Title: α -Helical peptidic scaffolds to target α -synuclein pathogenic species with high affinity and selectivity.

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Abstract

α -Synuclein aggregation is a key driver of neurodegeneration in Parkinson's disease and related syndromes. Accordingly, obtaining a molecule that targets α -synuclein pathogenic assemblies with high affinity and selectivity is a long-pursued objective. Here, we have exploited the biophysical properties of toxic oligomers and amyloid fibrils to identify a family of α -helical peptides that bind selectively to these α -synuclein species with low nanomolar affinity, without interfering with the monomeric functional protein. This activity is translated into an unprecedented anti-aggregation potency and the ability to abrogate the oligomers toxicity. With a structure-function relationship in hand, we identified a human peptide expressed in the brain and in the gastrointestinal tract with exceptional binding, anti-aggregation, and detoxifying properties, which suggests it might play a protective role against synucleinopathies. The chemical entities we describe here represent a new therapeutic paradigm and are promising tools to assist diagnosis by selectively detecting α -synuclein pathogenic species in biofluids.

Introduction

α -Synuclein (α S) is a 140 amino acid protein whose aggregation into amyloid fibrils in a subset of neuronal and glial cells lies behind the onset of a group of progressive and, ultimately, fatal neurodegenerative disorders, including Parkinson's disease (PD)¹⁻⁴, that are collectively referred to as synucleinopathies. A causative link between α S and disease is supported by the discoveries that multiplications and missense mutations in SNCA, the α S gene, cause dominantly-inherited familial forms of PD⁵.

Interfering with α S amyloid formation and abrogating the associated toxicity is considered a promising therapeutic strategy for synucleinopathies⁶⁻⁸. However, the design of molecular entities that target specific α S pathogenic assemblies is challenging because of the heterogeneous, dynamic, and transient nature of these species. High-throughput screening initiatives have rendered promising α S aggregation inhibitors⁹⁻¹¹. However, these selection procedures are blind to the ligand mechanism of action. In the absence of a structure-activity relationship (SAR), it is difficult to evolve the affinity and specificity of the identified hits to generate drugs that can reach the clinics. The lack of specific and sensitive molecules to detect the pathogenic forms of α S also constrains the early diagnosis of these diseases.

The *in vitro* aggregation of α S displays a sigmoidal growth profile, suggesting that it follows a nucleation-polymerization mechanism¹², where soluble α S undergoes a nucleation process that produces oligomers able to grow through further monomer addition to form insoluble amyloid fibrils. Oligomeric forms of α S have been detected in the brains and other tissues of patients suffering from PD, and growing evidence suggests that they constitute the primary pathogenic agents accounting for the gain-of-toxicity associated with α S aggregation, whereas both oligomers and fibrils would be responsible for pathology dissemination in the brain^{2,13-15}. We have recently identified the sequential population of two conformationally distinct types of oligomers during α S *in vitro* fibrillation. The initial non-toxic disordered oligomers, named as type A oligomers, undergo a structural reorganization to form more stable and compact β -sheet enriched, and proteinase-K resistant species that exhibit intrinsic cytotoxicity, named as type B oligomers¹⁶. Stable, trapped analogous of these two well-defined types of transient oligomers (referred to as type A* and type B* oligomers, where the star refers to the kinetically trapped nature of these isolated oligomeric forms) have been isolated and characterized in detail^{13,16} and, therefore, constitute important tools for the development of specific therapeutic and diagnostic strategies.

In this work, we have exploited our recent advances in the understanding of the structural determinants of toxicity of α S oligomers to rationally identify novel molecules able to target the pathogenic species of α S. By using a time-resolved single-particle fluorescence approach we demonstrate that short, amphipathic and cationic α -helical peptides bind toxic oligomers and fibrils with unprecedented specificity and affinity, resulting in the substoichiometric inhibition of α S aggregation and abrogation of oligomer toxicity in neuronal cell models. We then used a protein engineering approach to dissect the molecular determinants accounting for this interaction, which has allowed us to identify a human peptide, constitutively expressed in the brain and gastrointestinal tract, that binds with low nanomolar affinity and high specificity to α S pathogenic species, thus suppressing the aggregation cascade and its associated neurotoxicity, which suggests it might play a natural protective role against synucleinopathies.

