Small molecule inhibits α-synuclein aggregation, disrupts amyloid fibrils, and prevents degeneration of dopaminergic neurons


Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain; Max Planck Institute for Experimental Medicine, 37075 Göttingen, Germany; Institute Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; Department of Experimental Neurodegeneration, University Medical Center Göttingen, 37073 Göttingen, Germany; Center for Bistriuctural Imaging of Neurodegeneration, University Medical Center Göttingen, 37073 Göttingen, Germany; Center for Nanoscale Microscopy and Molecular Physiology of the Brain, University Medical Center Göttingen, 37073 Göttingen, Germany; Laboratoire de Chimie Théorique, Sorbonne Universités, CNRS, F-75005 Paris, France; Department of Biochemistry and Molecular Cell Biology, University of Zaragoza, 50018 Zaragoza, Spain; Institute for Bio-computation and Physics of Complex Systems (BIFI), University of Zaragoza, 50018 Zaragoza, Spain; Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology (BIST), 08028 Barcelona, Spain; Department of Inorganic and Organic Chemistry, University of Barcelona, 08028 Spain; Institut Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain; Max Planck Institute for Experimental Medicine, 37075 Göttingen, Germany; Institute for Neuroscience, The Medical School, Newcastle University, Newcastle upon Tyne NE2 4HH, United Kingdom; Faculty of Medicine, University of Vic-Central University of Catalonia (U Vic-UCC), 08590 Vic, Spain; and Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

Edited by Gregory A. Petsko, Weill Cornell Medical College, New York, NY, and approved August 16, 2018 (received for review April 3, 2018)

Parkinson’s disease (PD) is characterized by a progressive loss of dopaminergic neurons, a process that current therapeutic approaches cannot prevent. In PD, the pathological hallmark is the accumulation of intracellular protein inclusions, known as Lewy bodies and Lewy neurites, which are mainly composed of α-synuclein. Here, we exploited a high-throughput screening methodology to identify a small molecule (SynuClean-D) able to inhibit α-synuclein aggregation. The compound significantly reduces the in vitro aggregation of wild-type α-synuclein and the familiar A30P and H5Q variants in a substoichiometric molar ratio. This compound prevents fibril propagation in a protein misfolding cyclic amplification assay and decreases the number of α-synuclein inclusions in human neuroglioma cells. Computational analysis suggests that SynuClean-D can bind to cavities in mature α-synuclein fibrils and, indeed, displays a strong fibril disaggregation activity. The treatment with SynuClean-D of two PD Caenorhabditis elegans models, expressing α-synuclein either in muscle or in dopaminergic neurons, significantly reduces the toxicity exerted by α-synuclein. SynuClean-D–treated worms show decreased α-synuclein aggregation in muscle and a concomitant motor recovery. More importantly, this compound is able to rescue dopaminergic neurons from α-synuclein–induced degeneration. Overall, SynuClean-D appears to be a promising molecule for therapeutic intervention in Parkinson’s disease.


Significance

Parkinson’s disease is characterized by the accumulation of amyloid deposits in dopaminergic neurons, mainly composed of the protein α-synuclein. The disordered nature of α-synuclein and its complex aggregation reaction complicate the identification of molecules able to prevent or revert the formation of these inclusions and the subsequent neurodegeneration. By exploiting a recently developed high-throughput screening assay, we identified SynuClean-D, a small compound that inhibits α-synuclein aggregation, disrupts mature amyloid fibrils, prevents fibril propagation, and abolishes the degeneration of dopaminergic neurons in an animal model of Parkinson’s disease.

Interfering with α-Syn aggregation has been envisioned as a promising disease-modifying approach for the treatment of PD (1). However, the disordered nature of α-Syn precludes the use of structure-based drug design for the discovery of novel molecules able to modulate α-Syn aggregation. Therefore, many efforts have focused on the analysis of large collections of chemically diverse molecules to identify lead compounds (10). Recently, we have developed an accurate and robust high-throughput screening methodology to identify α-Syn aggregation inhibitors (11). Here, we describe the properties of SynuClean-D (SC-D), a small molecule identified with this approach (SI Appendix, Fig. S1). We first performed a detailed in vitro biophysical characterization of the inhibitory and disaggregation activities of SC-D and tested its performance in human neural cells. Finally, we validated the effects in vivo in two well-established Caenorhabditis elegans models of PD, which express α-Syn either in muscle cells or in dopaminergic neurons.
neurons. The inhibitor reduced α-Syn aggregation, improved motility, and protected against neuronal degeneration.

