






Review

Modulation of Alpha-Synuclein Conformational Ensemble and Aggregation Pathways by Dopamine and Related Molecules

Antonino Natalello^{1,*}, Stefania Brocca¹, Erika Ponzini^{2,3}, Carlo Santambrogio¹,
Rita Grandori^{1,4,*}¹Department of Biotechnology and Biosciences, University of Milano-Bicocca, 20126 Milan, Italy²Department of Materials Science, University of Milano-Bicocca, 20125 Milan, Italy³Optics and Optometry Research Center (COMiB), University of Milano-Bicocca, 20125 Milan, Italy⁴Institute for Advanced Simulations, Forschungszentrum Juelich, 52428 Juelich, Germany*Correspondence: antonino.natalello@unimib.it (Antonino Natalello); rita.grandori@unimib.it (Rita Grandori)

Academic Editors: Fabio Moda and Giorgio Giaccone

Submitted: 6 July 2023 Revised: 7 September 2023 Accepted: 25 September 2023 Published: 26 October 2023

Abstract

Dopaminergic neurons are constantly threatened by the thin boundaries between functional α -synuclein (AS) structural disorder and pathogenic aggregation, and between dopamine (DA) neurotransmitter activity and accumulation of cytotoxic by-products. The possibilities of developing drugs for Parkinson's disease (PD) depend on our understanding of the molecular mechanisms that cause or accompany the pathological structural changes in AS. This review focuses on the three interconnected aspects of AS conformational transitions, its aggregation pathways and ligand binding. Specifically, the interactions of AS with DA, DA metabolites, DA analogs and DA agonists are considered. Recent advances in the field are discussed with reference to the structural properties of AS and the methodologies employed. Although several issues are still object of debate, salient structural features of the protein, the aggregates and the ligands can be identified, in the hope of fueling experimental and computational approaches to the discovery of novel disease-modifying agents.

Keywords: intrinsically disordered proteins; ligand binding; oxidative stress; synucleinopathies; catecholamines

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (AD). This movement-related disorder is associated with a progressive loss of dopaminergic neurons in the brain - particularly in the *substantia nigra pars compacta* (SNpc) and in the *locus coeruleus* - and with the accumulation in nerve cells of β -sheet-rich protein inclusions, called Lewy bodies (LBs) or Lewy neurites (LNs) depending on intracellular localization and morphology [1]. LBs and LNs are constituted mainly of aggregated and modified α -synuclein (AS), the main protein implicated in PD etiology, and represent hallmarks of PD and related neurodegenerative disorders. In addition to AS fibrils, LBs and LNs can contain other proteins, lipids, and organelles [2].

Despite the strong effort devoted in the last decades to the investigation of PD etiology, the molecular mechanisms underlying the onset and progression of the pathology are still poorly understood. Several genes have been reported to play a role in the pathogenesis and several mutations in the AS-encoding gene (SNCA) have been linked to familial PD. However, the latter account for only 5% of the cases, while the large majority of patients develop the sporadic form. PD is, indeed, regarded as a multifactorial pathology, in which alterations in protein homeostasis, redox homeostasis, mitochondrial function, and membrane integrity are involved in a complex process leading to neu-

roinflammation and disease onset [3–5]. AS is known to be physiologically involved in neurotransmission and cognition, but the mechanisms of action have yet to be elucidated [6]. AS is expressed in vertebrates and particularly abundant in the brain, especially in the presynaptic terminals of dopaminergic neurons, but it can also be found in the central nervous system (CNS) and in peripheral tissues [7] or fluids, including serum [8], saliva [9,10] and tears [11,12]. The pathological accumulation of AS protein in specific populations of neurons and glia is the hallmark of several disorders identified as synucleinopathies. They can be classified in two major groups: Lewy body disease (characterized by LBs and LNs) and multiple system atrophy (MSA, characterized by glial cytoplasmic inclusions). Lewy body disease includes PD, PD dementia (PDD), dementia with Lewy bodies (DLB), and other neurodevelopmental and neurometabolic disorders, while MSA is subclassified into MSA with predominant cerebellar ataxia (MSA-C) and MSA with predominant parkinsonism (MSA-P) [13].

The difficulties in diagnosis and treatment of these pathologies are a mirror of the molecular complexity underlying it. The different molecular landscapes depicted in the literature suggest the involvement of distinct factors (Fig. 1) and underscore the importance of deep, individual, biochemical phenotyping for basic and translational studies [14]. Combined AS biomarkers, integrating structural, bio-



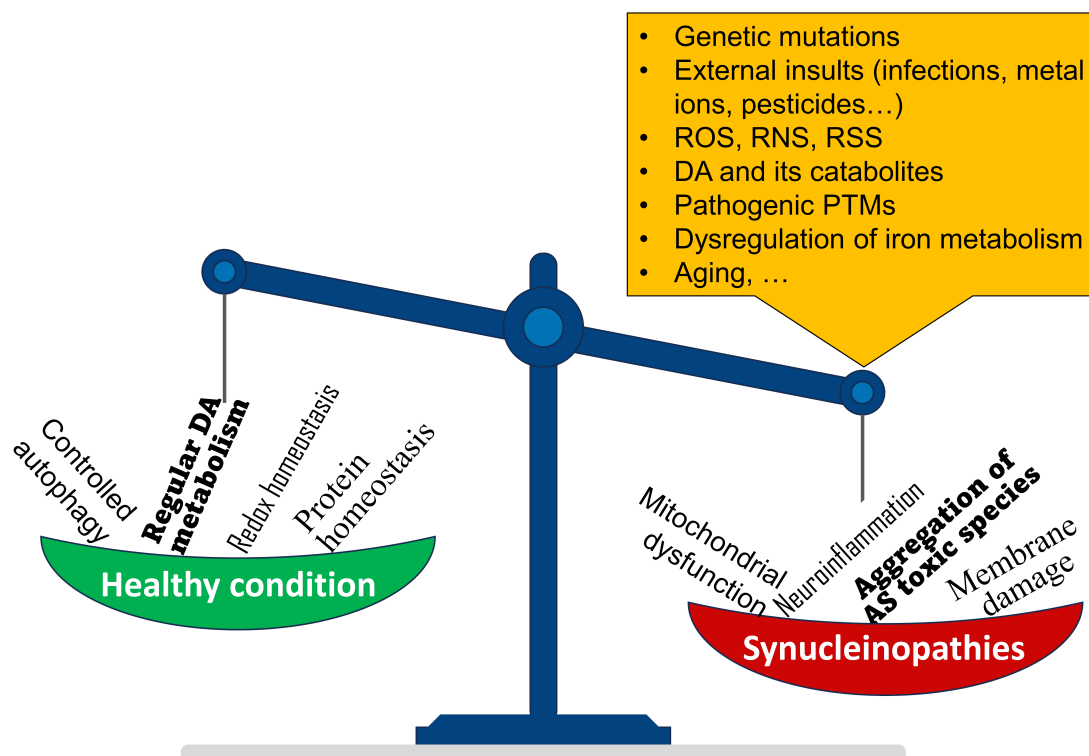


Fig. 1. Pictorial representation of the main factors affecting α -synuclein (AS) aggregation. DA, dopamine; ROS, reactive oxygen species; RNS, reactive nitrogen species; RSS, reactive sulfur species; PTMs, post-translational modifications.

chemical and aggregation properties, could contribute to diagnosis and typization of synucleinopathies [8]. Increasing interest is devoted to peripheral, non-invasive, AS biomarkers [8,12,15].

