Screening Cyclised Peptides Inside Cells Identifies a Short Helical Inhibitor of Alpha-Synuclein Aggregation

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1. Introduction

- Alpha-Synuclein (αSyn) aggregation is a hallmark of Parkinson's disease (PD), resulting in toxic oligomers and fibrils that drive neuronal death (Fig. 1a)[1].
- Lipid interactions induce an α-helical conformation in αSyn, essential for its synaptic role, with key residues in the N-terminal region (Fig. 1b, purple)[2]. Lipid interactions are also known to accelerate αSyn aggregation.

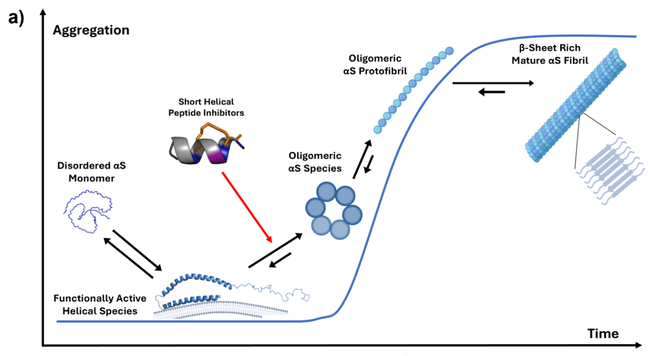


Fig. 1a) Schematic representation of αSyn aggregation.

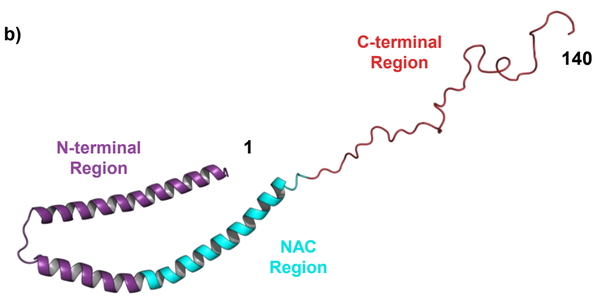


Fig. 1b) Domain organisation of αSyn: N-terminal region (purple), NAC region (blue), and C-terminal region (red)

- Short peptides can inhibit αSyn aggregation and toxicity[3,4]. We recently reported $\alpha S_{2\text{-}12}$(L6), an N-terminal-derived, α-helical peptide inhibitor of αSyn aggregation[5] (Fig. 1c-d).
- Here, we employ a novel intracellular cyclisation screening approach to improve upon $\alpha S_{2\text{-}12}$(L6) and identify a more potent inhibitor, $\alpha S_{2\text{-}12}$W.

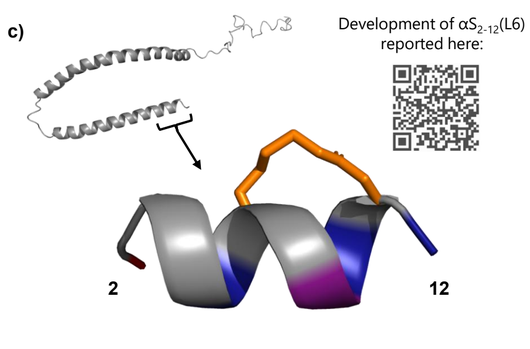


Fig. 1c) Solution NMR structure of $\alpha S_{2\text{-}12}$(L6) (PDB: 8OL8)

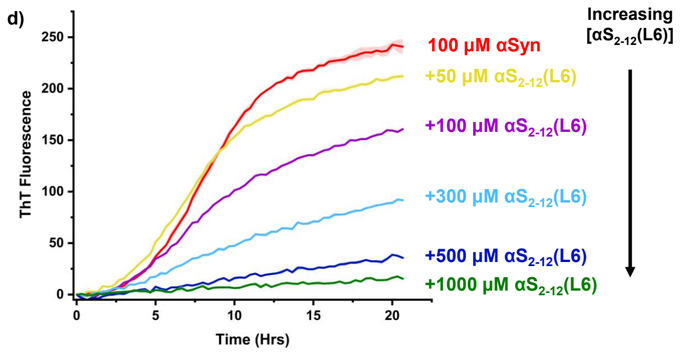


Fig. 1d) ThT fluorescence assay demonstrating dose-dependent inhibition of αSyn aggregation by increasing concentrations of $\alpha S_{2\text{-}12}$(L6).