In summary, here we describe the rational identification and characterization of a family of highly potent and α S species-specific peptidic ligands able to bind selectively to α S toxic species and abrogate their toxic effects in neuronal cells. This discovery opens previously unexplored avenues for the diagnosis and/or therapeutics of PD and related disorders.

Results

Identification of an α S species-specific peptide ligand.

We rationalized that the particular properties of the four main α S conformers identified during α S amyloid aggregation, namely monomers, non-toxic (type A/A*) oligomers, toxic (type B/B*) oligomers and fibrils, could be exploited to identify a selective ligand for the main species responsible for induction and propagation of toxicity, which are currently believed to be type B-like oligomers and amyloid fibrils, respectively¹⁷. In **Figure 1a**, we illustrate the dissection of the

differential traits of α S forms. Type B-like oligomers and amyloid fibrils share two features: (i) they expose relatively large lipophilic clusters to the solvent. These hydrophobic surfaces induce cellular toxicity and drive subsequent fibrillation^{16,18-20}. (ii) They possess a high anionic character, at neutral pH as a result of the stacking of α S monomers (net charge -9) with the highly negatively charged C-terminal regions exposed to the solvent. While the solvent exposure of hydrophobic surfaces seems to be a general feature of toxic pre-fibrillar oligomers^{13,21}, the combination of high exposed hydrophobicity and negative charge is likely unique to these two pathogenic α S assemblies. Thus, we hypothesized that hydrophobic patches embedded in an anionic environment might delineate a diffuse, but physicochemically-defined, binding surface in these two types of α S aggregates for a complementary molecule; ideally an amphipathic and cationic entity. A short α -helical peptide might provide a structurally stable scaffold to merge both features.

Short α -helical peptides tend to be only marginally stable in solution. Therefore, the *de novo* design of an amphipathic and cationic helical chassis that retains a stable secondary structure under physiological conditions is not trivial^{22,23}. Instead, we decided to identify a naturally occurring peptide bearing our desired properties, assuming that evolution would have selected for a stable α -helix fold. This is especially true for secreted peptides, which should remain folded in a harsh extracellular environment. Following this rationale, we selected PSM α 3 as a candidate peptide, since, theoretically, it fulfilled all our requirements. PSM α 3 is a 22 residues bacterial extracellular peptide that has been shown to remain in an α -helical conformation for weeks²⁴. It has a net charge of +2, a mean hydrophobicity (H) of 0.54, an α -helical hydrophobic moment (μ_H) of 0.56, and the helical wheel plot evidences its amphipathic character (**Figure 1b-c**). The far-UV circular dichroism (CD) spectrum of PSM α 3 confirms that it folds into an α -helix under our assay conditions (**Figure 1d**).

We engineered PSM α 3 to obtain a negative control peptide in which the formation of an α -helix is strongly disfavored. This will disrupt the peptide amphipathic character and, theoretically, abolish binding to α S type B* oligomers and amyloid fibrils. After a computational proline scanning of PSM α 3 using the AGADIR algorithm²⁵ (**Supplementary Figure 1a**), we selected the K9P and F11P mutations, as they have a significant impact in helical propensity and map to opposite faces of the α -helix (**Supplementary Figure 1b-c**). The characterization of the secondary structure of the K9P-F11P PSM α 3 peptide (further referred to as disrupted PSM α 3 or dPSM α 3) in solution by CD confirmed the disruption of the α -helix fold (**Supplementary Figure 1d**). Thus, dPSM α 3 constitutes a suitable negative control for further studies, as it keeps a sequence identity of 91 % with PSM α 3, but lacks its amphipathic character, a feature that we propose is key for the specific binding to the α S toxic species.

Selective interaction of PSM α 3 with α S pathogenic species.

