INTRODUCTION

Parkinson’s disease (PD) is a progressive neurodegenerative disorder that causes severe motor deficits. Common symptoms include rigidity, bradykinesia, tremor and postural instability [1]. With disease progression, nonmotor symptoms, such as depression, psychosis, falls, and sleep disturbance, also emerge [2]. Globally, 1.5% of the population over 65 years of age [3] and more than 5 million people [4] are affected by this devastating disease. PD is pathologically characterized by the substantial loss of dopamine (DA)-containing neurons in the midbrain [5] and the presence of intraneuronal cytoplasmic inclusions [6], known as Lewy bodies and Lewy neurites [7], both of which comprise alpha-synuclein (α-syn) aggregates.

A definitive diagnosis of PD has to rely on histopathological postmortem analysis and requires the detection of dopaminergic cell loss and the presence of Lewy bodies and Lewy neurites. For living patients, several clinical diagnostic criteria have been formulated, including the UK Parkinson’s Disease Society Brain Bank (UKPDSBB) [8], the National Institute of Neurological Disorders and Stroke (NINDS) criteria [9] and the Movement Disorder Society Clinical Diagnostic Criteria for PD [10]. However, the diagnostic accuracy obtained by using these criteria is only between 75% and 85% [11, 12]. Compared with the observation of clinical symptoms, neuroimaging may help to increase diagnostic precision. Clinically available imaging approaches include positron emission tomography (PET), single-photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI). PET and SPECT use a variety of radiotracers to quantitatively measure the metabolic and neurochemical changes in the brains. For example, fluorine-18-L-dihydroxyphenylalanine (18F-Dopa) PET can mark dopaminergic deficiencies in the brains of patients with PD [13]. MRI uses different sequences and contrasts to study brain structure and function [14]. Although substantial progress has been made in PD neuroimaging, currently, it can only be used as a supplementary tool to clinical examination [15].

Increasing evidence suggests that prior to the motor phase of classical PD there is a prodromal period that lasts for several years [16]. After the appearance of typical motor features, the disease can continue to progress for many years or even decades, although it is impossible to predict the trajectory of this progression at diagnosis [17]. The pathological changes in the central nervous system during the prodromal phase, such as the formation of α-syn aggregates, appear to mirror the occurrence of motor and nonmotor symptoms. A timeline of PD from onset to death has been proposed [17], which not only promotes a comprehensive understanding of the overall disease process but is also a reminder that before a patient shows any PD-related clinical symptoms, the disease may have progressed severely and possibly irreversibly as a result of neuronal dysfunction and cell loss [18, 19].

In the future, the traditional symptom-based clinical examination may be pre-empted by the use of biomarkers that may assist in identifying early disease-specific changes by early prodromal diagnosis, namely detecting specific changes in biomarkers during the early development of PD [20]. Since diagnosis informs treatment, novel therapeutics targeting the potential pathological culprits need to be developed.
Until now, no neuroprotective or neurorestorative therapy has been found for the treatment of PD. Existing treatments predominantly target dopamine-related symptoms. Few, if any, target nondopaminergic symptoms, which cause a severe burden for patients in the advanced stages of the disease. In addition, current PD treatments provide little or no relief for disease progression because they do not alter the rate or extent of neuronal cell loss [21, 22]. Therefore, for the future direction of PD medications, the priority is the development of neuroprotective or neurorestorative drugs that can stop or at least relieve disease progression and the relevant nonmotor symptoms [23]. To realize this goal, it is necessary to identify and target the key culprit underlying the pathogenesis of PD.

**ALPHA-SYNUCLEIN AND ITS FUNCTIONAL ROLE**

Native α-syn is a small protein encoded by the gene SNCA, with a molecular mass of 14.5 kDa (140 amino acids) [24]. Its primary structure (Fig. 1) shows three different regions: the N-terminal region (residues 1–60) that is characterized by repetitions of a highly conserved lysine-rich motif KTK(eQ)/GVG, the hydrophobic central part (residues 61–95, known as the non-amyloidogenic component (NAC) region), and the acidic C-terminal region (residues 96–140). The amphipathic N-terminal region has a structural alpha-helix propensity similar to apolipoprotein-binding domains, suggesting that α-syn is a membrane-bound protein [25]. The NAC region is believed to be involved in protein aggregation by mediating the conformational changes of α-syn from random coil to a beta-sheet structure [24]. The C-terminal region, having no distinct secondary structure, has been reported to interact with the N-terminal region or the NAC region to maintain the natively unfolded state of α-syn [26]. Although it is widely believed that α-syn exists mainly as monomer, studies have also shown that endogenous α-syn can form tetramers [27, 28], which comprise an alpha-helical secondary structure and show little tendency to form aggregates [29]. All these findings suggest that there may be a dynamic equilibrium between different α-syn conformational states [30].

The exact normal functions of α-syn are not well understood. Although some animal studies suggest that an α-syn-knockout is not fatal, deficiencies in synaptic transmissions were observed in some knockout animal lines [31, 32]. In neurons α-syn is mainly localized at the presynaptic terminals [33, 34] and is associated with the reserve pool of synaptic vesicles [35–37]. α-Syn can promote the assembly of the presynaptic soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) protein complex, that mediates vesicle fusion, which is a vital step for the release of neurotransmitters, including dopamine [38]. In addition to its role in synaptic transmission, α-syn is also involved in the suppression of apoptosis [39], antioxidation [40] and even regulation of dopamine biosynthesis [41, 42].

**ROLE OF ALPHA-SYNUCLEIN IN PD PATHOGENESIS**

The first connection between α-syn and PD was made in 1997 when point mutations in the SNCA gene were identified in familial PD cases [43]. To date, six missense mutations in SNCA are known to lead to autosomal dominant PD: A53T [43], A30P [44], E46K [45], H50Q [46], G51D [47], and A53E [48]. Meanwhile, the link between α-syn and PD was further elucidated with the discovery of duplications and triplications of the SNCA gene in PD families, which also suggested that increased expression of α-syn was a causative factor of PD [49, 50]. Moreover, certain polymorphisms in SNCA, such as the dinucleotide repeat REP1 located in the SNCA promoter (SNCA-REP1), a ~770 and ~116 base-pair (bp) single-nucleotide polymorphism, are major risk factors for sporadic PD, and are thought to result from altered expression of the gene product [51, 52]. Genome-wide association studies have also consistently revealed highly significant regions of genetic variation around the SNCA gene as contributors to the risk of PD. A meta-analysis of genome-wide association studies has identified 17 new PD risk loci [53].

As mentioned earlier, the NAC region of monomeric α-syn mediates the misfolding from a random coil to a beta-sheet structure, leading to the formation of oligomers, protofibrils and eventually mature fibrils [54]. This pathway is also shared by other amyloid-forming proteins such as beta-amyloid (Aβ), tau and human islet amyloid polypeptide (hIAPP). The fluorescent dye thioflavin-T (ThT) is a widely used "gold standard" for staining and identifying fibrils of these amyloid proteins [55]. Kinetics studies of α-syn aggregation using ThT[56, 57] revealed a sigmoidal curve of fibril growth, which is composed of the lag phase representing the nucleation stage, an exponential phase representing the elongation stage and a plateau phase corresponding to the completion of fibril formation [58].