**Results**

**SynuClean-D Inhibits α-Syn Aggregation in Vitro.** The formation of α-Syn amyloid fibrils can be reproduced in vitro by incubating the recombinant protein. However, fibril growth is very slow and highly variable, complicating drug screening (12). We have implemented a robust high-throughput kinetic assay to screen large chemical libraries in the search for α-Syn aggregation inhibitors (11). The assay uses thioflavin-T (Th-T) as readout of amyloid formation, completing highly reproducible reactions in 30 h. Approximately 14,400 chemically diverse compounds of the HitFinder Collection from Maybridge were screened with this approach. SC-D [2-hydroxy-5-nitro-o-(3-nitrophenyl)-4-(trifluoromethyl)nicotinonitrile], a small aromatic compound, was identified as one of the molecules of potential interest (SI Appendix, Fig. S1). Many compounds with promising pharmacological characteristics never become drugs because they are rapidly metabolized in the liver and therefore have low oral bioavailability. SC-D was metabolically stable in the presence of human hepatic microsomes, with an intrinsic clearance of <5 μL·min⁻¹·mg⁻¹ (SI Appendix, Fig. S2).

Incubation of 70 μM α-Syn with 100 μM SC-D impacted α-Syn aggregation, as monitored by Th-T fluorescence (Fig. 1A). The analysis of the aggregation curves indicated that the autocatalytic rate constant in the presence of the compound ($k_0$, 0.25 h⁻¹) was 25% lower than in its absence ($k_0$, 0.33 h⁻¹). SC-D decreases $k_0$ by 1.5 h and reduces by 53% the amount of Th-T positive material at the end of the reaction. By measuring light scattering, we confirmed that the observed changes in Th-T fluorescence reflected an effective decrease in the levels of α-Syn aggregates, with a reduction of 48 and 58% in the scattering signal at the end of the reaction in the presence of SC-D when exciting at 300 and 340 nm, respectively (Fig. 1B). Nanoparticle tracking analysis indicated that the presence of SC-D increased the number of particles of <100 nm and decreased the formation of large aggregates (150 to 300 nm) (SI Appendix, Fig. S3). Finally, transmission electron microscopy (TEM) images confirmed that samples incubated with SC-D contained smaller and much fewer fibres than untreated samples (Fig. 1C and D). The inhibitory activity of SC-D was dose-dependent and still statistically significant at 10 μM (1:7 compound:α-Syn ratio), where it reduces the final Th-T signal by 34% (Fig. 1E).

We further investigated whether SC-D was active against the aggregation of α-Syn variants associated with PD (1). SC-D was able to reduce by 45 and 73% the amount of Th-T–positive aggregates at the end of the reaction for the H50Q and A30P α-Syn familial variants, respectively (Fig. 1F).

The inhibitory activity of SC-D was also assessed using protein-misfolding cyclic amplification (PMCA) (13). Conceptually based on the nucleation-dependent polymerization model for prion replication, PMCA has been recently adapted to amplify α-Syn amyloid fibrils (14). The PMCA technique combines cycles of incubation at 37 °C, to grow fibrils, and sonication, to break fibrils into smaller seeds. In our conditions, a single cycle of amplification was sufficient to generate amyloid-like protease K (PK)-resistant α-Syn assemblies, but the highest levels of protection were attained after four rounds (Fig. 1G). When the same experiment was performed in the presence of SC-D, we observed a substantial decrease in the amount of PK-resistant material (Fig. 1H), indicating that the molecule was interfering with α-Syn template seeding amyloid formation.

**SynuClean-D Disrupts Preformed α-Syn Fibrils.** The progress of α-Syn PMCA reactions can also be monitored by using the Th-T signal as the readout for fibril assembly (15). Consistent with PK resistance analysis, Th-T fluorescence of α-Syn increased significantly after four cycles of PMCA (Fig. 2A). Surprisingly, in the presence of SC-D, the Th-T signal not only did not increase, but
We did not detect any perturbations in chemical shifts or peak intensities with respect to the original α-Syn spectrum in the presence of 100 μM concentration of the molecule (SI Appendix, Fig. S4), indicating that SC-D does not bind α-Syn monomers.