We then addressed the interaction of PSM α 3 and dPSM α 3 with the four major α S species generated during the process of amyloid aggregation. As multiple peptides are expected to bind multiple α S molecules in the aggregated states, the binding process can only be well described if both the stoichiometry of the complex and the affinity of the peptide for the α S molecules in a particular conformer is known. In order to obtain good estimates of both parameters, we exploited the power of dual-color fluorescence cross-correlation spectroscopy (dcFCCS), a time-resolved fluorescence fluctuation technique that allows the direct observation of co-diffusing fluorescent species arising from interactions between differently labeled molecules or assemblies in solution^{26,27}. To this end, α S species were cys-labeled with maleimide-AlexaFluor488 (AF488), with each α S molecule of the different species containing one fluorophore at position 122, and the peptides were cys-labelled with maleimide-Atto647N at the N-terminus (see Methods section for details). Simultaneously, we assessed complex formation by single-particle fluorescence spectroscopy (SPF) analysis, including Förster Resonance Energy

Transfer (FRET) and donor/acceptor stoichiometry (S), in order to validate and complement the dcFCCS approach. These approaches allow us to monitor distinct individual species simultaneously by avoiding measurements of ensemble averages (**Supplementary Figure 2**) and have been previously used for the characterization of α S aggregation pathways and the study of α S interactors²⁸⁻³⁰.

We first assessed the binding of PSM α 3 to monomeric α S and found by dcFCCS analysis that these molecules were unable to interact when mixed in an equimolar ratio (15 nM of each molecule) (**Figure 2a**), as reflected by a flat cross-correlation curve comparable to that of the negative control of cross-correlation (**Supplementary Figure 3**). We then analyzed the interaction of PSM α 3 with the different α S aggregated species by means of dcFCCS at approximately equimolar ratios of peptide and α S molecules (1-5 nM). Due to the different stability of the various aggregated species upon single-molecule dilution and their differential adsorption to the surface of the coverslips, the total α S concentration of each aggregated sample was adjusted between 1 and 5 nM (mass concentration) so that the frequency of events in the diffusion single-particle measurements was very similar between the various aggregated samples. Under these conditions, a marginal cross-correlation amplitude was observed for the type A* oligomers (**Figure 2b**), whereas a clear cross-correlation was obtained for the interaction of the peptide with both type B* oligomers and fibrils (**Figure 2c and d**, respectively), already indicating a stark difference in the binding ability of the peptide to the different aggregates. Consistently, a high number of FRET events were detected by single-particle burst-wise analysis, thus validating the direct interaction of the peptide with these two α S species. In contrast, the same analysis yielded either a statistically insignificant number of FRET events or none at all for the interaction between PSM α 3 and α S type A* oligomers and monomers, respectively (**Supplementary Figure 4a-c**). These results offer a single-particle

understanding of a complex binding scenario and further reinforce the observations derived from dcFCCS experiments.

We next performed a titration experiment for analyzing the binding of the peptide to the three α S aggregated species. For this, we developed a model-independent saturation binding curve, based on the theoretical framework previously developed by Kruger and coworkers³¹. This analysis allowed us to quantify the number of peptide molecules bound to each α S species (N_p) as a function of the concentration of unbound peptide ($C_{p,Free}$) (**Figure 2e**). Using a simplistic Langmuir isotherm model, we estimated the single-state dissociation constant (K_D) of the interactions and the average maximum number of peptide binding sites (N_{max}) in each type of α S species. Interestingly, while the K_D values for the peptide- α S interaction obtained for type A* oligomers, type B* oligomers and fibrils are very similar, in all cases in the very low nM range (3.07 nM, 6.67 nM and 7.8 nM, respectively), the average maximum number of peptides per aggregate varies remarkably, being 3, 30 and 120, respectively. This indicates that the main difference between the three aggregated species in terms of PSM α 3 interaction is the number of binding sites per aggregate rather than the affinity of the peptide for them. It is remarkable that the average maximum number of binding sites obtained for the type B* oligomers and fibrils nearly matches the average number of α S molecules per aggregate species (19, 33 and 107 for type A*, type B* oligomers and fibrils, respectively, estimated by comparing the molecular brightness of the aggregated species to that of the α S monomer, not shown), while for the type A* oligomers represents only one sixth of the average α S molecules in this type of aggregate. This data is in agreement with the single-particle fluorescence analysis obtained for the different complexes, where only very few FRET events were observed for the binding of PSM α 3 to type A* oligomers, in contrast to the numerous FRET events with a defined FRET efficiency (E) distribution observed for the binding to type B* oligomers and fibrils (**Supplementary Figure 4**). Note that a 500-fold excess of unlabeled monomeric α S does not interfere with the binding of PSM α 3 to type B* oligomers (**Figure 2f**) which indicates a high

specificity towards aggregated species and negligible monomer binding. Together, the dcFCCS and single-particle fluorescence data demonstrate that PSM α 3 is a high affinity α S species-specific ligand, with affinities in the low nanomolar range, and with a remarkable avidity and selectivity for the type B* oligomers and fibrils. Interestingly, when we analyzed the binding of the PSM α 3 analogous, but disordered peptide, dPSM α 3, we could not detect any interaction with any of the four α S species (**Supplementary Figure 5**), indicating that an amphipathic distribution of the peptide residues, achieved through an α -helical conformation, is a requirement for the interaction.