It is natural to deduce that α-syn can cause toxicity to neurons, as cell death is a major hallmark of PD and α-syn plays a causal role in the disease. Given that all known clinical mutations are thought to be linked to increased α-syn aggregation and that the main components of Lewy bodies and Lewy neurites are aggregated α-syn fibrils, it was initially believed that α-syn fibrils were the toxic species. Later, increasing evidence from both in vitro and in vivo studies has supported the proposal that oligomeric species are the most pathogenically relevant [59–63]. As a result, the oligomers may be a better therapeutic target, as the aggregated Lewy bodies themselves might be protective and represent a form of an aggresome [64, 65].

α-Syn oligomers are believed to exert toxicity both intracellularly and extracellularly. Within the cytoplasm, α-syn oligomers can inhibit the Hsp 70 chaperone system which is important for protein refolding [66]; α-Syn oligomers can also bind to and inhibit the activities of proteasomes [67], thus affecting protein degradation. Consequently, proteostasis is impaired, leading to endoplasmic reticulum (ER) stress and finally cell death. In vivo, α-syn oligomers have been detected within the ER lumen, and treatment with an inhibitor of ER stress, dramatically delayed the onset of motoric symptoms and decreased the accumulation of α-syn oligomers [68], suggesting that ER stress is involved in the toxicity of α-syn oligomers. In addition, α-syn plays an important role in the assembly of the presynaptic SNARE protein complex. The formation of oligomeric α-syn can inhibit SNARE-mediated vesicle docking and consequently reduce exocytosis, suggesting that inhibition of dopamine release is a potential mechanism in PD [63]. α-Syn oligomers can also induce the dysfunction of synapses by compromising the axonal transport of critical presynaptic proteins [69]. Other potential intracellular targets of α-syn oligomers include mitochondria [70], lysosomes [71] and microtubules [72].

Extracellular sources of α-syn oligomers can form pores in cell membranes, causing an increase in intracellular calcium levels that leads to cell death [59]. Exposure of neurons to exogenous α-syn oligomers can activate glutamatergic receptors, resulting in long-term potentiation (LTP) and excitotoxicity that leads to neuronal death [73, 74]. Moreover, there is significant evidence that α-syn oligomers can transfer from neuron to neuron or to glial cells via a prion-like process [75, 76], which may explain the spread of Lewy pathology throughout the brain in PD [77].
Overall, the accumulation of α-syn aggregates is a major pathological hallmark of PD and a priority target for drug development given its hypothesized contribution to neurodegeneration. In vivo imaging of α-syn pathology could be useful as a biomarker of the presence of the disease, disease progression and as a pharmacodynamic tool for drug development. Therefore, α-syn imaging is a critical need for PD research [78]. Moreover, since α-syn aggregation is regarded as a major pathogenic process in PD, several strategies exist for the prevention of α-syn toxicity [79]. Among them, inhibition of α-syn aggregation remains an extremely attractive target for drug development [30]. The representative progress in the development of imaging probes and aggregation inhibitors over the past decade will be discussed further.

ALPHA-SYNUCLEIN IMAGING PROBES

Radiotracers

Although several PET/SPECT tracers targeting the dopamine system have been used clinically, as reviewed by Politis [15], Braak staging of the PD brain suggests that the severe loss of dopaminergic neurons occurs at stage 4, while Lewy body pathology appears as early as stage 1. As such, imaging α-syn pathology, rather than dopaminergic changes, would be more suitable for early diagnosis in the prodromal period of PD. While great progress has been made toward developing imaging probes for other amyloid-forming proteins [80] (and notably three Aβ imaging probes have gained FDA approval), the development of α-syn imaging probes is still at an early stage. Despite the fact that there are no selective α-syn PET or SPECT probes for clinical PD diagnosis, several α-syn imaging probes have been developed and tested over the past decade (Fig. 2, Table 1).

Amyloid proteins, such as Aβ and α-syn, tend to form similar beta-sheet structures upon aggregation. As a result, probes that can interact with Aβ aggregates also have the potential to bind to aggregated α-syn. Following this rationale, some established Aβ PET probes have been evaluated against α-syn. One well-known example is 11C-Pittsburgh compound-B (PIB, 1), a derivative of thioflavin-T (ThT) which is the gold standard for staining all types of amyloid proteins. Probe 1 exhibited a similar binding affinity \( K_d = 4 \text{ nM}^* \) for in vitro generated α-syn fibrils compared to Aβ [81], but failed to bind to brain homogenates containing Lewy bodies [82]. Additionally, probe 1 displayed poor selectivity for α-syn in the staining of brain sections where Aβ was also present [82].

Benzoxazole BF227 is another established Aβ-binding probe. \(^{18}F\)-BF227 (2) was proven to bind to α-syn fibrils at an equimolar concentration compared to Aβ\(_{1-42} \) fibrils, but the binding affinity for α-syn fibrils \( (K_d = 9.63 \text{ nM}) \) was approximately sevenfold lower [83]. However, like \(^{11}C\)-PIB, probe 2 also failed to bind to brain homogenates containing Lewy bodies [83]. A follow-up in vivo study using an accelerated mouse model of synucleinopathy failed to observe a significant difference in the brains of α-syn transgenic mice compared with α-syn-KO mice after treatment with probe 2 [84]. These results suggest that it is necessary to develop specific α-syn PET probes.

To discover selective ligands for α-syn aggregates, Yu et al. synthesized a series of phenothiazine derivatives and evaluated their binding affinities for α-syn fibrils with a ThT competition assay. Compounds 11b, 16a and 16b exhibited \( K_i \) values of less than 60 nM (32.10 nM for 11b, 48.96 nM for 16a and 57.94 nM for 16b) and were considered for further study [85]. Later, \(^{125}I\) labeled 16b (3), denoted \(^{125}I\)-SIL23, was synthesized and tested by the same research group. Probe 3 can bind to recombinant α-syn fibrils and brain homogenates from PD patients and α-syn transgenic mice. Probe 3 exhibited a relatively high binding affinity for α-syn fibrils compared with Aβ\(_{1-42} \) fibrils (fivefold lower than α-syn) and tau fibrils (twofold lower than α-syn) but the selectivity was not high enough for in vivo imaging [86]. Using α-syn aggregation and fibril formation in transgenic mice as a model for PD, Yu et al. synthesized a series of compounds with different binding affinities for α-syn fibrils (Table 1).