Particularly relevant to this regard is the dual-hit hypothesis, also known as the Braak's hypothesis, stating that sporadic PD initiates in the peripheral nervous system [16]. It is hypothesized that after exposure to external insults, including viral or bacterial infections [16–18], the pathology propagates from peripheral tissues - neurons of the gut and nasal cavity - to and within the CNS [16,18]. Dysregulation of either redox or protein homeostasis induces a toxic response in dopaminergic neurons [4,5]. The accumulation of AS, in turn, may enhance the redox stress, thereby generating a vicious cycle that leads to cell death and neuroinflammation [19]. Thus, perturbations of redox and protein homeostasis enhance each other in degenerating neurons in PD (Fig. 1).

Proteostasis is a finely regulated process aimed at controlling the quality of protein synthesis, from transcription to folding. Alterations in this surveillance lead to the accumulation of abnormal products. Autophagy plays a key role in this context by removing dysfunctional elements and facilitating the natural turnover of cellular components. Dysfunctions in this mechanism can result in accumulation of aggregation products of misfolded AS. In turn, AS misfolding may further inhibit its degradation, generating another vicious cycle [20–25].

Reactive oxygen, nitrogen, and sulfur species (reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS), respectively) are produced by cell metabolism and play a fundamental role as second messengers in several metabolic processes, but also pose a threat to cellular structures and functions. Both enzymatic and non-enzymatic actors form a delicate and intricate network aimed at protecting cells from oxidative or reductive damage to DNA, lipids, and proteins. Another source of oxidative stress is dysregulation of iron metabolism, which is well-documented to be impaired in PD [26,27]. Neurons are particularly exposed to redox stress due to dopamine (DA) metabolism. The inherent toxicity of DA and AS could be a key to understanding PD pathogenesis [6,28].

The two crucial factors DA and AS are not only functionally but also directly linked to each other by the interactions that the protein and its aggregation products can establish with DA and its metabolites. Thus, it is of interest to elucidate the effects of such ligands on conformational and aggregation properties of AS. In this review, the recent literature on the interactions of AS with DA and its metabolites is commented, with regards to their effects on the protein conformational ensemble and aggregation process.

2. DA Metabolism and Oxidative Stress

DA and AS are two central actors of neurotransmission by dopaminergic neurons [3]. DA transmits stimuli

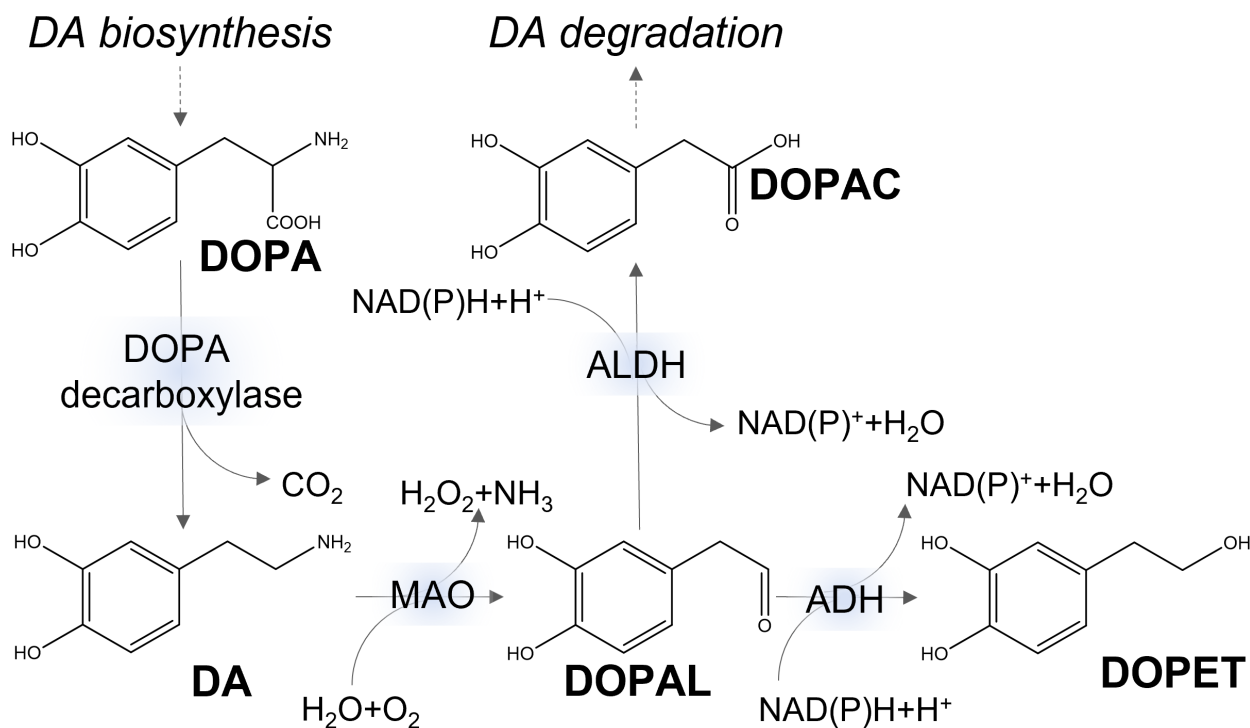


Fig. 2. Simplified metabolic pathways of dopamine (DA). DOPA, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetate; DOPAL, 3,4-dihydroxyphenylacetaldehyde; DOPET, 3,4-dihydroxyphenylethanol; NAD, nicotinamide adenine dinucleotide; ALDH, aldehyde dehydrogenase; MAO, monoamine oxidase; ADH, alcohol dehydrogenase.

from SNpc to striatum to activate motor function [29], while AS is involved in storage, release and recycling of the neurotransmitter [30]. In spite of this concerted action, the two molecules have restricted interactions under physiological conditions [6,27]. AS is a presynaptic, cytoplasmic protein. DA, too, is synthesized in the cytoplasm of dopaminergic neurons from the precursor tyrosine, through the sequential activities of tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC). However, its cytoplasmic concentration is kept low by efficient loading into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2).

Cytoplasmic DA can be catabolized by potentially toxic reactions having ROS as side products (Fig. 2). One pathway involves the enzymatic oxidation of DA to 3,4-dihydroxyphenylacetaldehyde (DOPAL), with concomitant production of hydrogen peroxide, by the monoamine oxidase isoforms MAO-A and MAO-B of the outer mitochondrial membrane. The highly reactive metabolite DOPAL has long been recognized as neurotoxic. It is further converted to the non-toxic metabolite 3,4-dihydroxyphenylacetate (DOPAC) by aldehyde dehydrogenase (ALDH). The catecholaldehyde hypothesis points to DOPAL as the main trigger of neurodegeneration in PD [31,32].

Cytoplasmic DA is also prone to auto-oxidation, a fate that is prevented inside synaptic vesicles by the low pH [33]. This reaction is accelerated by metal ions, particularly

Fe^{2+} [34,35], and can lead to intracellular accumulation of dopamine quinone, aminochrome, 5,6-indolequinone, 5-S-cysteinyl-dopamine, hypochlorite-oxidized cysteinyl-dopamine, and 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid. These products, themselves, exert recognized cytotoxic functions [36,37] and are produced along with superoxide, hydrogen peroxide and hydroxyl radicals, threatening redox homeostasis [38].