2. Peptide Screening: Protein-fragment Complementation Assay

- We screened a peptide library using a Protein-fragment Complementation Assay (PCA), in the presence of cell-penetrant bis-alkylating agents[6], selecting peptides that bind to αSyn by enabling bacterial survival in the presence of the antibiotic, trimethoprim (TMP) (Fig. 2).
- TMP inhibits bacterial dihydrofolate reductase (DHFR), but survival is restored when a split exogenous murine DHFR (mDHFR) is reformed via a peptide–αSyn interaction.
- The strongest peptide binder is identified as the fastest-growing colony during liquid passaging.

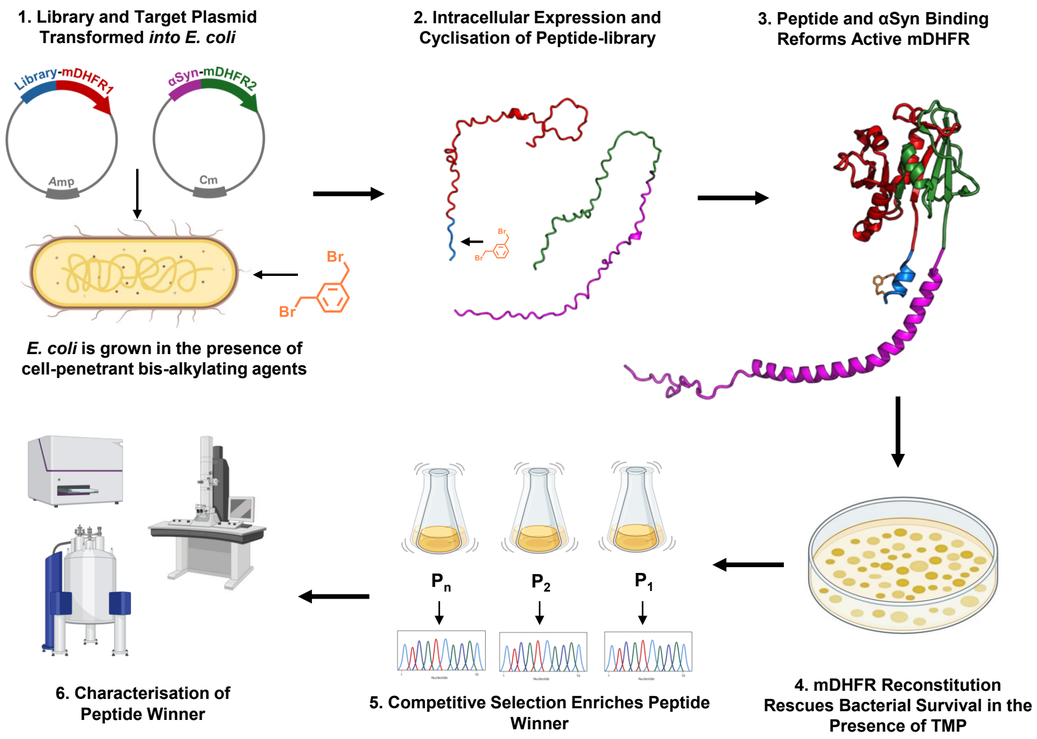


Fig. 2) Protein-fragment Complementation Assay used to screen the peptide library.

