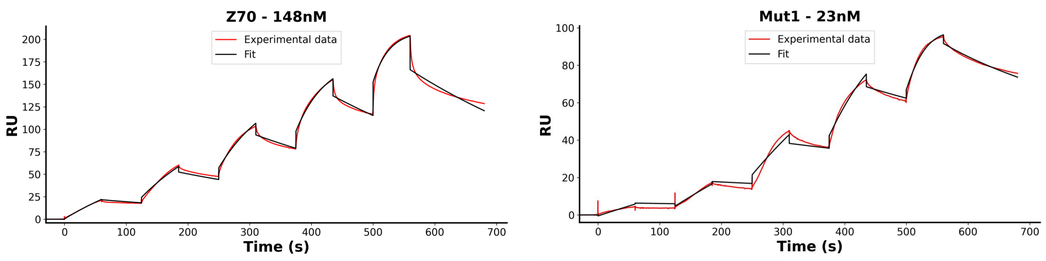
b

VHH	Substitutions	Domain
Mutant 1	G115E	FR4
Mutant 3	R47K	FR2
Mutant 5	T96S	FR3
Mutant 9	P101S + G115E	CDR3 + FR4
Mutant 12	S23C + G115E	FR1 + FR4
Mutant 14	T32I + E56G + G115E	CDR1 + CDR2 + FR4
Mutant 15	R90G + P101S + G114E	FR3 + CDR3 + FR4
Mutant 20	R47K + G115E	FR2 + FR4

8 mutants were selected to represent the diversity of substitutions.

Confirming mutants affinity and interaction site

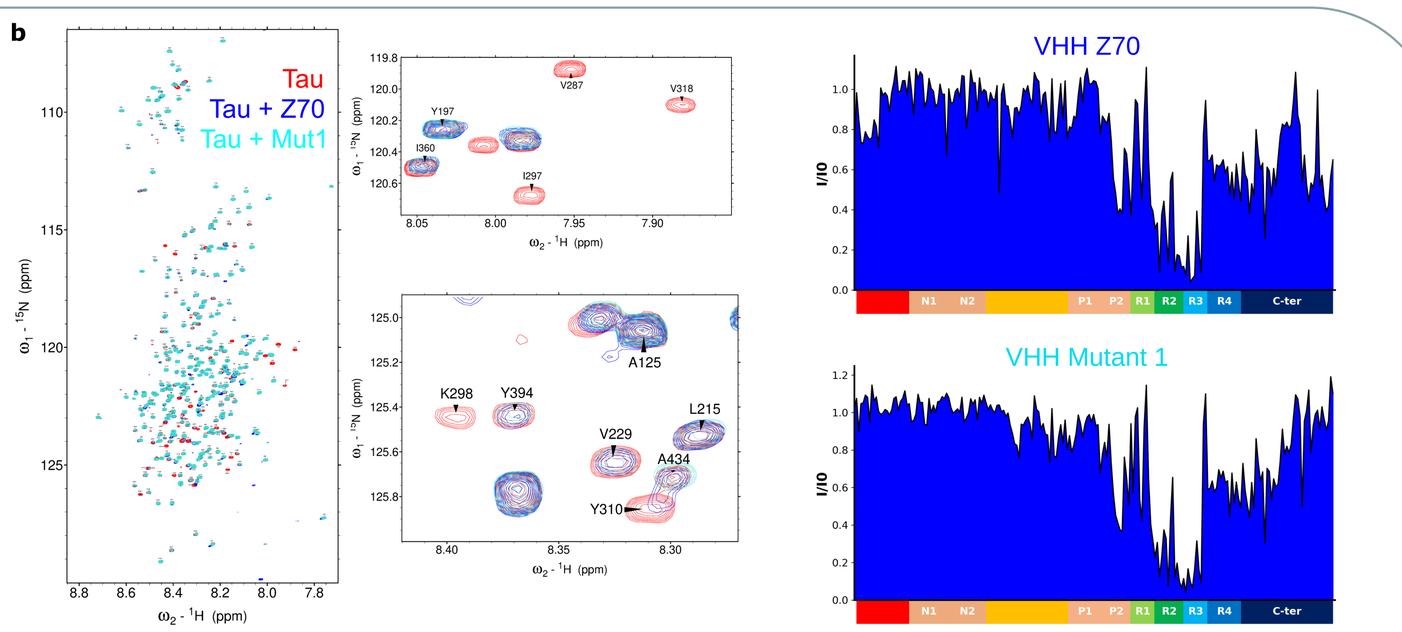
SPR was used to determine the affinity constant between VHH Z70 mutants and full length Tau 2N4R immobilized on a chip