Overall, our dcFCCS-derived binding analysis indicates that PSM α 3 binds with low nanomolar affinity to all α S aggregated species. The degree of binding is limited by the number of available interaction sites, which is likely associated with the extent of solvent-exposed hydrophobic surface per aggregate, which depends on both the size and the lipophilicity of the aggregate, in agreement with our initial reasoning.

PSM α 3 inhibits α S amyloid aggregation.

We hypothesized that the high affinity and number of binding sites of type B* oligomers for PSM α 3 might result in the partial or full coverage of the surface of these assemblies and likely their structurally homologous type B oligomers, thus preventing their progression to fibrils during the α S amyloid aggregation process. To assess if this was the case, we set up *in vitro* α S aggregation reactions in the absence and presence of an equimolar concentration of PSM α 3 (70 μ M) and followed its progression by monitoring the increase in thioflavin-T (Th-T) fluorescence. After 32 hours of incubation, a ~90 % decrease in Th-T fluorescence emission, relative to the untreated sample, was observed in the presence of PSM α 3, suggesting that the peptide acts as a potent inhibitor of α S amyloid aggregation (**Figure 3a**). Transmission electron microscopy (TEM) images confirmed that samples incubated with PSM α 3 contained very few

fibrils per field, in comparison to untreated samples (**Figure 3b**). The inhibitory activity of PSM α 3 was concentration-dependent and significant even at a substoichiometric 20:1 ratio (α S:PSM α 3) (**Figure 3c**), in agreement with its selective binding to low populated oligomeric species formed during the early stages of α S amyloid aggregation, in line with the time-resolved fluorescence spectroscopy data. The observation that dPSM α 3 exhibited a negligible anti-aggregative activity (**Supplementary Figure 6**) reinforces the connection between the binding of the amphipathic (hydrophobic/cationic) helical peptide to α S oligomers and its potent amyloid inhibition activity.

Remarkably, PSM α 3 is a better inhibitor of α S amyloid formation than SynuClean-D (**Figure 3a**), a small molecule with high neuroprotective activity in *Caenorhabditis elegans* models of PD that we have discovered recently⁹. Indeed, a survey of the literature indicates that the inhibition potency of PSM α 3 exceeds that reported for most non-covalent small molecules, including the diphenylpyrazole Anle138b, already in clinical trials¹⁰.

PSM α 3 protects cells from α S oligomer-induced toxicity.

As it occurs with toxic oligomers from other amyloidogenic systems, the toxicity of type B* oligomers relies on their ability to interact and disrupt cellular membranes¹³. In α S this activity is encoded in two of their characteristic structural elements¹³: (i) an exposed N-terminal region that acts as the initial anchor to the membrane surface, similarly as with the monomeric functional form of the protein, and (ii) a β -sheet core, composed primarily by the central region of the protein, with significant hydrophobic surface exposed to the solvent that then inserts into the lipid bilayer causing major perturbations. The highly negatively charged C-terminal region of the protein remains disordered without significant interactions with the membrane.

We hypothesized that the binding of PSM α 3 to type B* oligomers, likely mediated by the solvent exposed hydrophobic regions of the β -sheet core together with the proximal negatively charged C-terminal regions, would block their exposed lipophilic elements, thus decreasing its ability to insert to and perturb the membrane bilayer and, therefore, to induce cellular toxicity. We treated human SH-SY5Y neuroblastoma cells with 10 μ M of oligomers and observed that, as previously reported³², they possess a high affinity for cellular membranes (**Figure 4a-b**). When the oligomers were preincubated with an equimolar concentration of PSM α 3, we observed a ~60 % reduction in the amount of α S bound to cells relative to untreated oligomers, indicating that PSM α 3 binding to type B* oligomers directly affects the binding of the oligomers to cellular membranes.