3. Library Design and Selection of $\alpha S_{2\text{-}12}$W

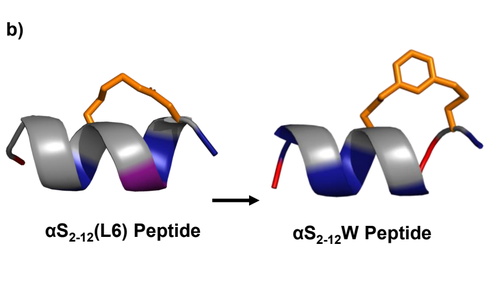
- A 20-million-member library based on $\alpha S_{2\text{-}12}$(L6) was designed with charged, polar, and hydrophobic residues at each position (Fig. 3a).
- The design enabled intracellular *i→i+4* cyclisation, locking peptides into an α-helical conformation using a cell-penetrating bis-alkylating agent.

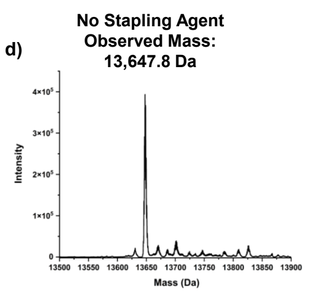
a)

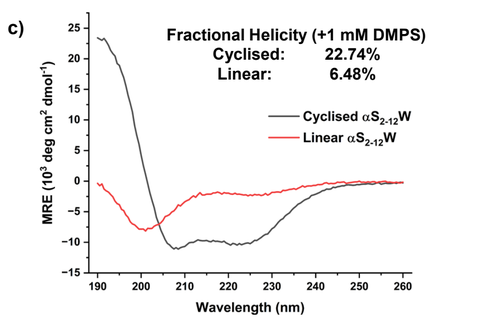
	2	3	4	5	6	7	8	9	10	11	12
Native $\alpha S_{2\text{-}12}$ Sequence	D	V	F	M	K	G	L	S	K	A	K
$\alpha S_{2\text{-}12}$(L6) Sequence	D	V	F	M	K	K	L	S	K	D	K
Library Options	D	V	F	M	K	C	L	S	K	C	K
	V	I	L	V	I		V	I	I		I
	I	G	V	G	G		M	L	G		G
	N	E	Y	E	V		Q	V	V		V
	E	K	D	R	E		E	G	E		E
	K	R	H	K	R		K	N	R		R
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Fig. 3a) Peptide library design

- Screening identified the hit peptide, $\alpha S_{2\text{-}12}$W (Fig. 3b).
- Circular Dichroism (CD) confirmed that cyclisation induced helicity in $\alpha S_{2\text{-}12}$W (Fig. 3c), while mass spectrometry verified successful intracellular cyclisation of the purified SUMO-fused peptide (Fig. 3d).







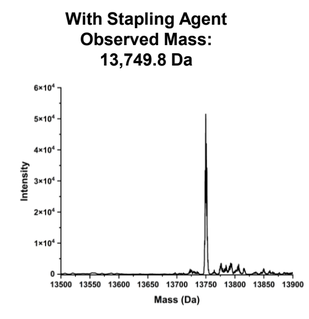


Fig. 3b) Solution NMR structures of $\alpha S_{2\text{-}12}$(L6) (PDB: 8OL8) and $\alpha S_{2\text{-}12}$W (PDB: 9SG1). **3c)** CD spectra showing helical induction of $\alpha S_{2\text{-}12}$W by addition of stapling agent in the presence of lipid (DMPS). **3d)** MS spectra showing mass increase of 102 Da for purified $\alpha S_{2\text{-}12}$W-SUMO fusion when expressed in the presence of the stapling agent, confirming intracellular cyclisation.

4. $\alpha S_{2\text{-}12}$W Inhibits αSyn Aggregation

- ThT fluorescence assays demonstrated that $\alpha S_{2\text{-}12}$W inhibits αSyn aggregation at substoichiometric concentrations, indicating greater inhibitory potency than $\alpha S_{2\text{-}12}$(L6) (Fig. 4a).
- Removal of the stapling agent reduced the level of inhibition (Fig. 4a).