A third pathway in DA metabolism is the polymerization of its auto-oxidation products and inclusion into neuromelanin (NM). NM is a pigment accumulating in dopaminergic neurons, containing DA oxidation products, iron, lipids, proteins (including AS) and different toxic compounds and metals, particularly iron, zinc, aluminum chromium, molybdenum, lead and mercury [39–41]. These components are bound to the polymer matrix and enclosed in a lipid bilayer membrane, forming intracellular NM organelles [39]. Thus, NM synthesis is thought to be protective of neurons by sequestering potentially toxic compounds from the cytosol. However, NM can also have toxic effects when released from degenerating neurons in PD, promoting microglia activation and neuroinflammation [42].

3. Structural Properties of AS

AS is an intrinsically disordered protein (IDP) composed of an N-terminal, lipid-binding domain (residues 1–66) [43], a central, non-amyloid β component (NAC,

residues 67–95) promoting cross- β structures [44], and a negatively charged, C-terminal domain (residues 96–140) playing a key role in determining AS (in)solubility and protein-protein interactions in a pH-dependent way [45] (Fig. 3A). Different states have been reported for AS (Fig. 3B): (i) monomeric, soluble, cytoplasmic and highly disordered [46,47]; (ii) monomeric, lipid-bound, with a helical N-terminal region and a disordered C-terminal tail [48]; (iii) helical, tetrameric, soluble state resistant to aggregation [49]; (iv) oligomeric, lipid-bound, forming pathological fibrils [50]. The first three can be considered physiological conformations, while the latter triggers pathological conditions [46–50]. Note that the physiological tetramer has been described only once, while several manuscripts have been published afterwards reporting contrasting evidence in this regard [51].

The soluble, disordered monomer is the most common state, in which AS is found in the cytoplasm of neuronal cells [47]. *In-vitro* studies indicate that AS forms rapidly interconverting conformers, whose nature and biased distribution can promote specific aggregation pathways [52]. The disordered nature of cytosolic AS has been experimentally assessed by in-cell nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopies [47]. NMR and EPR signals show that the protein remains highly flexible, transiently interacting with various cytoplasmic components and undergoing rapid molecular reorientation. *In-vivo* cross-linking (XL) coupled to mass spectrometry (MS) is another approach that probes protein conformation inside the cell, yielding information on intra- and inter-molecular contacts and inter-residue distances. Such kinds of measures can feed computational modeling by experimental constraints. This method describes AS conformational ensemble as dominated by rather compact, globular conformations with transient secondary structure elements. Compaction is determined by contacts between the N- and C-terminal regions, with a transient β -hairpin structure involving the so-called NAC and C-terminal regions that could provide a nucleation site for oligomerization [53]. XL-MS has also pointed out a set of rather compact, “hairpin-like” conformations of soluble AS *in vitro* with the positively charged N-terminal region interacting with negatively charged C-terminal region and thus shielding the NAC domain [54]. On the other hand, XL-MS analyses highlight open, “elongated” conformations in AS condensates, likely because they promote inter-protein contact underlying liquid-liquid phase separation (LLPS) [54].

Multiple and transient conformations are also captured by fluorescence resonance energy transfer (FRET), which also allows *in-vivo* conformational studies on individual molecules with high spatio-temporal resolution. Time-resolved FRET (trFRET) experiments have been performed on a set of eight AS mutants [55]. The structural transitions simulated by molecular dynamics (MD) based on FRET trajectories are heterogeneous and occur on the

millisecond timescale. Secondary-structure composition of AS conformers has been captured by surface-enhanced Raman spectroscopy (SERS) coupled with *in-situ* optical tweezers [56]. This experimental setting allows micro-sampling of aqueous solutions, in which AS is present in a non-aggregated form, at concentrations comparable to physiological concentrations in the neuronal cytosol (1 μ M) [56]. Secondary structure has been detected at quasi-single molecule level from 200 measurements performed in parallel on a same AS preparation. The majority of molecules (>75%) have been found in random-coil conformation, with minor fractions exhibiting α -helix and β -sheet structures (15% and 10%, respectively). Such a skewed conformational distribution could explain the lag periods required for the formation of fibrils observed macroscopically. Post-translational modifications (PTMs), such as phosphorylation, nitration, acetylation, arginylation, methylation, glycation, truncation, ubiquitination, SUMOylation and O-GlcNAcylation, have strong impact on AS properties *in-vivo* [57,58]. They can affect conformational transitions, biasing conformational equilibria towards some specific subpopulations and altering its seeding properties [58,59]. PTMs have been also implicated in AS aggregation propensity [58,60] and degradation [61], as well as interactions with membranes [62] or other proteins [63], including chaperones [64], small molecules [65] and metal ions [66]. Most studies have focused on pathological species extracted from tissues of patients with synucleinopathies [67,68]. However, some evidence shows that PTMs occur at varying degrees on soluble, monomeric AS in the brain of healthy individuals, too [2,69–71]. How the composition of the conformational ensemble affects the aggregation pathway and, consequently, the types of fibrils represents a salient aspect of structure-function relationship [72]. This is also relevant from a patho-physiological point of view, since the rate of disease progression can be affected by the local structures explored by monomeric AS in solution [52]. Sequence variants, PTMs and environmental conditions shape the conformational ensemble. However, exposure to the same environment can also lead to fibrils with different morphology, solubility, and stability [73]. In light of all this, the characterization of conformational ensembles and conditions stabilizing potentially pathological species is of utmost relevance for biomedical studies.

4. AS Aggregation Pathways

The general scheme of the protein aggregation mechanism involves misfolded conformers assembling into low-molecular weight oligomers, followed by the formation of prefibrillar aggregates and mature fibrils (Fig. 3B).

Despite the large amount of research, most of the details of these molecular events are unknown, including the structures of AS monomers and aggregation intermediates, the relationship among the different aggregation pathways reported in the literature, and the interplay between the pro-