One of the earliest effects of type B oligomer-mediated membrane perturbation is the substantial increase in the levels of intracellular reactive oxygen species (ROS)²¹, which in turn elicits mitochondrial dysfunction³³. We assessed if the blockage of the oligomer regions involved in membrane perturbation by PSM α 3 binding could protect membrane integrity and therefore prevent its associated increase in intracellular ROS levels. Treatment of neuroblastoma cells with 10 μ M of oligomers induced a drastic increase in ROS levels (**Figure 4c-d**). However, when these oligomers were preincubated with equimolar (1:1) and substoichiometric (1:0.2) concentrations of PSM α 3, the ROS levels of treated cells approached those of healthy, untreated, cells, indicating that PSM α 3 protects against oligomers induced damage. This detoxifying activity seems to be associated with the particular structural and physicochemical properties of this peptide since treatment with equimolar concentrations of dPSM α 3 failed to exert any protective effect.

Dissection of PSM α 3 aggregation-inhibitory determinants.

To this point, we have assigned the α S binding, anti-aggregation and cytoprotective properties of PSM α 3 to its helical, amphipathic and cationic character. To confirm that this is the case, we reverse-engineered PSM α 3 into a non-natural peptide scaffold with low sequence complexity that keeps its critical properties. We employed a set of bioinformatics tools to predict the helical propensity, helical hydrophobic moment, and thermodynamic stability of our successive designs using AGADIR²⁵, HELIQUEST³⁴, and FOLDX³⁵, respectively. Data regarding those predictions are displayed in **Supplementary Table 1**. Then, we evaluated the anti-aggregative properties of these new molecules, under the assumption that the inhibitory capacity is connected with the oligomer-peptide interaction affinity.

A first requirement for binding is a continuous hydrophobic face to interact with the surface of oligomers or fibrils. In our view, the specific sequence of this helical side would be irrelevant, as long as it keeps its lipophilic character. To demonstrate that this assumption is correct, we mutated all the residues in the hydrophobic face of the PSM α 3 α -helix to leucine (All_Leu), generating an amphipathic peptide devoid of any sequence diversity in this side. Simultaneously, we designed a variant of All-Leu devoid of the last three C-terminal residues (All_Leu19) since they are not part of the α -helix, and thus they are not expected to contribute significantly to the binding. Both redesigned peptides folded into α -helices and retained the inhibitory activity, with a potency that approaches that of PSM α 3 (**Figure 5a-b**). Thus, we concluded that it is the generic hydrophobic character of the helical face and not its sequence or composition that is relevant for the binding.

Next, to further reduce the peptide sequence complexity, we redesigned the hydrophilic face in such a way that it only contained ionizable residues. We designed a new variant (Scaffold_19) with only four different amino acids (Leu, Asp, Glu, and Lys) by introducing two point-

mutations (A5E_G16K) in the All_Leu19 peptide. We decided to maintain the peptide net charge by introducing residues with opposed charges. Thus, Scaffold_19 only has Leu in the hydrophobic face and charged residues in the hydrophilic one. This variant folded into an α -helix and showed the same anti-aggregation activity than the parental variant (**Figure 5c**).

The simplicity of Scaffold_19 allowed us to redesign the net charge of the peptide, to validate the other physicochemical property theoretically contributing to binding: a net positive charge. We generated a peptide with a net charge of -2 by introducing two charge-reversing mutations (K6E_K12E). This anionic peptide (Anionic_scaffold) folds into an α -helix and has a helical hydrophobic moment (μ_H) of 0.65, indicative of an amphipathic nature, but does not inhibit α S amyloid aggregation, confirming that a cationic character in the hydrophilic face is a requirement for binding (**Figure 5d**).

Overall, we succeeded in dissecting the peptide features responsible for aggregation inhibition. In the process, we generated a short peptide with low sequence complexity whose inhibitory activity does not stem from the primary sequence, but instead from a defined spatial distribution of two physicochemical traits. Scaffold_19 constitutes a representative of a generic family of peptides with potential therapeutic applications.

LL-37 inhibits α S aggregation and oligomer toxicity.