### Table 1. Characteristics of representative radiotracers

| No. | \( K_d/K_i \) | Other targets | Scaffold Status | Ref. |
|-----|---------------|---------------|----------------|-----|
| 1   | \( K_d = 4 \text{ nM}^* \) | Aβ, tau | Benzothiazole | in vitro [81, 82] |
| 2   | \( K_d = 9.63 \text{ nM}^* \) | Aβ | Benzoxazole | in vivo [83] |
| 3   | \( K_d = 148 \text{ nM}^* \), \( K_i = 57.94 \text{ nM}^\text{a} \) | Aβ, tau | Phenothiazine | in vivo [85, 86] |
| 4   | \( K_d = 66.2 \text{ nM}^\text{c,d} \) | Aβ, tau | Phenothiazine | in vivo [87] |
| 5   | \( K_d = 19.9 \text{ nM}^\text{c,d} \) | Aβ, tau | Phenothiazine | in vivo [87] |
| 6   | \( K_d = 8.9 \text{ nM}^\text{a} \), \( K_i = 2 \text{ nM}^\text{a} \) | Aβ, tau | 3-(benzylidine) indolin-2-one | in vitro [88] |
| 7   | \( K_d = 5.4 \text{ nM}^\text{a} \) | Aβ | Chalcone | in vitro [89] |
| 8   | \( K_d = 99.5 \text{ nM}^\text{c} \) | Aβ | Benzimidazole | in vivo [90] |

* Determined by saturation binding assays
** Determined by ThT competition assays
\(^\text{a}\) Isotope unlabeled
\(^\text{c}\) Determined by \(^{125}I\)-SIL23 competition assays

![Fig. 2 Radiotracers for α-syn](image-url)
α-Synuclein imaging probes and aggregation inhibitors

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probe 3-based competition binding assay, the binding affinities ($K_i$ values) of the aforementioned 11b (renamed 2a) and 16a (renamed 2b) were calculated (66.2 nM for 2a, 19.9 nM for 2b), further confirming the potency determined previously [87]. In addition, $^{1}C$-2a (4) and $^{1}F$-2b (5) were synthesized and proven to cross the blood–brain barrier (BBB) in Sprague–Dawley (SD) rats. Both radiotracers exhibited high initial uptake (0.953% ID/g for 4, 0.758% ID/g for 5), homogeneous distribution and rapid washout kinetics. The authors believed that these two radiotracers could be good leads for the discovery of selective imaging probes for α-syn [87].

Identifying new chemical scaffolds is necessary for developing specific radiotracers with high selectivity for α-syn fibrils versus other amyloid proteins such as Aβ and tau. Recently, a series of compounds based on the 3-(benzylidene)-indolin-2-one scaffold were synthesized and the most potent compound was 46a, having a high affinity ($K_i = 2$ nM) and very good selectivity for α-syn versus Aβ ($K_i = 142.4$ nM) and tau fibrils ($K_i = 80.1$ nM). The binding affinity of $^{18}F$-labeled 46a (6) was determined from a saturation binding assay. The $K_d$ of probe 6 for α-syn fibrils was 8.9 nM while the values for Aβ and tau fibrils were 271 and 50 nM, respectively. However, probe 6 is not believed to be a suitable PET tracer for in vivo imaging due to poor druggability [88].

Flavonoids are a common source for the discovery of inhibitors of Aβ aggregation as well as α-syn. Chalcone is a good example and Ono et al. synthesized a series of chalcone derivatives in the hope of discovering new scaffolds for α-syn imaging. Of all four synthesized compounds, IDP-4 showed the most selective binding to α-syn aggregates ($K_i = 5.4$ nM, $K_d = 16.24$ nM for α-syn). Fluorescent staining of PD brain sections confirmed the affinity of IDP-4 for Lewy bodies. Unfortunately, $^{125I}$-IDP-4 (7) displayed the lowest initial uptake of the four compounds synthesized (0.45% ID/g) in normal mice, making it difficult to use for in vivo imaging [89].

More recently, the same group also synthesized three novel radiiodinated benzoimidazole (BI) derivatives based on the previous findings: (1) the benzoimidazole scaffold could bind to α-syn aggregates; (2) the diene moiety helps to increase the affinity for α-syn aggregates; and (3) introduction of large substituents increases the selectivity for α-syn. Compound BI-2 showed the highest selectivity and binding affinity for α-syn aggregates ($K_i = 99.5$ nM, $K_d = 727$ nM for α-syn). Fluorescent staining of PD and AD brain sections using BI-2 was conducted and BI-2 clearly stained Lewy bodies but failed to label Aβ aggregates, further confirming its selectivity for α-syn. However, the promising compound $^{125I}$-BI-2 (8) exhibited low initial uptake (0.56% ID/g) and poor washout kinetics in normal mouse brains, suggesting that the introduction of bulky substitution groups may affect penetration into the BBB [90]. The authors also mentioned that criteria exist for ideal αβ and tau imaging probes according to previous reports [91, 92]: (1) an initial brain uptake up to 4% ID/g at 2 min postinjection in mice; and (2) a remaining amount of less than 1% ID/g at 30-min postinjection in normal mouse brains. Clearly, none of the reported probes for α-syn met these criteria, including probe 8.

In the past decade, significant progress toward discovering useful α-syn radiotracers has been made; however, an ideal, druggable probe possessing high affinity and selectivity for α-syn has yet to be identified [93].

Fluorescent probes

Fluorescence imaging presents a promising alternative technique to radiotracers. In contrast to PET/SPECT techniques, fluorescence imaging is monitored in real time, is inexpensive, is nonradioactive and is of high-resolution when using near-infrared fluorescence (NIRF) imaging probes. As a technique, fluorescence imaging is progressively being explored for the neuroimaging of Aβ aggregates [94]. The ideal fluorescent probe will not only possess high selectivity and binding affinity, penetrate rapidly into the BBB and be cleared quickly from normal brain regions, but will also have low background fluorescence (ideal emission wavelength of greater than 650 nm) in addition to producing a significant increase in fluorescence upon binding to target proteins. Given that aggregated α-syn is far less abundant in the brain than Aβ, α-syn fluorescent probes will require very high selectivity for α-syn over Aβ and tau. Since the majority of α-syn is found intracellularly, in addition to penetrating the BBB, such a probe has to cross the cell membrane [78].

ThT is a widely used fluorescent dye for the nonselective staining of protein aggregates. Its in vivo utility is limited due to its positive charge which affects its penetration through the BBB and its emission wavelength is not suitable for in vivo imaging. To search for improved fluorescent probes for α-syn, Celej et al. evaluated the abilities of several N-arylaminonaphthalene sulfonate (NAS) derivatives to be used as fluorescent markers for α-syn aggregates since the NAS scaffold has a long history of being applied to study the molecular microenvironment and conformation of proteins. Compounds 2,6-ANS (Fig. 3, 9), 2,6-TNS (10), bis-ANS (11), and bis-TNS (12) exhibited slightly improved binding affinity ($K_i$ values: 8.8, 11.7, 8.6 and 11.6 μM, respectively) for α-syn fibrils compared with ThT ($K_i = 14.9$ μM) [95]. Although these molecules also displayed advantages over ThT in terms of providing structural information during α-syn fibrillation, they are still not useful as imaging probes as a result of their charged properties and unideal emission wavelengths.