Induced-fit docking simulations of α-Syn–SC-D revealed four major poses for its interaction with α-Syn fibrils (16): two internal, with SC-D fully inserted in the fibril (poses 1 and 2), and two external, with SC-D partially exposed (poses 3 and 4) (SI Appendix, Fig. S5). In the internal poses, the ligand is sandwiched between two parallel β-sheets of the Greek-key motif and interacts with the side chains of ALA53, VAL55, THR59, GLU61, THR72, and GLY73. The only difference between pose 1 and 2 lies in the orientation of the compound in the binding pocket. PELE (17) interaction energies are stronger for the internal poses, where SC-D binds essentially through dispersion interactions into a solvent-excluded cavity, than for external ones, where SC-D inserts into a surface groove of the fibril. In light of these calculations, we predict that SC-D binds into the core of α-Syn fibrils.

MM/GBSA calculations (SI Appendix, Table S1) (18, 19) show that internal binding pose 1 (Fig. 3) exhibits the largest binding energy with the fibril, the computed ΔG_{bind} being −18.4 ± 4.1 kcal mol⁻¹. The main contribution comes from the van der Waals term, representing roughly 80% of the interaction. This is not surprising given the nature of SC-D, a planar aromatic molecule. Plots of the reduced density gradient versus the density (SI Appendix, Fig. S6A) provide information on the nature of the noncovalent interactions in the system (20). Peaks in the negative and positive regions of the x axis are indicative of attractive and repulsive interactions, respectively. The region around zero corresponds to the weakest noncovalent van der Waals contacts. Though weak, these interactions are present in large number and involve the whole body of the molecule, being the largest contribution to the binding energy. Their spatial extension is shown in SI Appendix, Fig. S6B. For pose 1, the noncovalent interaction plot shows that began to decrease after the third cycle. This suggested that SC-D might disrupt newly formed amyloid fibrils.

To address the time window in which SC-D is active, we set up aggregation reactions with a constant amount of SC-D at different time intervals. As presented in Fig. 2B, the effect of SC-D on the final amount of amyloid structures was independent of whether it was added at the beginning (4 h), in the middle (12 h), or at the end (18 h) of the exponential phase, or even when the reaction had already attained a plateau (24 h). These results suggested again ability to disrupt/destabilize fibrils.

α-Syn Fibrils Can Accommodate SynuClean-D. To assess if SC-D can bind monomeric and soluble α-Syn, the recombinant protein was isotopically labeled and NMR ¹H–¹⁵N-HSQC spectra of 70 μM [¹⁵N]α-Syn were recorded in the absence and presence of SC-D.

Fig. 2. Disaggregational capacity of SynuClean-D. (A) Th-T fluorescence of the different PMCA passes of both treated (blue) and untreated (black) samples with SC-D. (B) Aggregation kinetics of α-Syn after the addition of SC-D at different time points. (C and D) Th-T–derived fluorescence (C) and light-scattering (D) assays before and after the addition of SC-D to preformed α-Syn fibrils. (E and F) Representative TEM images in the absence (E) and presence (F) of SC-D. Th-T fluorescence is plotted as normalized means. Error bars are represented as SE of mean values; ***p < 0.001.
besides van der Waals, an H-bond contact is responsible for the binding of SC-D (SI Appendix, Fig. S6A).