Once we elucidated the determinants of this novel mechanism of α S amyloid inhibition, we wondered if this activity could also be encoded in natural human peptides. First, we screened the EROP-Moscow oligopeptide database³⁶ for human cationic peptides longer than 10 residues (≥ 3 helical turns), obtaining 287 hits. Next, we run AGADIR on them to exclude peptides with a low helical propensity, which resulted in 25 peptides, from which only 9 peptides were predicted to have at least a partial amphipathic character, according to their helical

hydrophobic moment (μ_H) (**Supplementary Table 2**). Then we screened the literature for candidates whose tissue distribution overlapped with that of α S and selected LL-37, the only human member of the cathelicidin family of antimicrobial peptides, for its further characterization. LL-37 is a 37-residue peptide resulting from a post-translational cleavage at the C-terminus of cathelicidin hCAP18³⁷. This peptide is constitutively expressed in the brain and the gastrointestinal tract; its presence in both tissues is engaging, as the brain-gut axis connection is gaining momentum in PD³⁸⁻⁴⁰.

First, we confirmed that LL-37 adopts an α -helical conformation under our assay conditions. With α -helical hydrophobic moment (μ_H) of 0.52, the helical-wheel projection and the available 3D-structures³⁴ indicate that this α -helix would be both cationic and amphipathic (**Figure 6a-b**). Then, we titrated the anti-aggregative activity of LL-37, confirming that it suppresses α S amyloid formation at substoichiometric concentrations (**Figure 6c**). Next, we labeled LL-37 with maleimide-Atto647N at a single engineered cysteine at the N-terminus, and we performed time-resolved dual-color fluorescence spectroscopy experiments as described previously for PSM α 3. Both dcFCCS and spFRET (**Figure 6d-f and Supplementary Figure 7**) reported a strong binding to type B* oligomers and fibrils, a weak interaction with type-A* oligomers and the absence of any interaction with the α S monomer, indicating that LL-37 and PSM α 3 share a very similar binding mechanism (**Figure 6g**), with LL-37 displaying slightly higher affinities than PSM α 3 (K_D = 3.62 nM for type B* oligomers, K_D = 5.14 nM for sonicated fibrils and K_D = 1.92 nM for type A* oligomers), and significant higher number of binding sites in type B* oligomers (N_{max} = 64) and sonicated fibrils (N_{max} = 181), while remain the same as PSM α 3 for the number of binding sites in the type A* oligomers (N_{max} = 3). Consistent with the LL-37 ability to bind type B* oligomers with high affinity, the preincubation of these toxic species with the human peptide at an equimolar concentration completely abolished the production of ROS in neuroblastoma cells (**Figure 6h-i**). LL-37 is not related in sequence to PSM α 3 or Scaffold_19, but the three peptides

share the same structural and physicochemical traits. This confirms that a linear combination of these properties suffices to identify, and potentially design, potent inhibitors of α S aggregation.

Whether LL-37 is actually involved or not in the pathogenesis of PD remains unexplored.

However, our results suggest that small peptides able to interact actively with α S pathogenic species might cohabitate with this protein in tissues relevant to the disease. These human peptides would display, in principle, low immunogenicity and may open novel avenues for PD treatment, either by their direct administration or by stimulating their endogenous expression.

Discussion

Because of its involvement in PD and other synucleinopathies, α S aggregation remains a promising target for therapeutic intervention. Herein, we propose a novel strategy for targeting the α S species behind the onset of these neurodegenerative diseases selectively. By binding to α S toxic oligomers and fibrils, the described collection of peptides inhibits the progression of α S aggregation, while suppressing the toxicity of pathogenic intermediates. Importantly, because the binding determinants are structurally encoded, these peptidic molecules do not recognize monomeric α S. From a therapeutic perspective, this is a significant advantage, as they are not expected to interfere with the physiological functions of the soluble protein. Furthermore, the avidity of these peptides for early non-toxic oligomers is more than one order of magnitude lower than the one for toxic oligomers and fibrils, indicating that they are extremely selective for the α S pathogenic species.

To the best of our knowledge, this is the first time that α S species-specific biomolecules have been rationally predicted, identified, and engineered. PSM α 3 is a *first-in-class* hit molecule that sets the ground for a new generation of leads for disease modification in PD and other

synucleinopathies. The requisites for a high peptide binding specificity and affinity are relatively simple: hydrophobic and positively charged surfaces with opposed orientations in the space. This is best exemplified by Scaffold_19, a short and low complexity peptide that fulfills those conditions. This defined SAR, usually absent or difficult to dissect in small molecules, should help in the development and diversification of novel candidates with increased activities employing protein engineering.