In an endeavor to search for novel specific fluorescent probes for α-syn, Volkova and coworkers studied the potential of a series of monomethine and trimethinecyanines to detect the formation of α-syn fibrils. The most potent dyes, T-284 (13) and SH-516 (14), had similar binding affinities to ThT but showed large increases in fluorescence (~9.5-fold for 13 and ~7.6-fold for 14) upon binding to α-syn fibrils relative to ThT (2.9-fold). A notable improvement was that the emission wavelengths of probes 13 and 14 after binding to α-syn fibrils were determined to be 570 and 580 nm, respectively, compared with ThT (478 nm) [96]. The specificity of these two dyes was not evaluated by assays against other amyloid proteins.

Later, carbocyanine compound JC-1 (15) was introduced to conduct real-time analyses of α-syn fibril formation. Probe 15 could bind to α-syn monomers as well as fibrils. Interestingly, the maximum emission wavelengths of probe 15 after binding to monomers and fibrils were different (590 nm for monomers, 538 nm for fibrils), indicating that probe 15 is able to distinguish between monomeric and fibrillar α-syn. Furthermore, probe 15 did not interact with either monomeric or fibrillary Aβ. The high selectivity of probe 15 might be explained by its interaction with the acidic C-terminal region of α-syn [97]. The results also suggested that promising selective probes for α-syn may be discovered by searching for compounds that can interact with the monomeric form of α-syn, since different amyloid proteins have different primary structures but share similar beta-sheet structures once aggregated.

Some fluorescent probes previously used as sensors for other biomolecules or microenvironments were tested to monitor α-syn aggregation. One of the compounds, Coumarin 6 (16), displayed a similar sigmoidal curve as ThT when monitoring the aggregation process of α-syn. The lag phase (58 h) was shorter than that of ThT (74.9 h) and the concentration required (50 nM) was much lower than that of ThT (10 μM), suggesting that probe 16 is more sensitive than ThT. The other compound 1,6-diphenyl-1,3,5-hexatriene (DPH, 17), failed to show a sigmoidal curve and was believed to interact with hydrophobic environments without selectivity, while probe 16 could bind to the beta-sheet structure more specifically [98].

Emerging evidence indicates that α-syn oligomers are more toxic and more pathologically connected to PD than fibrils. Consequently, recent research has focused on developing

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selective fluorescent dyes for oligomeric α-syn. A number of benzothiazolemethines and pentamethinecyanines were investigated by Kovalska and coworkers. The pentamethine cyanine dye SL-631 (18) displayed the highest selectivity for oligomeric α-syn (fluorescence increase: 14.58-fold) over fibrillary α-syn (fluorescence increase: 1.88-fold) [99]. The emission wavelength of probe 18 after binding to α-syn oligomers was determined to be 664 nm, showing its potential to be used in vivo as a NIF imaging probe. However, it remains questionable whether the bulky probe 18 could penetrate the BBB.

Another novel probe aimed at detecting α-syn oligomers is tetraphenylethene tethered with triphenylphosphonium (TPE-TPP, 19). Unlike SL-631, probe 19 is able to detect the monomeric, oligomeric and also fibrillar forms of α-syn. At the same concentration, probe 19 emitted over four times more fluorescence than ThT after incubation with α-syn fibrils. The calculated $K_d$ for probe 19 was 4.36 μM, lower than that of ThT (8.48 μM) [100]. Compound selectivity among different amyloids has yet to be determined. Huge challenges still exist in the discovery of probes targeting α-syn oligomers and major breakthroughs in terms of the 3D structure of oligomeric α-syn and the synthesis of high contrasting and selective imaging probes are urgently needed.

A known modulator of α-syn aggregation, anle138b (20), was found to exhibit a significant increase in fluorescence upon binding to α-syn fibrils with high affinity ($K_d = 190$ nM) [101]. The study of probe 20 indicates not only the feasibility of discovering fluorescent probes from known inhibitors of α-syn aggregation but also the possibility of developing drugs with both therapeutic and diagnostic functions.

Until now there have not been any useful α-syn fluorescent probe reported for in vivo imaging (Table 2). In fact, the great progress made in the development of Aβ fluorescent probes might negatively affect the discovery of a specific imaging probe for α-syn because naturally the same strategies to design Aβ probes have been applied to the development of α-syn probes [102]. Since most of the existing Aβ fluorescent probes share the classical push–pull structure [94], which shows little selectivity for the different amyloid protein aggregates, it has been recommended to work on novel strategies to search for or design specific ligands for α-syn.

**SMALL MOLECULE INHIBITORS AGAINST A-SYNUCLEIN AGGREGATION**

The idea of inhibiting α-syn aggregation, especially oligomerization, using small molecules to combat α-syn toxicity and prevent neurodegeneration has been gaining increasing attention. It has been proposed that the identification of aggregation inhibitors from screening compound libraries should be a priority [30]. Until now, a variety of small molecules have been discovered to inhibit α-syn aggregation (Table 3). In many cases, however, the inhibitory effects have also been translated to other amyloid proteins.

**Antibiotics**

In addition to their roles as antimicrobial agents to fight infectious diseases, antibiotics have demonstrated other properties, such as neuroprotective activity. Rifampicin (Fig. 4, 21) stabilized α-syn as a monomer and blocked the fibrillation process. Moreover, rifampicin was also able to disaggregate existing fibrils [103]. Another study confirmed the efficacy of compound 21 by discovering that rifampicin could prevent 1-methyl-4-phenylpyridinium (MPP⁺)-induced toxicity in PC12 cells, increase their survival and reduce α-syn oligomer formation [104]. Another molecule tetracycline (22) is also able to inhibit the formation of fibrils and destabilize preformed fibrils. Compound 22 exhibited moderate effective concentrations (EC$_{50}$ values) for the formation (6.06 μM) and destabilization (18.78 μM) of α-syn fibrils while the values for Aβ$_{1-40}$ and Aβ$_{1-42}$ were higher (10 and 10 μM respectively for fibril formation; 23 and 45 μM respectively, for fibril destabilization), indicating a slight selectivity for α-syn over Aβ [105].