**SynuClean-D Inhibits the Formation of Intracellular α-Syn Aggregates in Cultured Cells.** We tested the potential toxicity of SC-D for human neuroglioma (H4) and human neuroblastoma (SH-SY5Y) cells. For both cell lines, the molecule was innocuous at concentrations as high as 50 μM (Fig. 4A and SI Appendix, Fig. S7). We used a well-established cell model that enabled us to assess α-Syn inclusion formation. H4 cells were transiently transfected with C-terminally modified α-Syn (synT) and synphilin-1, which results in the formation of LB-like inclusions, as we previously described (9). The formation of α-Syn inclusions was assessed 24 h after treatment by immunofluorescence (Fig. 4D). Upon treatment with 1 and 10 μM SC-D, we observed a significant increase in the number of transfected cells devoid of α-Syn inclusions (SC-D, 1 μM: 42.4 ± 1.0%; SC-D, 10 μM: 49.5 ± 4.5%) relative to untreated samples (control: 28.7 ± 2.0%) (Fig. 4B). SC-D treatment also promoted a significant decrease in the number of transfected cells displaying more than five aggregates (SC-D, 1 μM: 35.5 ± 5.0%; SC-D, 10 μM: 32.5 ± 6.6%) relative to control cells (control: 49.6 ± 5.6%) (Fig. 4C).

**SynuClean-D Inhibits α-Syn Aggregation in a C. elegans Model of PD.** Next, we tested SC-D in a living system. We used a well-studied nematode model of PD, the strain NL5901, in which human α-Syn fused to the yellow fluorescent protein (YFP) is under control of the muscular unc-54 promoter, transgene pks2386 [P::unc-54::α-SYN::YFP] (21). Muscle expression has been used successfully to model protein-misfolding diseases and to identify modifier genes without considering neuronal effects (21, 22). To determine the effects of SC-D in α-Syn accumulations, animals at the fourth larval stage (L4) (23) were incubated with and without the compound, to analyze the inhibitor efficiency in aged worms at 9 d posthatching (L4 + 7), which mimics aged human PD (24). We avoided a compound burst at L1 (25) because this treatment mimics a preventive rather than a disease-modifying intervention. Quantification of the number of α-Syn aggregates revealed that, in treated animals, the number of visible α-Syn aggregates decreased by 13.2 units, relative to untreated worms (18.3 ± 2.8 vs. 31.5 ± 1.1, respectively) (Fig. 5 A and D). In some animals, treatment resulted in a near-complete loss of protein aggregates (SI Appendix, Fig. S8). In this assay, SC-D is as effective as epigallocatechin gallate (EGCG), a polyphenol able to inhibit α-Syn aggregation and to disentangle preformed α-Syn aggregates (26, 27). EGCG has also been shown to reduce the deposits of the amyloid β-peptide in a C. elegans muscular model of AD (28). In our PD model, EGCG treatment reduced the number of visible aggregates by 13.4 units (18.1 ± 0.7) (Fig. 5A).

Major defects in regular bending have been used to identify modifiers of protein aggregation (21). Indeed, C. elegans thrashing can be measured in liquid media by counting the number of body bends per unit of time (29). By using this method, we confirmed an improved motility in SC-D–treated animals in comparison with nontreated worms (Fig. 5 B and E). We observed a decrease of bending of 72.2% in YFP:α-Syn animals compared with the N2 wild-type strain (18.1 ± 2.5 vs. 90.3 ± 6.7, respectively). This motility decrease was reverted in YFP:α-Syn animals treated with SC-D. In these animals, the average bending increased by 27-fold, compared with nontreated animals (18.1 ± 2.5 vs. 49.1 ± 4.4) (Movies S1 and S2).

**SynuClean-D Prevents Degeneration of Dopaaminergic Neurons in a C. elegans Model of PD.** PD is characterized by the degeneration of dopaminergic (DA) neurons. There exist four pairs of DA neurons in C. elegans hermaphrodites, three of them (CEPD, CEPV, and ADE) located in the anterior part, and one pair, the PDE, in the posterior part of the nematode (30). To investigate the neuroprotective role of SC-D in dopaminergic cell death, we sought to analyze its effect in a C. elegans model of PD in which DA neurons undergo age-dependent neurodegeneration (31). In this model (strain UA196), animals express both human α-Syn and GFP in DA neurons, according to the simplified genotype Pdat-1::GFP; Pdat-1::α-SYN (the full genotype is detailed in SI Appendix). This strain has been successfully used for the investigation of human PD-related mechanisms (24). When human α-Syn is expressed in these animals’ DA neurons, the six DA neurons within the anterior region of the worm display progressive degenerative characteristics (32). To model the aging contribution to PD, we determined the inhibitor capacity of SC-D in dealing with DA cell death induced by human α-Syn at 9 d (L4 + 7) posthatching. Cell bodies and neuronal processes were assessed to determine whether these structures displayed morphology changes. At 9 d posthatching, only 14.0 ± 1.5% of nontreated animals showed six wild-type DA neurons. In contrast, 44.4 ± 2.8% of treated animals showed the six intact DA neurons (Fig. 5 C and F), which evidenced the ability of SC-D to protect against α-Syn–induced DA neuron degeneration. In contrast to SC-D, the administration of EGCG did not have any beneficial impact on neurodegeneration, since only 12.0 ± 0.8% of EGCG-treated animals exhibited six intact DA neurons, a proportion that is fairly similar to that in untreated worms (14.0 ± 1.5%) (Fig. 5C).