Many small bioactive peptides are derived from larger precursors and generated after proteolytic cleavage⁴¹. In some cases, these peptides are encrypted inside globular proteins, and their processing results in the manifestation of a new biological function. LL-37 is a cathelicidin-derived peptide constitutively expressed in the human brain⁴². Here, we show that LL-37 is a tight binder of α S pathogenic assemblies, with exceptional anti-aggregation and cytoprotective properties. LL-37 has been reported to inhibit the amyloid aggregation of two other disease-linked peptides, A β -42⁴³ and IAPP⁴⁴. However, the mechanism behind this activity is drastically different from the one we describe here since LL-37 binds to A β -42 and IAPP monomers, and the interaction is driven by a certain degree of sequence homology between short linear stretches in these molecules. Thus, it is assumed that LL-37 binds to A β -42 and IAPP in an, at least, partially unfolded conformation. Despite speculative, it is tempting to suggest that LL-37 is a first representative molecule of a previously unexplored regulatory system, where small peptides act as silent guardians of the proteome by targeting aggregation-prone proteins.

Apart from their therapeutic implications, the ability of the amphipathic cationic helical peptides to bind to α S toxic species with high affinity and selectivity might find a direct application in diagnosis. The presence of α S aggregates in biofluids is considered a biomarker for PD and other synucleopathies^{45,46}. However, current detection methods are not specific and sensitive enough for their clinical implementation. For instance, ELISA approaches based on the

so-called conformation-specific antibodies, perform better when the detection is normalized relative to the total levels of α S or when the same epitope-blocking antibody is used for both capture and detection⁴⁷. This indicates that a major limitation of these methods is the unwanted cross-reaction of the antibodies with the large excess of α S monomer in the fluid. In contrast, the peptides we describe here do not interact with monomeric α S, and the presence of up to 500-fold excess of monomeric α S does not interfere with the detection of nanomolar amounts of toxic oligomers. This conformational specificity, together with their close-to-antibody affinities, make of these molecules powerful tools for the implementation of diagnostic strategies in which they may act as nanosensors of pathogenic α S species in biological fluids.

Overall, the molecular entities we describe in this work represent a new conceptual framework to develop therapeutic and diagnostic strategies for the synucleinopathies.

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Authors contribution

S.V. conceived the project. J.S., I.P. and S.V. designed all the peptide sequences. J.S., S.P-D. and J.P. performed in vitro experiments. J.S. and S.N. designed, conducted and analyzed cellular experiments. P.G. and N.C. performed and analyzed time-resolved fluorescence spectroscopy

experiments. J.S., I.P. and S.V. analyzed the data. J.S., P.G., N.C., I.P. and S.V. prepared the manuscript with contributions from all the authors.

Competing interest

Authors are elaborating a patent application on the bases of the here presented research.

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Figures

Figure 1. Rational identification of a peptide ligand for αS pathogenic species. (a) Main molecular features of the four isolated αS species. Values with a dagger (†) represent extrapolations based on the average number of monomers in each species. (b) Helical wheel projection of PSMα3 sequence (red, hydrophobic residues; blue pallet, hydrophilic residues depending on their character). (c) Surface representation of the three-dimensional structure of PSMα3 with hydrophobic residues in red and hydrophilic residues in blue. (d) Far-UV circular dichroism spectra of PSMα3.

Figure 2: Interaction of PSMα3 with different αS species by FCCS. (a-d) Representative auto-correlation curves for αS (blue line) and PSMα3 (red line) and cross-correlation curves for interacting molecules (purple line). The amplitude (G) error is shown as faint colored area for the corresponding correlation curves. Samples contained (a) ~15 nM αS monomer and ~15 nM PSMα3, (b) 1 nM type A* and ~5 nM PSMα3, (c) 1nM type B* oligomers and ~5 nM PSMα3 or (d) ~5 nM sonicated fibrils and ~5 nM PSMα3. (e) Titration binding curves for the interaction of PSMα3 with type A* oligomers (red circles), type B* oligomers (blue circles) or sonicated fibrils (grey circles) obtained by FCCS experiments, showing their corresponding analysis assuming a model of n identical and independent binding sites (referred in equation 7 as N_{max}) per αS aggregated species (solid lines). (f) Auto-correlation curves (αS in blue, PSMα3 peptide in red) and cross-correlation curve for the interacting molecules (in purple) obtained in samples containing ~1 nM αS type B* oligomers and ~2 nM PSMα3 in the absence (solid lines) or presence (dashed lines) of a 500-molar excess of unlabeled monomer with respect to the particle concentration of oligomers. The inset shows the number of bound peptides (N_p) per aggregate in both conditions.