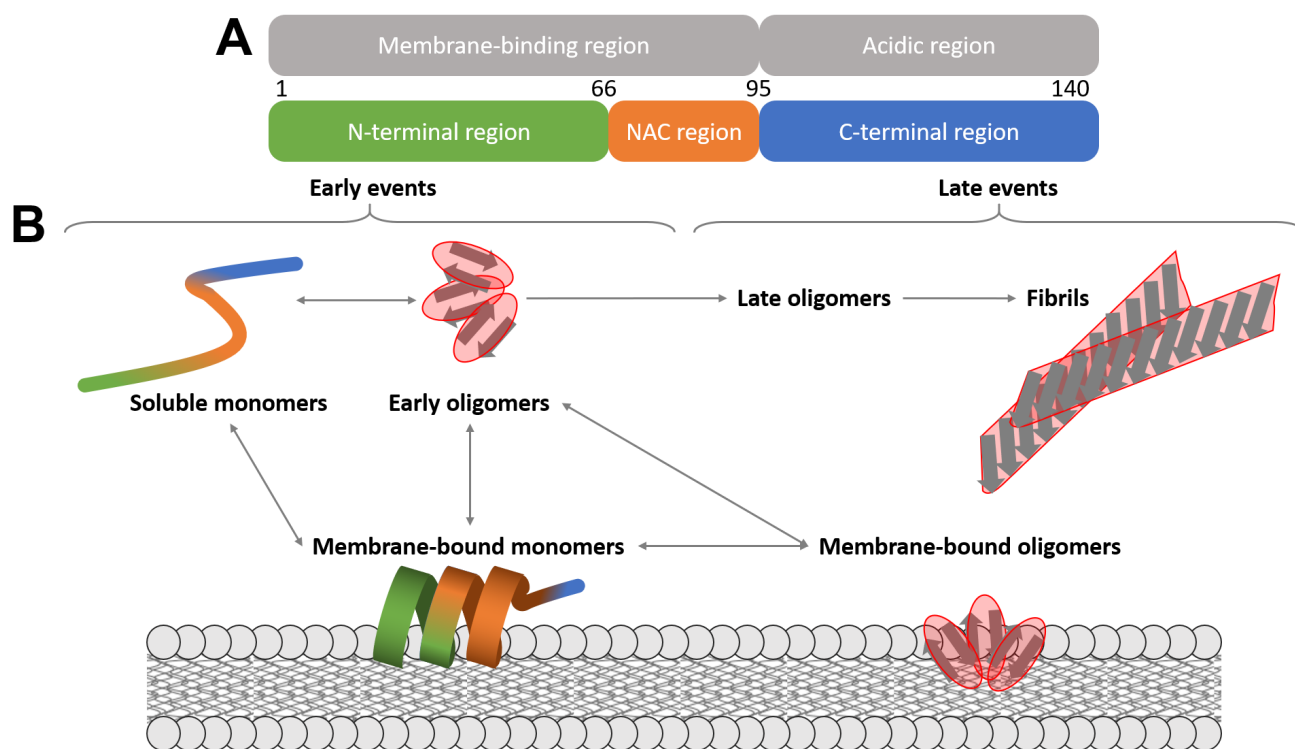


Fig. 3. Schematic representation of AS structural plasticity. (A) Domain organization depicted on AS primary structure. (B) Main conformational transitions of AS. NAC, non-amyloid β component.

tein structure and the cellular/extracellular contexts. Fig. 4 gives an outline of the AS aggregation pathways described in the literature, where steps modulated (triggered or inhibited) by DA and related molecules are indicated (see also the next sections).

As reported in the previous section, AS monomers are highly dynamic and populate extended and partially folded conformers, whose equilibrium can be shifted by environmental factors, including ligand binding. AS is partitioned between cytosolic and membrane-bound forms. It has been shown that the presence of free monomers in solution (high AS/membrane ratio) promotes primary nucleation on the surface of vesicles already saturated with AS by up to three orders of magnitude [74].

Starting from the heterogeneous ensemble of AS conformers, two main interconnected aggregation pathways have been described [75], both inhibited by DA, as discussed in the next paragraph. In the classical nucleation-dependent polymerization process [76], aggregation-prone conformers of AS [77] form oligomeric structures, which are on- or off-pathway of fibrils formation. Oligomers with spherical, ellipsoidal and annular morphologies have been described. Conformational transitions from random coil to α -helix and β -sheets have been reported [78,79]. AS oligomers are metastable, highly heterogeneous and are regarded as the most toxic species. To better understand the structure-toxicity relationship, two types (-A* and -B*) of oligomers characterized by different ability to perturb biological membranes and disrupt cellular function

have been compared [80]. Type-A* oligomers were prepared by incubating AS monomers with epigallocatechin gallate (EGCG). The isolated oligomers contained an average of approximately 24 monomers. Type-B* oligomers were, instead, prepared by AS incubation in phosphate buffered saline at high protein concentration without agitation. The isolated material consisted of two populations of oligomers, each containing an average of approximately 18 or 29 monomers. Type-A* and type-B* display similar size and morphology but only type-B* can be considered toxic in consideration of their ability to disrupt synthetic and cellular membranes, to increase intracellular ROS concentration, and to reduce the mitochondrial activity in neuronal cells. The biophysical characterizations of the two oligomer types disclosed substantial structural differences. Type-A* oligomers displayed negligible secondary structure content while type-B* contained considerable β -sheet structures, with a more dynamic and accessible N-terminal region than in type-A*. Different models for the interaction with cellular membranes have been proposed for the two oligomer types: type-A* binds exclusively to the membrane surface; type-B* binds the membrane surface *via* the folding of the N-terminal regions into amphipathic α -helices while the rigid β -sheet regions intercalate into the lipid bilayers [80]. It has been shown that oligomer toxicity correlates directly with surface hydrophobicity and inversely with oligomer size, with the most toxic oligomers characterized by high hydrophobicity and small size [81]. Thus, hydrophobicity, β -sheet content, and also α -helical

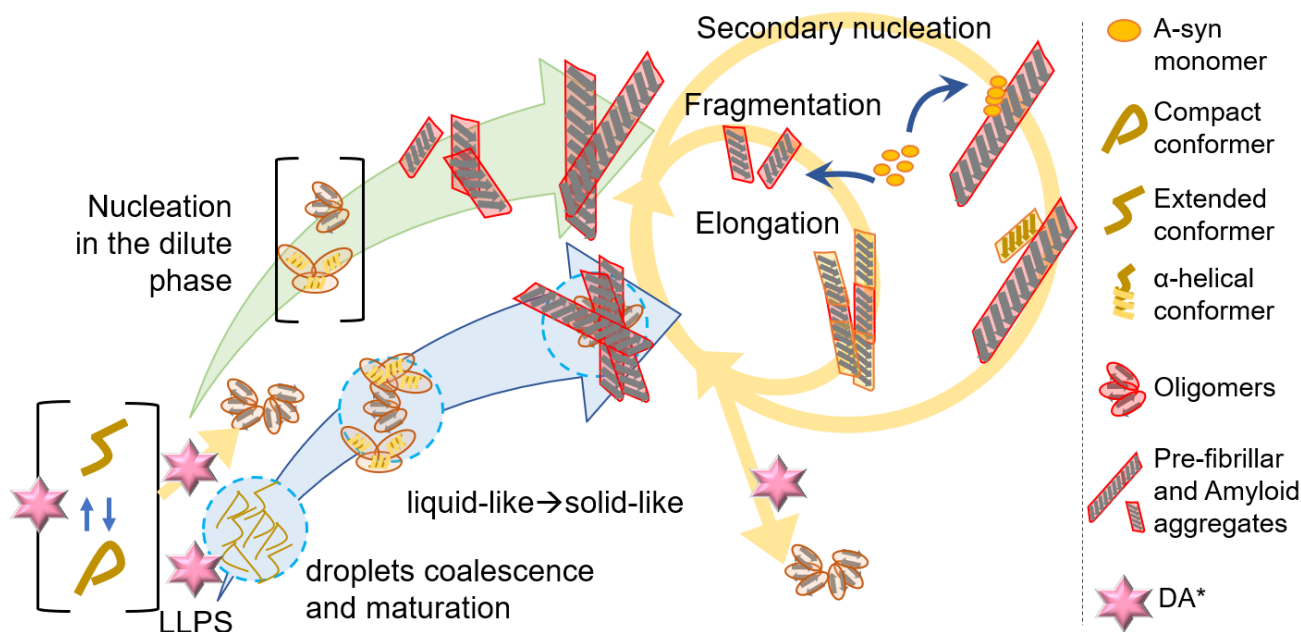


Fig. 4. Aggregation pathways of AS. DA* indicates events known to be modulated (triggered or inhibited) by DA and related molecules. Off-pathway intermediates and amorphous final aggregates are not shown. LLPS, liquid-liquid phase separation.