**Discussion**

α-Syn aggregation plays a major pathophysiological role in the development of PD. Since its discovery and subsequent identification as the most abundant protein in Lewy bodies (3), α-Syn was shown to be important for a number of cellular processes (7). Therapeutic strategies targeting the aggregation of α-Syn thus hold the promise to result in disease modification and mitigate pathology in PD (33).
The screening of large chemical libraries has rendered promising molecules that inhibit the progression of PD by targeting the aggregation of $\alpha$-Syn, like BIOD303 (34) or anle138b (35). Here, by exploiting a previously developed high-throughput methodology, we identified SC-D as a metabolically stable compound able to inhibit $>50\%$ of $\alpha$-Syn amyloid formation in vitro when employed in a 0.7:1 (protein:SC-D) ratio. The ability of SC-D to inhibit $\alpha$-Syn aggregation was confirmed by light-scattering and TEM assays. SC-D was also active against the aggregation of $\alpha$-Syn mutants that cause familial forms of the disease. The activity of SC-D was concentration-dependent and still evident at a substoichiometric 7:1 protein:compound ratio. This already suggested that, unlike other compounds, SC-D does not bind to monomeric $\alpha$-Syn, supported by NMR experiments. From a therapeutic perspective, this is an important advantage, as SC-D is not expected to interfere with the physiological functions of the soluble protein.

PMCA experiments indicated that SC-D might disrupt $\alpha$-Syn amyloid assemblies, a property that was confirmed by the ability of SC-D to reduce the amount of formed amyloid fibrils, almost independent of the stage of the aggregation reaction at which it was added. Indeed, SC-D is very effective at disassembling clusters of aged $\alpha$-Syn amyloid fibrils, conceptually similar to the $\alpha$-Syn amyloid inclusions recurrently observed in the dopaminergic neurons of PD patients. This is important, because the disassembly of preformed amyloid fibrils has been traditionally challenging, and very few molecules have been reported to break down amyloid fibrils (27) because of their high stability. Computational analysis suggests that the disrupting activity of SC-D is mediated by its binding to an inner cavity in $\alpha$-Syn fibrils. The disassembly of large fibrils has been seen traditionally as a risky strategy for the amelioration of aggregation-linked diseases, because it may increase the population of smaller toxic species.

![Fig. 5. Inhibition effect of the compound in the formation of $\alpha$-Syn inclusions and protection from the $\alpha$-Syn-induced dopaminergic cell death in C. elegans models of PD. (A) Quantification of $\alpha$-Syn muscle inclusions per area of NLS901 worms in the absence (white) and presence of SC-D (blue) or EGCG (gray). (B) Worm-thrashing representation as the number of bends per minute of N2 wild-type and NLS901 worms treated without (white) and with SC-D (blue). (C) Percentage of UA196 worms that maintain a complete set of dopaminergic neurons (four pairs located in the head) after treatment without (white) and with SC-D (blue) or EGCG (gray) for 7 d after the L4 stage. (D) Representative images of $\alpha$-Syn muscle aggregates obtained by epifluorescence microscopy of NLS901 worms treated without (Top, vehicle) and with SC-D (Bottom, drug). (E) Path representation of the mobility of N2 wild-type (Left, vehicle) and NLS901 worms grown without (Middle, vehicle) and with SC-D (Right, drug). (F) Representative worms expressing GFP-$\alpha$-Syn specifically in DA neurons without (Left, vehicle) and with SC-D (Right, drug) for 7 d after L4. Healthy neurons are labeled with white arrows, whereas neurodegenerated or missing neurons are labeled with red arrows. (Scale bars, 30 $\mu$m.) Between 40 and 50 animals were analyzed per condition in each experiment. Data are shown as means, and error bars are shown as the SE of means; ** $P < 0.01$ and *** $P < 0.001$.]