Figure 3. Effect of PSM α 3 on *in vitro* α S amyloid fibrillation. (a) Inhibition of α S amyloid aggregation as measured by Th-T fluorescence after 32 h incubation in the presence of equimolar concentrations of PSM α 3, SynuClean-D (SC-D), and dPSM α 3. **** p-value < 0.0001 relative to untreated α S (b) TEM micrographs of α S aggregated for 32 h in the absence (left) and presence of an equimolar concentration of PSM α 3 (right). (c) Aggregation kinetics of 70 μ M α S and titration of the inhibitory activity of PSM α 3 at different concentrations: 35 μ M (green), 14 μ M (orange), 7 μ M (blue), 3.5 μ M (gray) and in the absence of PSM α 3 (black). Error bars represent the standard error of the mean (SEM).

Figure 4. Suppression of the toxicity of αS oligomers in neuroblastoma cells. (a) Representative confocal images showing the αS load per cell after the treatment with 10 μM of type B* oligomers pretreated with an equimolar concentration of PSMα3. (b) Quantification of the αS load per cell. Error bars represent the standard deviation (SD). **** p-value < 0.0001 relative to untreated cells. **** p-value < 0.0001 relative to cells treated with αS type-B* oligomers. (c) Quantification of the levels of intracellular ROS of SH-SY5Y cells incubated with 10 μM of type B* oligomers preincubated with different concentrations of PSMα3 and dPSMα3. Error bars represent the standard error of the mean (SEM). **** p-value < 0.0001 relative to cells treated with αS type B* oligomers. (d) Representative confocal images of the analysis of panel c.

Figure 5. Redesign of PSMα3 variants to dissect the molecular determinants of the anti-aggregative activity. (a,b,c,d) Helical wheel (up), circular dichroism spectra (mid) and titration of the inhibitory activity of PSMα3 at different concentrations (down): 35 μM (green), 14 μM (orange), 7 μM (blue) and in absence of PSMα3 variants (black). Variants: All_Leu (a), All_Leu19 (b), Scaffold_19 (c) and Anionic_scaffold (d). Error bars represent the standard error of the mean (SEM).

Figure 6. Characterization of the interaction of LL-37 with the α S pathogenic species. (a) Helical wheel projection of LL-37 sequence (red, hydrophobic residues; blue pallet, hydrophilic residues; green, proline). (b) Far-UV circular dichroism spectra of LL-37 in PBS pH 7.4. (c) Aggregation kinetics of 70 μ M α S and titration of the inhibitory activity of LL-37 at different concentrations: 35 μ M (green), 14 μ M (orange), 7 μ M (blue), 3.5 μ M (gray) and in the absence of peptide (black). Representative auto-correlation curves for α S and LL-37 peptide and cross-correlation curves for interacting molecules are shown in blue, red and purple lines, respectively. The amplitude (G) error is shown as faint colored area for the corresponding correlation curves. Samples contained (d) ~15 nM α S monomers and ~15 nM LL-37, (e) 1 nM type B* oligomers and ~5 nM LL-37 or (f) ~5 nM sonicated fibrils and ~5 nM PSM α 3. (g) Titration binding curves for the interaction of LL-37 with type A* oligomers (red circles), type B* oligomers (blue circles) or sonicated fibrils (grey circles) obtained by FCCS experiments, showing their corresponding analysis assuming a model of N_p independent binding sites per α S aggregated species (solid lines). (h) Representative confocal images of SH-SY5Y cells treated with 10 μ M of type B* oligomers in the presence of an equimolar concentration of LL-37. (i) Quantification of the intracellular ROS of the experiment displayed in panel h. Error bars represent the standard deviation (SD). **** p-value < 0.0001 relative to untreated cells. **** p-value < 0.0001 relative to cells treated with α S type B* oligomers.