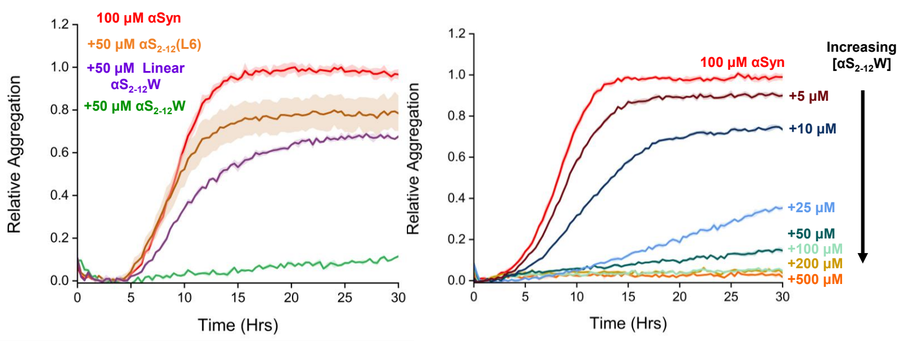


Fig. 4a) (Left) ThT fluorescence assay showing inhibition of αSyn aggregation by the $\alpha S_{2\text{-}12}$W peptide compared to the initial $\alpha S_{2\text{-}12}$(L6) peptide and linear, unstapled version. **(Right)** $\alpha S_{2\text{-}12}$W induces a strong concentration-response.

- TEM confirmed that $\alpha S_{2\text{-}12}$W significantly reduced αSyn fibril formation (Fig. 4b).

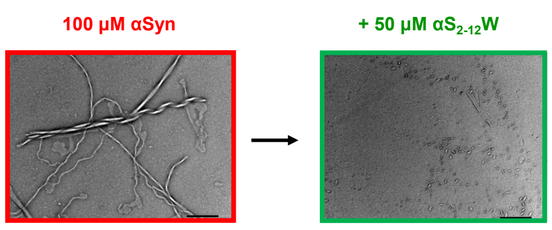


Fig. 4b) TEM micrographs of endpoint ThT assay samples, showing no αSyn fibril formation with $\alpha S_{2\text{-}12}$W addition.

5. $\alpha S_{2\text{-}12}$W Binds the C-terminal Region of αSyn

- αSyn chemical shift perturbations were tracked in the presence of increasing concentrations of $\alpha S_{2\text{-}12}$W (Fig. 5a).

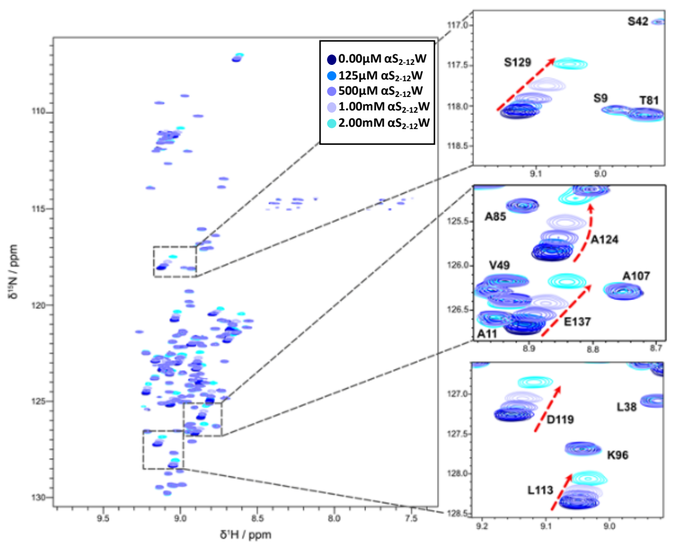


Fig. 5a) ^{1}H-^{15}N HSQC spectra of αSyn in the presence of $\alpha S_{2\text{-}12}$W at increasing concentrations. Residues in the C-terminal region show the largest chemical shift perturbations.

- The $\alpha S_{2\text{-}12}$W peptide interacts with the C-terminal region of αSyn based on CSP analysis (Fig. 5b).