VHH	kon (1/M.S)	koff (1/s)	Kd (nM)
Z70	18072 ± 42	0.00267 ± 0.00001	148 ± 0.9
Mutant 1	106744 ± 1720	0.00242 ± 0.00003	23 ± 0.5
Mutant 3	26042 ± 354	0.00210 ± 0.00003	81 ± 1.5
Mutant 5	10918 ± 32	0.00088 ± 0.00002	81 ± 1.5
Mutant 9	40110 ± 149	0.00205 ± 0.00002	51 ± 0.6
Mutant 12	33865 ± 236	0.00207 ± 0.00001	61 ± 0.6
Mutant 14	18799 ± 46	0.00140 ± 0.00002	74 ± 0.8
Mutant 15	65291 ± 706	0.00277 ± 0.00003	42 ± 0.7
Mutant 20	21285 ± 180	0.00089 ± 0.00001	42 ± 0.7

All the selected mutants showed a better affinity than Z70 towards full length Tau in this assay, further validating the selection process

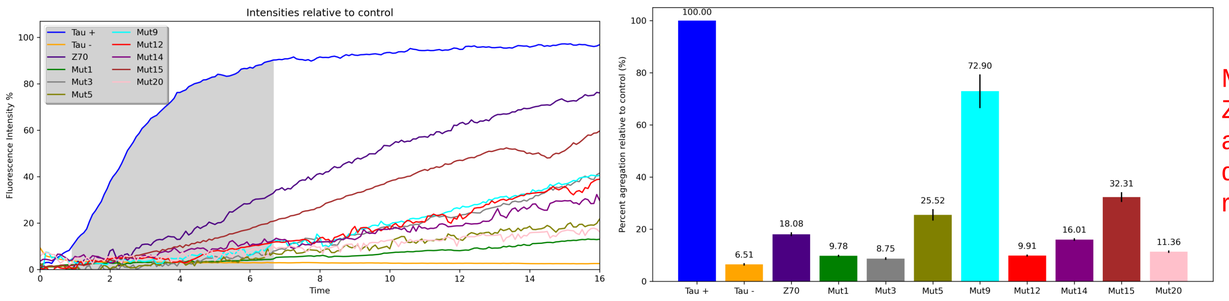
b



We used ^{1}H,^{15}N resonance intensity in 2D NMR spectra of Tau as reporters of the interaction at each amino acid position in Tau sequence for the 8 different mutant VHHs. The intensity profile is well conserved between the different VHHs with a major loss of intensity for resonances corresponding to residues located in the R3 repeat, similarly to the effect of Z70 binding to Tau PHF6 motif, confirming that the interaction site was kept intact in the different mutants.

In vitro Aggregation assay

VHH Z70 was already assessed for its capacity to interfere with Tau *in vitro* aggregation. Due to its high efficiency we used subequimolar ratios to discriminate between Z70 and its mutants efficiency. The assays were carried out with Tau recombinant protein in the presence of heparin, using thioflavin T as a dye whose fluorescence is increased in presence of aggregates.



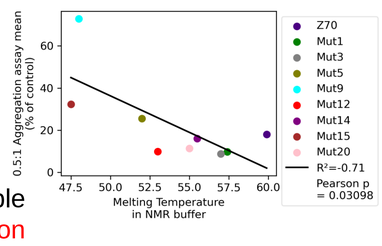
Most mutants keep Z70's efficiency in this assay, with 4 of them displaying even better results.

To facilitate comparison between the different VHH, the data from the different aggregation test were analyzed as a ratio of Thioflavin T fluorescence intensity in the presence of VHH compared to the positive control (Tau alone) for all points in the dynamic range of fluorescence corresponding to an intensity between 10 and 90% of the maximum fluorescence obtained for the positive control, in the absence of any VHH (grey area).

In vitro VHH stability

Some mutants showed a propensity to self aggregate in the *in vitro* aggregation assays giving rise to higher fluorescence than Tau alone. We have checked their thermal stability using a fluorescent reporter whose fluorescence increases once it binds the hydrophobic regions exposed during denaturation.