structures are all relevant in the structure-toxicity relation of AS oligomers [78,80,82]. A high-resolution structural model of membrane-bound AS oligomers, based on solution NMR and cross-linking mass spectrometry, has been recently reported [83]. The formation of early aggregates by primary nucleation events is followed by aggregate elongation, leading to mature fibrils characterized by a cross- β structure and further assembly into LB and LN. Fibril fragmentation and secondary nucleation (i.e., nucleation mediated by the fibril surfaces) contributes to the growth of the fibrillar material [84]. In addition to the prefibrillar species generated by the primary nucleation events, fibrils also represent a source of potentially toxic oligomers, contributing to their heterogeneity [85]. A different pathway of fibril formation has been recently reported *in vitro*, applying 10% of PEG800 as a crowding agent [86]. In this model, AS undergoes LLPS in the nucleation step. During droplets coalescence and maturation, several AS structural changes occur. Initial liquid droplets mainly contain low-molecular weight, disordered AS species (~90%), whose relative amount decreases during maturation, while fibrillar material increases. Oligomeric species were also observed and, interestingly, they displayed a helical structure. In the late stages, mature fibrils were observed within droplets, which can promote secondary nucleation events. These structural changes were accompanied by a liquid-to-solid transition [86].

It has been reported that the nucleation-dependent polymerization process occurs at low AS concentrations, while an increase in protein concentration above a threshold value is necessary, at least locally, for phase transition [86,87]. PD-promoting conditions, such as AS mutations

and PTMs, oxidative stress, exposure to metal ions and pesticides, have been reported to impact on AS aggregation. Under the above-mentioned conditions, to which molecular crowding is also added, a conspicuous lowering of the critical concentration for LLPS could occur [86,87], suggesting the inclusion of PD in the list of so-called condensopathies. Pathogenic AS species deriving from these pathways spread from cell to cell, propagating amyloid aggregation similarly to prions. However, since infectivity of synucleinopathies has not been proved, AS is referred to as a “prion-like protein” or “prionoid”, rather than “prion” [88]. The AS spread is considered an important factor determining PD progression, as supported by the spatial and temporal spreading pattern of AS in the brain at different stages of the disease [89].

Intercellular AS transmission requires two fundamental steps: (1) release of seeding-competent AS species from donor cells and (2) internalization of these protein species by healthy cells where they act as aggregation nuclei for the endogenous AS monomers. Several mechanisms have been reported in the literature. Processes that have been implicated in the first step are: diffusion across intact cell membranes of AS monomers and small oligomers, diffusion through damaged cell membranes, non-classical exocytosis pathways independent of the endoplasmic reticulum (ER)-Golgi apparatus, exosomes, and tunneling nanotubes [88]. Processes that have been reported for the second step are: diffusion across intact cell membranes, diffusion through damaged cell membranes, transmembrane amyloid pore-like channels, the classic endocytosis pathway, exosome-mediated uptake, and receptor-mediated internalization. An in-depth overview of these mechanisms has been recently reported [88].

Different polymorphs and “strains” of AS could arise from these pathways with a potential role in the phenotypic heterogeneity of PD and other synucleinopathies [13,90]. Thanks to solid-state NMR spectroscopy and cryo-electron microscopy (cryo-EM), polymorphic amyloid structures can be described at atomic resolution [91]. AS fibrils obtained *in vitro* using recombinant wild type (WT) and disease-associated mutants display structural differences. These observations and the implication of AS in several distinct neurological disorders support the “strain hypothesis”, where distinct pathogenic AS conformations determine the specific diseases [92]. The AS fibril structures derived from the brains of individuals affected by synucleinopathies, such as MSA, PDD and DLB, have been recently reported [1,93]. AS inclusions from MSA brain are made of two types of filaments (each consisting of two protofilaments) that are different from those of individuals with DLB and from those obtained *in vitro* by recombinant AS [93]. AS filaments from the brains of PD, PDD and DLB patients, instead, are made of a single protofilament [1]. These results [1,93] strongly support the strain hypothesis, indicating the presence of distinct AS assemblies in different synucleinopathies. Cryo-EM structures of AS in presence of lipids shows that phospholipids induce alternative arrangement of protofilaments and fill the central cavities of AS fibrils [94].

In addition to membrane destabilization by oligomers [80], several other mechanisms of AS toxicity have been reported in the literature, including synaptic dysfunction, impairment of the normal quality control systems (molecular chaperones, ubiquitin proteasome system, phagosome/lysosome system), disruption of microtubule dynamics and axonal transport, endoplasmic reticulum/Golgi dysfunction, nucleus malfunction, and microglia activation leading to neuroinflammation [95]. Among these, mitochondrial dysfunction, leading to oxidative stress, is considered one of the main mechanisms of AS-induced toxicity [95]. Indeed, AS affects mitochondrial function by several mechanisms, including impairment of mitochondrial electron transport chain complexes, dysregulation of mitochondrial calcium levels, dysfunction of the mitochondrial quality control systems, and impairment of mitochondrial protein import machinery [95].

5. AS Interactions with DA and Related Molecules

The interest in small molecules able to bind AS is highly motivated by the goal of developing new drugs for synucleinopathies and understanding the mechanisms of disease onset and development. Such studies have been focusing on the assessment of direct or indirect interaction between AS and the ligand, identifying the structural determinants of intermolecular recognition, investigating the effects of ligand binding on protein conformation and aggregation, as well as computational modeling of supramolecular complexes and conformational ensembles.

5.1 Interactions with DA

It has long been recognized that DA and its oxidation products can inhibit and even reverse AS fibrillation *in vitro* and *in vivo*, deviating the aggregation pathway towards spherical, soluble oligomers [96]. This effect does not depend on protein covalent modification and is, rather, associated to protein conformational changes [96,97]. DA oxidation, too, is not absolutely required, as shown by control experiments, in which the effect is retained while inhibiting oxidation by NaBH₄. DA-induced AS oligomers slow down fibrillation in a dose-dependent manner [98]. Circular dichroism (CD) and infrared (IR) spectroscopy indicate that the conformational ensemble of oligomeric AS is shifted in the presence of DA towards a predominantly disordered state with some elements of α - and β -secondary structure, resulting in an average β -sheet content in between that of monomeric and fibrillar structures [96,97]. The role of pH on the effects of DA on AS aggregation has also been investigated [99]. At acidic pH, a condition in which DA is stable, AS undergoes amyloid aggregation regardless of the presence of DA. DA impacts, instead, on AS aggregation at pH >7, an effect that is counteracted by antioxidants (ascorbic acid, glutathione, or homocysteine) and reducing agents (β -mercaptoethanol and dithiothreitol) to different extents, depending on the specific agent [99]. These results support the hypothesis that DA oxidation products are more effective than DA itself in inhibiting AS amyloid aggregation.

In-vitro characterization of serial AS deletion products and competition experiments by designed peptide have led to first identification of the main AS-DA interaction site within the C-terminal pentapeptide ₁₂₅YEMPS₁₂₉ [97]. That this sequence is required *in vivo* for AD-dependent inhibition of AS fibrillation has been demonstrated in SH-SY5Y cells expressing a multiple mutant carrying five amino acid substitutions in the 125–129 region, while controlling catechol levels by the expression of the biosynthetic, rate-limiting enzyme TH [100]. Such an evidence has been later extended to mice and *Caenorhabditis elegans* [26].