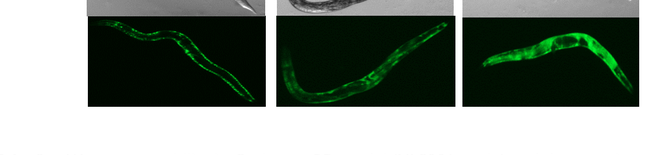


Fig. 5b) The chemical shift perturbation per residue at 2 mM $\alpha S_{2\text{-}12}$W.

6. $\alpha S_{2\text{-}12}$W is Cell-Penetrant and Non-Toxic

- Rhodamine B-labelled $\alpha S_{2\text{-}12}$W shows strong dose-dependent uptake in SH-SY5Y neuronal cells versus $\alpha S_{2\text{-}12}$ and $\alpha S_{2\text{-}12}$(L6) (Fig. 6a-b).
- $\alpha S_{2\text{-}12}$W is also non-toxic at concentrations tested (Fig. 6c-d)

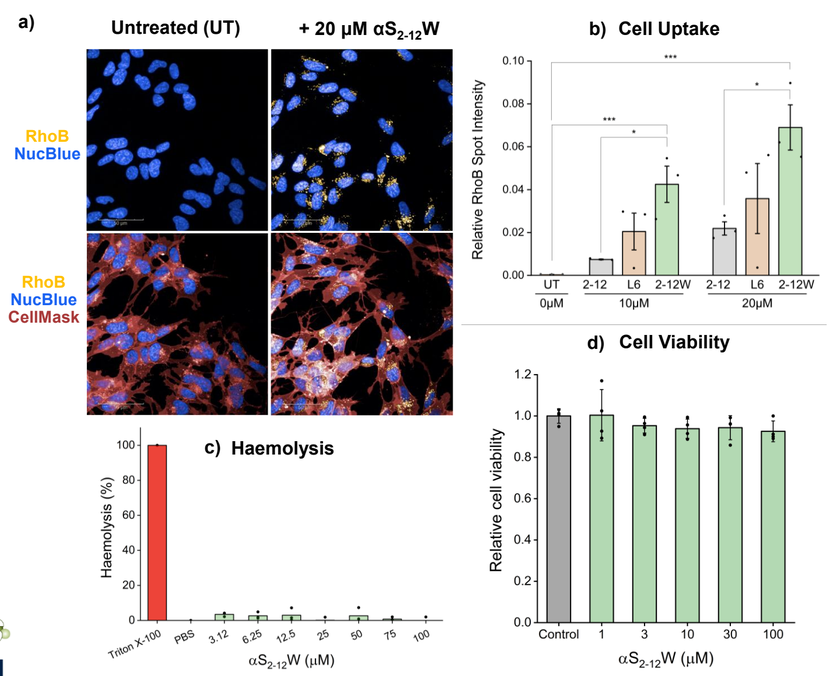


Fig. 6a) RhoB-$\alpha S_{2\text{-}12}$W (Yellow) uptake into SH-SY5Y cells. 6b) Quantitative comparison of uptake between native $\alpha S_{2\text{-}12}$ sequence, $\alpha S_{2\text{-}12}$(L6) and $\alpha S_{2\text{-}12}$W. 6c-d) Cell viability and haemolysis not impaired by $\alpha S_{2\text{-}12}$W up to 100 μM.

7. $\alpha S_{2\text{-}12}$W Rescues Mobility in *C. elegans* PD Model

- $\alpha S_{2\text{-}12}$W rescues thrashing (bending) in αSyn-mobility impaired *C. elegans* model (Fig. 7a).
- $\alpha S_{2\text{-}12}$W reduces αSyn inclusions in YFP-αSyn overexpressing worm (Fig. 7b-c).

Fig. 7a) $\alpha S_{2\text{-}12}$W rescues mobility in *C. elegans* PD model (NLS901 strain) and does not impact control strain. 7b-c) $\alpha S_{2\text{-}12}$W treatment reduces αSyn inclusion formation in *C. elegans* PD model.

References and Acknowledgments

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