![Graphs showing the number of apparent aggregates, worm thrashing, and percentage of animals with wild-type DA neurons treated with different compounds.](image-url)
However, our in vivo experiments demonstrate that this is not the case for SC-D.

The ability of SC-D to target preformed fibrils might have important implications for the prion-like pathological spreading of α-Syn aggregates in the brain (4). By disentangling transmissible fibrillary assemblies, SC-D might reduce templated seeding and thus aggregate-catalyzed conversion of soluble α-Syn molecules into their insoluble forms. It has been suggested that to attain a sustaining spreading and prevent dilution of aggregates as they propagate from cell to cell, a process of aggregate amplification is required, in addition to templated seeding (36). PMCA-SC-D mimics these particular conditions in vitro. The potential of SC-D in blocking PMCA-induced amplification of α-Syn fibrils is thus promising.

SC-D displayed low toxicity for neural cells and displayed cellular permeability. Indeed, SC-D treatment at concentrations as low as 1 μM significantly reduced the number of α-Syn inclusions and the number of inclusions per cell. These data prompted us to assess the effects of SC-D treatment in in vivo models of α-Syn aggregation. First, we selected a well-validated C. elegans model of PD that expresses human α-Syn in muscular cells (21). When worms were treated with SC-D at predautophagy stages, we observed a significant decrease in the number of α-Syn inclusions and a significant recovery of motility in adult PD worms. However, independent of whether a compound targets the early stages of aggregation or disrupts mature protein inclusions, or both, the final aim of a PD-oriented therapy is not to interfere with α-Syn aggregation per se but instead to prevent the neuronal degeneration associated with this phenomenon. It is in this therapeutic context where SC-D stands out, since it is able to increase by more than threefold the number of animals with intact DA neurons in a C. elegans model in which the expression of human α-Syn is directly connected to dopaminergic degeneration (31).

Conclusions

In the present study, we describe how drug-screening efforts have crystallized in the discovery of a molecule able to inhibit α-Syn aggregation, both in vitro and in vivo, without interacting significantly with functional, monomeric, and soluble α-Syn. SC-D is a nontoxic molecule that exhibits a unique capability to interact with and disassemble amyloid fibrils, a property that is likely connected to its ability to prevent the α-Syn–promoted degeneration of dopaminergic neurons. Taken together, SC-D constitutes a very promising lead compound for the development of a novel therapeutic molecule for disease modification in PD and other synucleinopathies.

Materials and Methods

Protein purification, metabolic stability assays, in vitro aggregation studies, protein-misfolding cyclic amplification, molecular dynamics simulations, NMR studies, cytotoxicity assays, in-cell aggregation studies, and the C. elegans models of PD are described in detail in SI Appendix.

ACKNOWLEDGMENTS. We thank the Infrastructure Científica y Técnica Singular NMR facility at Centres Científics i Tecnològics de la Universitat de Barcelona for help with NMR, Amable Bernabé at Instituto de Ciencia de Materiales (CSIC-Consejo Superior de Investigaciones Científicas) for help with nanoparticle tracking analysis, and Anna Villar-Piqué for help with plasmid construction. The worm strain UA196 used for neurodegeneration assays was a generous gift of Dr. Guy A. Caldwell. S.V. and T.F.O. are supported by Fundación La Marató de TV3 (Ref. 20144330). T.F.O. is supported by the Deutscher Forschungsgemeinschaft Center for Nanoscale Microscopy and Molecular Physiology of the Brain and by SFB1286. S.V. is supported by Ministerio de Economía y Competitividad (MINECO) (BIOMOLE2011-28581-P) and Gobierno de Aragón (E45_179). E.D. is supported by Instituto de Salud Carlos III (HF161383/ERDF/EFS). J.G. and X.S. are supported by MINECO (BIOP2015-67522-P) and the European Research Council (Contract 648201).