Dopamine and its analogs

Dopamine (23) is believed to react with α-syn covalently to form α-syn-quinone adducts which are primarily large molecular mass.
Dyes and pigments
Some common dyes, such as lacmoid (29), Congo red (30) and phthalocyanine tetrasulfonate (31), have been reported to inhibit α-syn fibrillation [109]. Compounds 29 and 30 were found to be nonspecific inhibitors; however, their effects were mediated by the formation of aggregates of these compounds which can interact with different regions of α-syn monomer [110]. Unlike these two nonspecific inhibitors, the inhibition of α-syn fibrillation by compound 31 is believed to be mediated by specific interactions with the N-terminus of α-syn [111]. This molecule has also been tested in animal models for the treatment of scrapie disease and exhibited anti-prion activity and showed low toxicity [112]. Therefore, compound 31 could be a potential therapeutic candidate for amyloid protein-related diseases. Recently, another dye, Coomassie brilliant blue R (32) not only exhibited significant inhibition of α-syn fibrillation but also prevented the formation of oligomers that caused notable neurotoxicity in cells, making it a potentially useful candidate for future in vivo studies [113]. The red blood pigment hemin (33) is a well-known inhibitor of Aβ aggregation [114]. Until recently, compound 33 was also thought to interfere with α-syn aggregation [115, 116]. The effects of compound 33 on α-syn remain controversial; however, it has not been confirmed whether compound 33 inhibits ThT fluorescence via inhibition of α-syn aggregation or by obstructing the interaction between ThT and the proteins [117].

Polyphenols
Polyphenols are the largest group of inhibitors of α-syn aggregation and many of them can also inhibit the aggregation of other amyloid proteins such as Aβ. We have categorized representative polyphenols into the following groups.

Curcuminoïds. The interaction of curcumin with α-syn seems complicated in that different mechanisms of anti-aggregation by curcumin (34) have been reported. Early studies from the ThT assay and TEM showed that compound 34 not only inhibited the formation of α-syn fibrils but also destabilized the preformed fibrils [105]. Later, the anti-aggregation ability of compound 34 was confirmed by Western blot analysis and in a cell model of α-syn aggregation [118]. Interestingly, another report stated that compound 34 does not interact with α-syn monomers but binds to oligomers and fibrils, causing morphological changes and consequently reducing their toxicity. Moreover, the interaction of compound 34 with early oligomers could impact the toxicity by converting the preformed oligomers into the less toxic fibrils [119]. Although compound 34 has been widely studied, its instability and poor bioavailability limits its medical use.

With the aim of discovering more drugable molecules with a scaffold similar to that of compound 34, Ahsan et al. first synthesized two analogs, curcumin pyrazole (35) and curcumin isoxazole. As 35 exhibited the most potent inhibition of α-syn aggregation (% inhibition of α-syn aggregation: 45.57 for 34, 37.28 for curcumin isoxazole and 62.21 for 35), they proceeded to synthesize 16 derivatives of 35. Among them, compound 6 (36) and 15 (37) showed stronger aggregation inhibition than curcumin (% inhibition of α-syn aggregation: 90.05 for 36, 63.93 for 37). Compound 36 displayed the lowest IC50 value (9.21 μM) compared with compounds 34 (161.8 μM) and compound 37 (89.13 μM). Surprisingly, unlike compound 37, compound 36 failed to reduce the cytotoxicity caused by α-syn oligomers [120]. While compound 37 is a promising therapeutic agent for the potential treatment of PD, the case of compound 36 strengthens the importance of evaluating the anti-oligomerization ability of any molecules that are expected to show neuroprotection in cells and/or animals.

Another group focused on modifications of the two aromatic rings of curcumin to increase the hydrophobicity. Of the nine reported analogs, only two (C2 and C4) showed lower stability than curcumin, and one (C3) exhibited significant cytotoxicity. Among the remaining compounds, C6 (38), which was synthesized by replacing all the hydroxyl groups of compound 34 with -OCH2Ph groups, showed the highest reduction in cytotoxicity.

| No. | λex/λem (nm) | Kd (μM) | Other targets | Scaffold | Status | Ref. |
|-----|--------------|---------|--------------|----------|--------|-----|
| 9   | 320/471b, 320/417b | 8.8 | N/A | N-arylaminonaphthalene sulfonate | in vitro [95] |
| 10  | 320/475b, 320/437b | 11.7 | N/A | N-arylaminonaphthalene sulfonate | in vitro [95] |
| 11  | 395/493b | 8.6 | N/A | N-arylaminonaphthalene sulfonate | in vitro [95] |
| 12  | 395/505b | 11.6 | N/A | N-arylaminonaphthalene sulfonate | in vitro [95] |
| 13  | 443/580a, 441/570b | 0.56 | N/A | Monomethine cyanine | in vitro [96] |
| 14  | 562/568b, 570/580b | 0.65 | N/A | Trimethine cyanine | in vitro [96] |
| 15  | 490/590a, 490/538b | 2.6 | N/A | Carboxcyanine | in vitro [97] |
| 16  | 450/508a | N/A | Bovine carbonic anhydrase | Coumarin | in vitro [98] |
| 17  | 360/423b | N/A | Bovine carbonic anhydrase | Hexatriene | in vitro [98] |
| 18  | 650/665a, 672/683b, 650/662c, 664/678d | N/A | N/A | Pentamethine cyanine | in vitro [99] |
| 19  | 350/480b | 4.36 | μM | N/A | N/A | N/A |
| 20  | 300/345b | 190 nM | N/A | Diphenyl-pyrazole | in vitro [101] |

*aWavelengths of unbound probes
bWavelengths of probes bound with α-syn fibrils
cWavelengths of probes bound with α-syn monomers
dWavelengths of probes bound with α-syn oligomers
Table 3. Characteristics of representative aggregation inhibitors