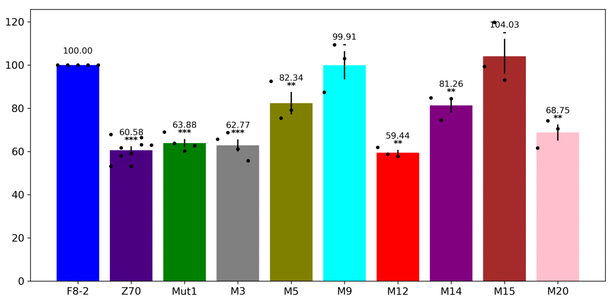
Z70	M1	M3	M5	M9
59.9 ± 0.9	57.4 ± 1.4	57.0 ± 0.5	52.0 ± 0.5	48.0 ± 0.3
M12	**M14**	**M15**	**M20**	
53.0 ± 1.0	55.5 ± 0.4	47.5 ± 0.4	55.0 ± 0.8	-



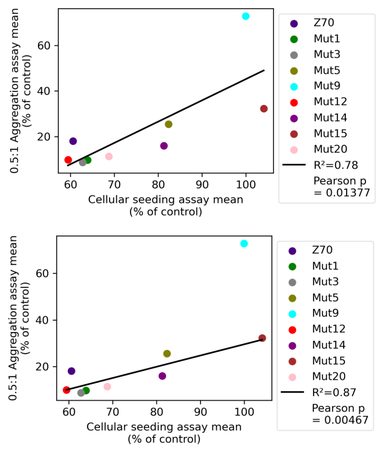
While no mutants were found to be more stable than VHH Z70, we could find a correlation between the VHHs melting temperature and their efficiency in the *in vitro* aggregation assay

Cellular Tau seeding assay

We previously reported that Z70 is able to inhibit Tau seeding in a cellular assay, using HEK293 Tau RD P301S FRET Biosensor reporter cell line model. We thus evaluated the potency of the different mutants in this assay.



Tau aggregation can be followed by the apparition of a FRET signal. VHH-transfected cells are monitored by the presence of mCherry fluorescence. We evaluate the efficiency of the different mutants compared to a non relevant VHH, F8-2 in the mCherry positive cell population.

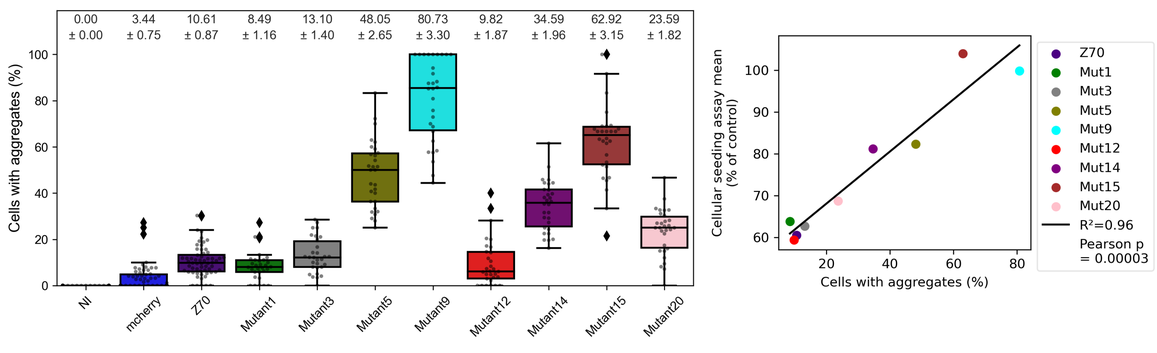


There is a high correlation between the VHH inhibition capacity in the cellular seeding assay and the *in vitro* aggregation assay (R^2 = 0.78) with mutant 9 being a clear outlier probably due to its self-aggregation in the *in vitro* assay (without mutant 9 R^2 = 0.87)

VHH Z70 and 4 mutants drastically reduce FRET signal in the mCherry positive cell population.
(2 mutants lost their efficiency while 2 others showed a reduced efficiency)
The efficiency of the mutants could be influenced by their stability inside cells

Cellular self aggregation assay

To evaluate the aggregation of the Z70-derived VHH series expressed in the cellular environment, we used HEK cells transfected with pMCherry constructs that produced VHH-mCherry The presence of aggregates is noticed by the appearance of "puncta" inside the cells in contrast with a uniform fluorescence across the cell in the absence of aggregates



The results we obtained for the *in vitro* VHH stability test and Cellular Tau seeding assay correlate with this self aggregation experiment.
Some VHHs self aggregate, explaining their differences in ability to inhibit Tau aggregation

Conclusion : In this study, we have selected 2 VHHs with aggregation inhibition activity on par with VHH-Z70. This lead us to question whether the mutants were actually not better than the parent VHH or if we hit a limit of the system. Through these experiments, we have selected best VHHs to be tested in a mice brain experiment in order to give us the opportunity to use it as a diagnostic or therapeutic tools.