MD simulations of AS in aqueous solutions and clustering analysis on experimental NMR structures indicate that DA binds preferentially to the ₁₂₅YEMPS₁₂₉ region and to a few residues of the NAC region, establishing hydrophobic contacts as well as H-bonds [101,102]. Those results also show that the AS-DA complex is stabilized by electrostatic interactions with the E83 residue of the NAC region. Mutation of this residue to alanine impairs fibrillation inhibition by DA, confirming an important role of this interaction in AS affinity for this ligand and hinting for the first time to a non-linear binding site [101]. The AS-DA complex in the different structural clusters appears to fluctuate around rather large average values (16.3–25.8 Å), indicating that the complex maintains large conformational flexibility, in spite of some induced structural compaction

and induced structural order (Fig. 5, Ref. [102]). MD simulations on preformed AS44–96 protofibrils and DA have shown disruption of the Greek-key-like core by hydrophobic and electrostatic interactions at multiple AS-DA interaction sites [103].

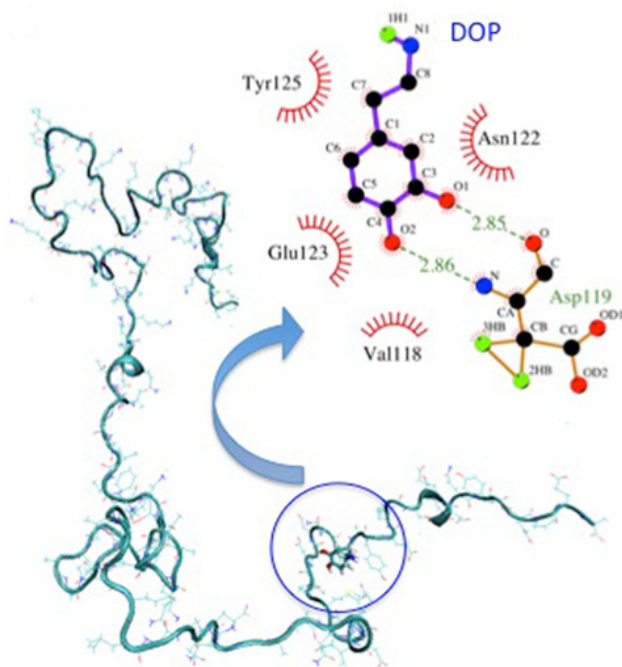


Fig. 5. Representative structure of the AS-DA complex from molecular dynamics (MD) simulations. Hydrogen bonds are represented by dashed lines and hydrophobic interactions by arcs with spokes (adapted with permission from [102]).

In-vivo AS conformational properties and their response to DA concentration have been explored by the FRET-based assay for molecular proximity called fluorescence lifetime imaging microscopy (FLIM) [104]. This technique is based on the observation that the fluorescence lifetime of a donor fluorophore decreases with the 6th power of the distance from a FRET acceptor. Mouse and rat primary neuronal cultures were transfected by a doubly-tagged Myc-aSyn-V5 to probe the intramolecular distance between N- and C-termini by FLIM, based on immunocytochemistry with fluorescent labeled secondary antibodies. A first interesting observation was an inhomogeneous intracellular distribution of AS conformers, with more extended conformations in the cell body and more compact ones in the neurites. Furthermore, DA uptake significantly reduces the average distance, indicating that DA itself or some DA metabolite modulates AS conformational ensemble *in vivo* in favor of partially structured states. No effect was observed with DA agonists or antagonists. Very minor changes in CD spectra have been interpreted as an increase in random-coil and loss of β -structures, but these effects seem hardly significant [104].

Other reports by CD and/or FTIR failed to detect a significant effect of DA on monomeric AS secondary structure in solution [96,105,106].

DA-induced changes in AS conformational ensembles *in vivo* are mirrored by peculiar structural and functional features of the resulting oligomers [26]. Indeed, the oligomeric aggregates that accumulate in mice expressing the human A53T mutant, upon induction of catecholamine production, are larger (up to 122 Å) than in the control system (up to 65 Å) and do not display seeding activity on AS aggregation, as assessed by *in-vitro* Thioflavin T (ThT) assays and analysis of trans-synaptic spreading to motor cortex. Furthermore, AS oligomers formed in the presence of DA are known to be sodium dodecyl-sulfate (SDS)-resistant [100,107] and this feature has been reported to be modulated by the concomitant presence of copper and DA [108]. DA-stabilized oligomers are suspected to have toxic effects by multiple possible mechanisms [109], such as inhibition of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex [110], impairing chaperone-mediated autophagy [111], and causing mitochondrial dysfunction [112]. However, a recent article on SH-SY5Y cells and rat synaptosomes reports negligible toxicity of purified AS oligomers formed in the presence of DA [98].

In line with previous evidence, it has been shown that DA counteracts the effect of psychosine on AS conformation and aggregation *in vitro* [113]. Psychosine promotes AS fibrillation in ThT assays and has a fingerprint in ^{15}N AS Heteronuclear Single Quantum Coherence (HSQC) NMR spectra, consistent with interactions with the C-terminal domain of the protein. AS incubation with both ligands abolishes these effects, although no comparison with the effect of DA alone is reported. The authors of that study speculate about interconversion between an “open” and a “closed” AS conformations, where the former (fibrillation-competent) would be stabilized by psychosine and the latter by DA.

Further NMR experiments have recently pointed out that ^{15}N AS HSQC spectra are very sensitive to pH changes and that previous AS-ligand binding studies might be exposed to the risk of false-positive results, due to small pH changes induced by the components of commercial ligand formulations, rather than by ligand binding [114]. In particular, DA-induced changes in ^{15}N AS HSQC spectra become evident only at pH 5 and not at pH 7, upon subtraction of the pH effect, under the experimental conditions employed in that study.

Native MS can provide important additional information in the attempt to describe disordered conformational ensembles [115,116]. This technique captures conformational components coexisting in the original liquid sample by their different ionization propensity under mild electrospray conditions [117,118]. The possibility to preserve non-covalent interactions, along with conformation-dependent

protein ionization, makes it possible to depict conformational ensembles, discriminating conformational states by structural compactness. At the same time, non-covalent complexes are detected by their specific mass, providing a unique possibility to gather combined information on protein folding and binding [119,120]. Native MS on AS in aqueous solutions detects four main conformational components based on charge-state distributions [121,122]. These are referred to as compact, extended and intermediate (I and II), by comparison to the reference behavior of folded and unfolded proteins [118].

Native-MS analysis of AS-DA interactions by titration experiments at pH 7.4 [122] provides direct experimental evidence of the formation of non-covalent complexes and shows that no chemical modification of protein or ligand takes place in the time scale of such experiments. DA binds preferentially to a partially structured AS conformer with stoichiometry up to 1:4 and sub-millimolar apparent K_D , and slightly remodels the AS conformational ensemble by stabilizing the intermediate. Ligand footprinting [122] by electron-transfer dissociation (ETD) of the 1:1 complex locates the main interaction site in the $_{127}\text{MPSEE}_{131}$ region, in agreement with the above reported evidence. The apparent discrepancy with the NMR study failing to detect significant binding at pH 7 [114] could be explained by the peculiar properties of MS, which escapes averaging over the molecular population and, rather, describes folding and binding status of each sorted ion in the spectrum. This property makes MS more similar to single-molecule than to bulk spectroscopy methods [105], offering peculiar sensitivity and deepness in the description of heterogeneous samples. It is interesting to note that, at 1 mM DA and 20 μM AS, ~75% of the 15+ charge state (representing the intermediate state in the native MS spectrum) is bound to DA, while only ~29% of the 8+ charge state (representing the compact state) is bound [122]. In that study, the pH of the DA stock solution was controlled after DA addition [122]. Also relevant to this discussion is the large amount of evidence that rules out uncontrolled pH changes during nano-electrospray of buffered aqueous solutions [123,124].