| No. | EC_{50}/IC_{50} | Other targets | Status | Ref. | No. | EC_{50}/IC_{50} | Other targets | Status | Ref. |
|-----|----------------|--------------|--------|------|-----|----------------|--------------|--------|------|
|     |                |              |        |      |     |                |              |        |      |
| Antibiotics |              |              |        |      |      |                |              |        |      |
| 21  | EC_{50} = 6.96 \mu M | \alpha \text{S}, tau | a     | [103, 105] | 51–53 | N/A | N/A | a, b | [137–139] |
| 22  | EC_{50} = 6.06 \mu M | \alpha \text{S} | a     | [105] | 54  | IC_{50} = 30 \mu M | N/A | a     | [140] |
| 23  | IC_{50} = 7.1 \mu M | \alpha \text{S}, tau | a     | [105, 106, 109] | 55  | IC_{50} = 29 \mu M | N/A | a     | [140] |
| 24–28 | N/A | N/A | a     | [108] | 56  | IC_{50} = 18 \mu M | N/A | a     | [140] |
| Dopamine and its analogs |              |              |        |      |      |                |              |        |      |
| 29  | IC_{50} = 14.7 \mu M | \alpha \text{S}, tau | a     | [109] | 58–59 | N/A | \alpha \text{S} | b     | [141] |
| 30  | IC_{50} = 2.3 \mu M | \alpha \text{S}, tau | a     | [109] | 60–61 | N/A | N/A | b     | [142] |
| 31  | IC_{50} = 27.5 \mu M | \alpha \text{S}, tau | a     | [109] | 62  | N/A | \alpha \text{S} | b     | [145] |
| 32  | IC_{50} = 322 \mu M | N/A | a     | [113] | 63  | N/A | N/A | b     | [146] |
| Dyes and pigments |              |              |        |      |      |                |              |        |      |
| 34  | EC_{50} = 0.22 \mu M | \alpha \text{S} | a     | [105] | 64–65 | N/A | N/A | b, c | [148, 149] |
| 35  | IC_{50} = 126.77 \mu M | N/A | a     | [120] | 66  | N/A | \alpha \text{S} | a     | [150] |
| 36  | IC_{50} = 9.21 \mu M | N/A | a     | [120] | 67  | N/A | \alpha \text{S}, huntingtin | a     | [151] |
| 37  | IC_{50} = 89.13 \mu M | N/A | a     | [120] | 68  | N/A | N/A | a     | [152] |
| 38  | IC_{50} = 0.79 \mu M | \alpha \text{S}, amylin | b     | [117, 130] | 69  | EC_{50} = 2.7 \mu M | \alpha \text{S} | a     | [153] |
| 39  | N/A | \alpha \text{S} | a     | [127] | 70  | EC_{50} = 0.27 \mu M | \alpha \text{S} | a     | [154] |
| 40  | N/A | \alpha \text{S} | a     | [129] | 71  | N/A | N/A | b     | [155] |
| 41–42 | N/A | \alpha \text{S} | a     | [129] | 72  | IC_{50} = 4 \mu M | \alpha \text{S} | b     | [157, 158] |
| 43  | N/A | \alpha \text{S} | a     | [129] | 73  | EC_{50} = 2.8 \mu M | Prion, tau | b     | [159] |
| 44  | IC_{50} = 2.03 \mu M | \alpha \text{S}, tau | a     | [130] | 74  | N/A | N/A | a     | [160] |
| 45  | N/A | \alpha \text{S} | a     | [130] | 75  | N/A | N/A | a     | [166] |
| 46  | IC_{50} = 3.57 \mu M | N/A | a     | [130] | 76  | N/A | N/A | a     | [167] |
| 47  | IC_{50} = 77 nM | N/A | a     | [130] | 77  | N/A | N/A | a     | [166] |
| 48  | N/A | \alpha \text{S} | a     | [133] | 78  | N/A | N/A | a     | [168] |
| 49  | N/A | \alpha \text{S}, amylin | a     | [134] | 79  | N/A | N/A | a     | [166] |
| 50  | N/A | \alpha \text{S} | a     | [135] | 80  | N/A | N/A | a     | [168] |

N/A: not available
a: in vitro; b: in vivo; c: clinical stage
caused by the preformed oligomers and fibrils. Consistent with previous study [119], curcumin and its analog compound 38 were found to accelerate the process of α-syn aggregation into less toxic fibrils [121].

Flavanols. The green tea polyphenol EGCG (Fig. 5, 39) is perhaps the most studied inhibitor of aggregation of different amyloid proteins. It is also a common positive control in many studies aiming to develop new anti-amyloidogenic molecules. Ehrnhoefer et al. demonstrated that compound 39 can directly bind to monomeric α-syn and promote the formation of unstructured, nontoxic α-syn oligomers. NMR results suggested that the compound 39 binds randomly to the backbone of α-syn [122], which, along with another report using MS to study the binding of compound 39 with α-syn [123], may explain why the effects of compound 39 on α-syn can also be seen on Aβ. Additionally, compound 39 is able to bind to preformed α-syn fibrils ($K_d = 100$ nM) and transform them into smaller amorphous aggregates that are less toxic [124]. However, the mechanism by which compound 39 reduces the toxicity of α-syn oligomers is not the same as in the case of fibrils. One group found that treatment with compound 39 does not cause notable changes in the structure or size of α-syn oligomers. Instead, the binding of compound 39 to α-syn oligomers prevents permeabilization of the α-syn oligomers with cell membranes, thus reducing the toxicity [125]. In animal studies, tea polyphenols mainly containing compound 39 were reported to reduce the level of α-syn oligomers in a PD monkey model [126].

Theaflavins, which are abundant in black tea, exert anti-amyloidogenic effects in the same way as compound 39, promoting the assembly of monomeric α-syn and Aβ into nontoxic aggregates and converting the preformed fibrils into nontoxic aggregates [127]. Nevertheless, the tendency of compound 39 and theaflavins to be oxidized could compromise their...
ability to inhibit amyloidogenesis. Although preoxidized compound 39 is still able to significantly inhibit the aggregation process, a reduction in efficacy can be seen after long-term preoxidation in the case of compound 39 but not in the case of another theaflavin, TF3 (40), which is less rapidly oxidized than EGCG [127]. These results suggest that the anti-amyloidogenic abilities of common polyphenols may partially depend on the antioxidant properties. Developing polyphenols that are resistant to oxidation, such as compound 40, may help to discover promising therapeutic agents for amyloid protein-related disease.

Stilbenes. Previous evidence has demonstrated that some stilbenes can inhibit Aβ aggregation [128]. To explore the potential application of stilbenes in the discovery of inhibitors for α-syn aggregation, Temsamani et al. studied three wine stilbenes, piceatannol (41), ampelopsin A (42) and isoeheopeaphenol (43). Although all three stilbenes inhibited fibrillation, only compound 41 showed notable protection of cells treated with α-syn aggregates [129]. An explanation for this may be that compound 41 is also able to disaggregate preformed fibrils, and its relatively small size could enable compound 41 to easily penetrate the cell membrane to exert its protective effects.

Other phenolic compounds. Using confocal single-particle fluorescence techniques, Caruana et al. investigated the effects of different polyphenolic compounds. In addition to the above-mentioned theaflavins and compound 39, they also studied flavones (apigenin, baicalein and scutellarein), flavonols (myricetin and quercetin), phenolic acids (rosmarinic acid and tannic acid), a stilbene (resveratrol) and others (ginkgolide B, nordihydroguaiaretic acid). The most potent compounds, baicalein (44), scutellarein (45), myricetin (46), compound 39, nordihydroguaiaretic acid (47) and black tea extract (mainly theaflavins), were selected because they exhibited significant inhibition and disaggregation of α-syn at
low concentrations (IC\textsubscript{50} < 4 \textmu M). The structure activity relationship (SAR) study suggested that aggregation inhibitors should contain aromatic rings for interactions with monomeric and oligomeric \( \alpha \)-syn and adjacent hydroxyl groups on the same ring [130]. Moreover, the authors proposed that the selected compound would be promising for tests in PD animal models. In addition to compound 39, compound 44 has also been studied in vivo and should be considered a potential drug candidate for clinical trials. In MPP\textsuperscript{+}-treated SD rats, compound 44 reduced the increase of \( \alpha \)-syn aggregates and protected the nigrostriatal dopaminergic system in the brain [131]. Similar effects from compound 44 were observed in the rotenone mouse model, where \( \alpha \)-syn oligomers greatly decreased the striatal neurotransmitters, including dopamine, and rotenone-associated behavioral dysfunction was improved [132].

As a main metabolite of green tea polyphenols, protocatechuic acid (48) is able to inhibit \( \alpha \)-syn aggregation, disaggregate preformed \( \alpha \)-syn fibrils and protect PC12 cells from toxicity caused by \( \alpha \)-syn aggregates [133]. However, this phenolic acid can also exert similar effects on A\textsubscript{B}. Oleuropein aglycone (49), which is commonly found in olive oil, was found to protect SH-SY5Y cell viability via stabilization of \( \alpha \)-syn in the monomeric state, directing \( \alpha \)-syn to form nontoxic aggregates and obstructing the binding of \( \alpha \)-syn to cell membranes [134]. Another \( \alpha \)-syn aggregation inhibitor discovered from olives is hydroxytyrosol (50). Similar to compound 48, compound 50 can also inhibit \( \alpha \)-syn aggregation and destabilize preformed \( \alpha \)-syn fibrils. Notably, compound 50 at a concentration of 25 \textmu M almost completely reversed the toxicity caused by \( \alpha \)-syn aggregates [135] while compound 48 only rescued the cell viability to approximately 80\% at the same concentration [133]. The discovery of active anti-amyloidogenic compounds from olive indicates another good dietary source that may help to prevent neurodegenerative diseases, in addition to green tea.

Glucosides
Glucosylation of common organic compounds may help to increase their bioavailability [136]. Curcumin-glucoside (51), which was synthesized by replacing the two hydroxyl groups on the aromatic rings with glucose moieties, showed similar effects to inhibit \( \alpha \)-syn oligomerization as well as to destabilize preformed fibrils compared with compound 34 but was believed to be more potent. Importantly, the introduction of glucose increased the solubility [137]. There are also natural glucosides that have been reported to be anti-amyloidogenic agents. One common group is ginsenosides, the main biologically active extract from ginseng. Rb1 (52) is one of the most studied ginsenosides and can inhibit oligomerization and fibrillation as well as disaggregate preformed fibrils. Administration of compound 52 in cells can significantly attenuate the toxicity induced by \( \alpha \)-syn aggregates, thus making it worthwhile for compound 52 to be further tested in animal models [138]. Another glucoside, liquiritin (53), comes from Glycyrrhiza uralensis, a common traditional Chinese medicine for the treatment of PD. In vitro studies have demonstrated that compound 53 can inhibit both oligomerization and fibrillation of \( \alpha \)-syn. In a transgenic C. elegans expressing human \( \alpha \)-syn, compound 53 significantly inhibited \( \alpha \)-syn aggregation and extended the life span of the treated worms [139].

Quinones
It has been reported that several vitamins K, unlike many promiscuous inhibitors that bind to \( \alpha \)-syn via nonspecific hydrophobic interactions, can inhibit \( \alpha \)-syn fibrillation and disassemble preformed fibrils by specifically binding to the N-terminal region of \( \alpha \)-syn. These vitamins (Fig. 6, phylloquinone, 54, menaquinone, 55 and menadione, 56) are actual derivatives of 1,4-naphthoquinone (1,4-NQ, 57), indicating a potential role for the scaffold of compound 57 in the design of novel specific inhibitors for \( \alpha \)-syn [140]. Interactions of these vitamins K with other amyloid proteins have yet to be explored.

Tanshinone I (58) and tanshinone II A (59) are the main active components in the traditional Chinese medicine Danshen. Similar to EGCG, these two phenanthrenequinones have been demonstrated to reduce the formation of \( \alpha \)-syn oligomers and fibrils as well as destabilize the preformed fibrils. The benefits of these two molecules were further confirmed in a dye leakage assay where they managed to prevent the membrane damage caused by \( \alpha \)-syn aggregates. The good performance in vitro paved the way for later in vivo assays conducted in a C. elegans model expressing \( \alpha \)-syn. Compounds 58 and 59 both significantly prevented \( \alpha \)-syn aggregation and extended the life span of treated C. elegans [141]. Since other neuroprotective effects of these compounds have already been studied in different rodent models, compounds 58 and 59 are expected to target \( \alpha \)-syn aggregation in PD rodent models.

Hybrid molecules
In light of the complex pathogenesis of PD, designing molecules that are capable of targeting different factors related with the disease is an attractive approach. Compounds 18 (60) and 24 (61), which are 3-arylcoumarin-tetracyclic tetracine derivatives, were synthesized and selected as promising leads for the further development of potential PD therapeutics. Consistent with the design strategy, these two compounds exhibited multitargeted properties, including inhibition of \( \alpha \)-syn aggregation, antioxidation and enhancement of the content of dopamine [142]. The concept of designing hybrid compounds for multitargeted functions may contribute to discovering novel therapeutics for future PD treatment.

Aminosterol
It has been found that lipid vesicles can assist the nucleation of monomeric \( \alpha \)-syn, which is a key step in \( \alpha \)-syn aggregation [143]. The potential effects of the aminosterol compound squalamine (62) on \( \alpha \)-syn aggregation was studied as this compound is able to translocate proteins from the cell membrane to the cytoplasm [144]. In an \( \alpha \)-syn aggregation system containing lipid vesicles, compound 62 intervened in the interaction between \( \alpha \)-syn and the surface of the vesicles, thereby inhibiting \( \alpha \)-syn aggregation [145]. This mechanism of compound 62 also counteracted the toxicity of oligomeric \( \alpha \)-syn in SH-SYSY cells, reduced \( \alpha \)-syn aggregation and alleviated the immobility caused by \( \alpha \)-syn aggregates in C. elegans overexpressing \( \alpha \)-syn [145]. Later, the same group studied another structurally similar compound, trodusquemine (63). The side chain of compound 63 is spermine rather than spermidine as in compound 62. The introduction of additional positive charges was attributed to the increased ability of compound 63 to displace \( \alpha \)-syn from both lipid vesicles and preformed \( \alpha \)-syn fibrils, thus making this aminosterol able to inhibit not only the nucleation of \( \alpha \)-syn monomers but also fibril-induced aggregation [146]. Like compound 62, compound 63 also protected SH-SYSY cells from the toxicity of oligomeric \( \alpha \)-syn and improved the fitness of C. elegans overexpressing \( \alpha \)-syn [146]. Future investigations of these two aminosterols in rodent PD models are anticipated.
α-syn and lipid membranes, compound 64 reduced the formation of toxic α-syn oligomers in primary rat neurons. The long-term effects of compound 64 in a wild-type α-syn transgenic mouse model improved the performance of treated mice in motor behavioral assessments [148]. In the brains of these treated mice, the accumulation of α-syn was significantly reduced in different regions (neocortex, hippocampus, and striatum). A reduction in α-syn was also seen in the substantia nigra but this result was not significant. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that compound 64 decreased
the amount of α-syn dimers and oligomers, a phenomenon that was also seen in an oligomer-prone E57K α-syn transgenic mouse model [148]. Pathologically, neuronal death, synaptodendritic damage and astrogliosis were notably improved, indicating an association between inhibition of α-syn accumulation, recovered neurodegeneration, and ameliorated motor function [148]. The short-term effects of compound 64 were evaluated in real time in α-syn-GFP transgenic mice. The reduction of the α-syn-GFP signal at synapses could be seen between 30 and 60 min after administration, indicating the efficiency of compound 64 to inhibit α-syn accumulation at synaptic terminals [148]. Compound 64 suffered from poor oral bioavailability and poor brain penetration; therefore, this compound was not advanced further for clinical studies [149]. Based on the structure of compound 64, a new compound, NPT200-11 [65], with improved pharmacokinetic properties, was introduced. Compound 65 maintained similar efficacies compared to compound 64, including a decrease in α-syn accumulation, amelioration of neurodegenerative pathology and improvements in behavioral performance [149]. According to the authoritative database of clinical trials (ClinicalTrials.gov), the phase 1 study of compound 65 in healthy subjects was successfully completed in 2016. A phase 1b clinical study in both healthy subjects and PD patients is expected to be conducted in Europe. Without a doubt, compound 65 is the most promising small molecule drug candidate targeting α-syn aggregation.