Another biophysical method to explore conformational ensembles is single-molecule force spectroscopy (SMFS), in which individual molecules in solution are unfolded mechanically by the cantilever of an atomic force microscopy (AFM) instrument, relating pulling force to the structural features of the molecule [125]. By randomly reiterating this procedure, it is possible to characterize the statistical distribution of distinct protein conformers. Three main conformational states of AS in buffered solutions at pH 7.4 can be detected by this method based on the strength of the intramolecular interactions [105,126,127]. The addition of DA shifts the conformational ensemble towards the strong interactions, with loss of the fully unstructured component [105]. These interactions must be at the level of tertiary structure, since the secondary structure content

remains unaltered, as discussed above. The structural interpretation of MS and SMFS data has been corroborated by comparison to structural clusters obtained from a 73 μs -long MD simulation of the AS conformational ensemble in solution [128,129].

AS-DA binding at near-neutral pH has been detected by other orthogonal techniques, such as isothermal titration calorimetry (ITC) and nanopore-permeability analysis. One ITC study at pH 7.8 indicates relatively weak binding, with an estimated dissociation constant of about 280 μM [106]. On the other hand, another ITC study reports too weak binding at pH 7.4 to be quantitatively evaluated [130]. Apparent dissociation constants in the sub-millimolar or millimolar range can be considered biologically relevant, since DA concentrations are of the micromolar order in the cytosol and reach the millimolar range within storage vesicles and striatal nerve terminals [99]. Nanopore-permeability assays provide additional evidence of AS-DA interaction by detecting changes in the frequency distribution of pore translocation or bumping events for the protein in solution, before and after the addition of the ligand [106]. Translocation events are only considered possible for molecules in random-coil conformation, while folded or partially folded structures, instead, bumping away on the *cis* side of the membrane [131,132]. According to this interpretation, DA would stabilize a partially folded intermediate in WT AS, while stabilizing the random-coil state in the A30P mutant. Although the results are not of straightforward interpretation in terms of conformational effects, they suggest direct interaction between AS and DA [106]. Thus, the available data suggest that DA itself can bind to AS and affect its aggregation. DA oxidation would not be an absolute requirement, although, as described below, some DA metabolites, such as DA o-quinone and DOPAC, are more effective [97,100]. Further studies are needed to understand whether apparently conflicting reports in the literature can be interpreted and reconciled by the peculiarities of the different biophysical approaches.

5.2 Interactions with DA Agonists and Analogs

In order to find AS ligands that can inhibit AS fibrillogenesis and toxicity, several Authors have studied the interactions of this protein with DA-related molecules (catabolites, agonists, and analogs). DA analogues are molecules that structurally mimic DA, while DA “agonists” and “antagonists” are molecules able to activate or inactivate the biological response mediated by DA receptors.

One of the first systematic search for direct interactors has been carried out by *in-silico* screening of 70 compounds and led to the identification of 5 DA analogs that, similarly to 6-aminoindole, are expected to affect the nucleation/polymerization mechanism and possibly to bind oligomers [133] (Fig. 6). *In-vitro* assays by ThT and AFM has showed that 6-aminoindole and 5-hydroxyindole have the strongest inhibitory effects on AS aggregation, while

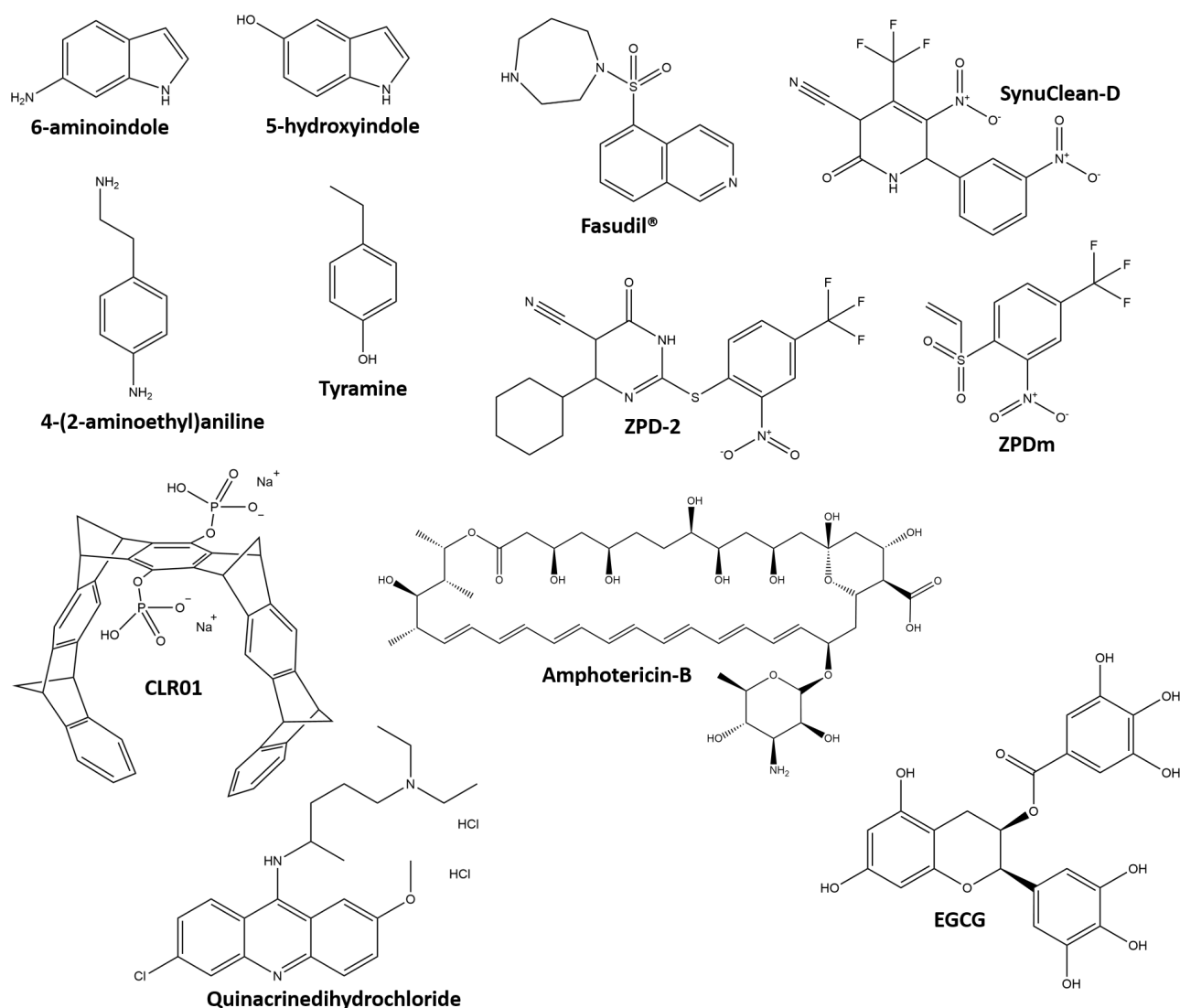


Fig. 6. Structural formulas of the DA analogs mentioned in the text. EGCG, epigallocatechin.