Others

There are still many other molecules that have been reported to be inhibitors of α-syn aggregation at the molecular level, such as aldehyd e 4-hydroxy-2-nonenal (HNE) [66] and scyllo-inositol [67]. Compound 66 has perhaps the simplest structures among all the reported inhibitors since it has no aromatic ring and the molecular mass is only 156 Da. Compound 67 is already well known for its efficacy to inhibit Aβ aggregation in vitro and in vivo.

In cell models, compounds such as dieckol [68], melatonin [69], selegiline [70], and synuClean-D [71] can exert neuroprotection against toxicity induced by α-syn aggregates. The discovery of compound 71 resulted from drug screening based on the ThT assay [156]. The ameliorative effects of compound 71 were also observed in PD C. elegans models. Treatment with compound 71 remarkably reduced the formation of α-syn aggregates and protected dopaminergic cells from death in C. elegans, attributing to the improved motility [155].

The following three molecules are promising compounds to be further developed into useful PD disease-modifying drugs for clinical applications. The first one, CLR01 (72), also termed molecular tweezer, was first studied systematically by Prabhudesai et al. In vitro assays including ThT assays and TEM demonstrated that compound 72 can inhibit α-syn aggregation as well as disassemble preformed fibrils. Compound 72 also exhibited significant protection in both HEK293 cells expressing α-syn endogenously and PC12 cells treated with aggregated α-syn. Excitingly, compound 72 managed to reduce apoptosis induced by α-syn aggregation in zebrafish embryos expressing α-syn, which contributed to increased survival of the treated embryos [157]. Both intracerebroventricular and subcutaneous administration of compound 72 to mice overexpressing α-syn can notably alleviate the motor deficits caused by α-syn pathology and is accompanied by a decrease in the soluble α-syn fraction. The long-term efficacy of compound 72 could be explained by its stable kinetics in vivo [158]. Modifications of this molecule to increase its ability to penetrate the BBB would be required before moving to human trials.

Anle138b [20] was discovered by a high-throughput screening system against the aggregation of the prion protein. Interestingly, compound 20 is also able to reduce the formation of α-syn oligomers. Administration of compound 20 in the rotenone mouse model successfully ameliorated motor dysfunction. In another transgenic mouse model expressing human A30P mutated α-syn, compound 20 was shown to improve motor performance, prevent the spread of deposited α-syn in the brain and reduce the level of α-syn oligomers [159]. Since compound 20 possesses ideal pharmacokinetic properties, it would not take long for it to be tested clinically to treat PD and prion diseases.

The identification of fasudil (73) as a potent inhibitor of α-syn aggregation is quite exciting, as it is already an approved drug for cerebral vasospasm. Therefore, it would not be difficult for compound 73 to be used clinically in the future for treatment of PD given that this molecule can effectively inhibit α-syn aggregation via specific binding to the C-terminal region and more importantly, treatment with compound 73 can improve motor performance and recognition memory in α-syn A53T mutated mice [160].

CONCLUSIONS AND FUTURE PERSPECTIVES

Lewy bodies were first identified over 100 years ago and their main component, α-syn, though seemingly discovered much later, has been investigated for over 20 years. It is believed that the aggregation process of α-syn plays a central role in PD pathogenesis and as a result, the past decade has seen a large number of studies focusing on α-syn aggregation and its role as a biomarker.

A variety of small molecule probes and inhibitors of α-syn have been developed over the past decade. Some of these inhibitors have been tested in vivo and therefore are promising candidates for clinical trials. Many of the discovered molecules are also able to affect the aggregation of other amyloid proteins, indicating their roles as nonspecific amyloid inhibitors. The reason for this may be explained by discussing the common techniques for the discovery of molecules capable of interacting with α-syn.

Similar to other biophysical techniques, ThT assays are not able to sensibly detect and individually characterize unique protein subspecies with which a small molecule ligand interacts [161]. A ThT assay can also be compromised if the small molecule can competitively bind to the dye-binding site on the protein or quench the emission of fluorescence via interaction with the dye [162].

The advantages of mass spectrometry (MS) over other screening assays include the rapid discovery of candidate inhibitors and, more importantly, the ability to identify binding modes and the particular protein conformations responsible for the interactions with the small molecules [116, 117, 163]. Based on previous studies, we have recently established an automated screening system for screening scaffolds that can bind to monomeric α-syn. By screening over 2500 pure compounds, we identified a new α-syn aggregation inhibitor (74) with a pyrazol o [1, 5-a] pyrimidine-5-carboxylic acid scaffold. This molecule exhibited similar effects in the ThT assay and circular dichroism (CD) and TEM experiments with the positive controls EGCG and hemin. Coincubation of α-syn with compound 74 protected SH-SY5Y cells from the toxicity of α-syn aggregates. MS techniques also allow the electron capture dissociation (ECD) fragmentation of the protein–ligand complexes, which can offer information about the binding regions of the ligands [164, 165]. Compound 74 displayed a more specific binding region on the primary sequence of monomeric α-syn and a clear binding preference for low-charge states, suggesting its higher selectivity for monomeric α-syn than EGCG and hemin [166].

In an endeavor to discover specific α-syn aggregation inhibitors, research has started to focus on developing molecules that target specific regions of monomeric α-syn, such as vitamins [54, 55 and 56] [140] and the two de novo synthesized pyrimido pyrazine derivatives [64 and 65] [148, 149]. The ability to detect the interactions between small molecules and monomeric α-syn...
makes MS a powerful technique to discover specific a-syn binding compounds and identify the specific regions to which the compounds bind. We also propose that the a-syn binding scaffolds screened by MS have the potential to be further developed into both fluorescent and PET imaging probes. This means that a single molecule may have both therapeutic and diagnostic functions. These types of theranostic reagents have already been investigated for cancer [167] and AD [168] and we believe that developing molecules with theranostic functions for PD will become a hot topic in the near future.

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MMX wrote the paper; PR, SR, RQJ, HYZ and GDM read and revised the paper.

ADDITIONAL INFORMATION
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