tyramine and 4-(2-aminoethyl)aniline have the poorest effects [133]. Structural comparison between tyramine and DA suggests that the loss of just one of the -OH groups (replaced by -H in tyramine) causes a dramatic loss in anti-fibrillogenic activity. It has been hypothesized that a benzene ring with two -OH or quinone groups (i.e., 1,2-benzoquinone moieties) may have great potential in AS binding. Stacking interactions and charge interactions appear to be relevant in determining the interaction with either soluble or aggregated AS. Below, DA analogs showing these key structural motifs are discussed. An example of DA analog directly interacting with soluble AS is provided by Fasudil®, an isoquinoline substituted by a (1,4-diazepan-1-yl)sulfonyl group at position 5 (Fig. 6). It also acts as an inhibitor of Rho kinases and serendipitously proved to have antifibrillogenic activity. Its use has been approved by the U.S. Food and Drug Administration (FDA) in clinical trials of disorders such as Raynaud's disease, atherosclerosis, and age-related or neurode-

generative diseases [134]. Direct interaction of Fasudil® with AS has been primarily demonstrated by NMR [135] and analyzed by MD simulations [136]. These studies offer an atomic-level description of a possible inhibition mechanism. Aromatic stacking and electrostatic interactions are formed through a process termed *dynamic shuttling*, in which one set of interactions breaks before another is formed nearby [136]. The interactions preferentially involve the C-terminal region of AS, which remains highly dynamic upon binding. Beside the tyrosine-glutamate (YE) residues $_{136}\text{YE}_{137}$, also the $_{125}\text{YE}_{126}$ pair appears to be involved in these interactions, although with lower affinity likely depending on the sequence context. The binding of Fasudil® in the C-terminal region may screen protein-protein electrostatic attractions or sterically prevent interactions between monomers.

The category of molecules that bind and dissolve aggregated AS includes three molecules recently isolated by *in-vitro* high-throughput screening based on ThT flu-

orescence, light-scattering and transmission electron microscopy (TEM), applied to a library of more than 14,000 compounds [137] (Fig. 6). These are named SynuClean-D [65,138], ZPD-2 [139] and ZPDm [140], and proved effective even at sub-stoichiometric concentrations in disaggregating mature fibrils of WT AS and familial variants A30P and H50Q. All three compounds are also able to reduce the aggregation of AS in PD models of *Caenorhabditis elegans* but do not interact with soluble AS, as indicated by NMR studies [140,141] (Fig. 6). Unfortunately, no structural data are available on the atomic details of the interactions between these disaggregating agents and AS fibrils. Although the three molecules have similar effects, they might have fine differences in their mechanism of action reflecting their structural differences.

For the purpose of drawing some rules about the structure and function of disaggregating molecules, SynuClean-D has been compared with diverse and much more complex compounds, such as EGCG [142] and the molecular tweezer CLR01 [143,144]. The hypothesis underlying this comparison is that greater structural complexity and larger hydrophobic contact surface (i.e., larger number of aromatic rings) lead to stronger effects in fibril disaggregation [145]. In fact, SynuClean-D, EGCG, and CLR01 contain, respectively, 2, 3, and 5 aromatic rings and this ranking also reflects their disaggregation efficacy (CLR01 > EGCG > SynuClean-D). Nonetheless, it has been observed that small, single-ring molecules can still be highly effective as disaggregating agents [145], while more complex molecules, such as EGCG and CLR01 can have pleiotropic effects combining fibril disaggregation with the ability to directly bind soluble AS and modulate its aggregation pathways, thus reducing the quantity and toxicity of aggregates [146–149]. This is also reflected in an enhanced ability to attenuate cytotoxic effects *in vivo* [143,150]. Furthermore, the effects of polyphenolic molecules may also derive from an indirect protective action against oxidative stresses due to chelation of metal ions [150–152]. Comparative structural and functional analyses of DA, amphotericin-B, EGCG, and quinacrine dihydrochloride unfortunately confirm our current inability to extract a general model, relating binding affinity and fibril inhibition to chemical structures [150]. The interactions of these compounds to soluble or fibrillar AS, analyzed by NMR and surface plasmon resonance (SPR), show differences in strength and position, with no evident structure–function relationship [150].

DA agonists include molecules that are commonly used to relieve symptoms of PD. Fig. 7 shows the structure of some DA agonists [153,154]. Among them, the neuroprotectant cabergoline has been suggested to directly interact with AS by molecular docking experiments [155]. Pergolide and bromocriptine have long been recognized by *in vitro* ThT assays to also destabilize preformed fibrils by direct interaction [156,157]. A small group of agonists can be considered bifunctional, in that they also exhibit the ability

to modulate AS aggregation by either direct interaction, or by affecting its PTMs and/or turnover [158]. Carbazole-based DA agonists are a recent example of development of such kind of multifunctional drugs [159]. Bifunctional molecules have been designed *ad hoc* by introducing hydroxyl groups into the structure of known agonists, so that their efficacy on the signaling cascade is not altered and the ability to interact directly with AS is newly conferred. Compounds such as D-519 and D-520 (Fig. 7) prove effective in reducing symptoms in *Drosophila melanogaster* and rat models of PD, having maintained their efficacy as strong agonists of D2/D3 receptors, and showing new properties derived from their ability to directly interact with AS [160]. In particular, D-520 can reduce the toxicity of preformed AS aggregates against rat pheochromocytoma cell cultures and modulate AS aggregation, as demonstrated *in vitro* by ThT and TEM [160]. Apomorphine (Apo) is a non-ergoline DA agonist, whose dual function is a downside. Apo binds with high affinity to DA receptors D2, D3, and D5, and can also directly interact with AS [160]. Apo was approved by the FDA in April 2004 for the treatment of hypomobility and dyskinesia associated with PD. Apo inhibits fibrillation and dissolves pre-formed fibrils, as assessed by ThT assays and TEM. Under oxidizing conditions, off-pathway oligomeric adducts, Apo-AS-O, are formed. AS oligomerization is faster in the presence of Apo than DA. Apo-derived AS oligomers are toxic to cultured neuronal cells, and form by recruiting innocuous AS monomers. NMR 1H-15N-HSQC experiments show that Apo binds monomeric AS in the $_{125}YEMPS_{129}$ region, as observed for other oxidized catechols. Apo establishes sufficiently stable hydrophobic interactions with monomeric AS to resist dilution during size exclusion chromatography (SEC) [160]. Further studies are needed to understand whether neurotoxic effects of Apo can also occur *in vivo* [160]. Given that anti-PD drugs can produce toxic intermediates, it is appropriate to reason on their role and the rationale behind their design. Indeed, the formation of specific neurotoxic intermediates, or even the persistence of oligomeric forms derived from fibril disgregation, can have a high neurotoxic potential, and hence should be considered as undesirable effects. Instead, it is desirable to develop drugs that reduce fibrillogenetic potential by inhibiting the formation of oligomers, as part of a general preventive and prophylactic strategy to be adopted throughout life, rather than drugs to be used at more advanced stages of a synucleinopathy, when oligomers or fibrils have already formed.

5.3 Interactions with DOPAL

The interaction of AS with DOPAL, the immediate metabolite of DA catabolism, has been proposed as a key factor in PD pathogenesis in the so-called catecholaldehyde hypothesis [32]. The aldehyde group generated by MAO-mediated conversion of the DA amine group makes DOPAL capable of quinonizing AS, forming co-

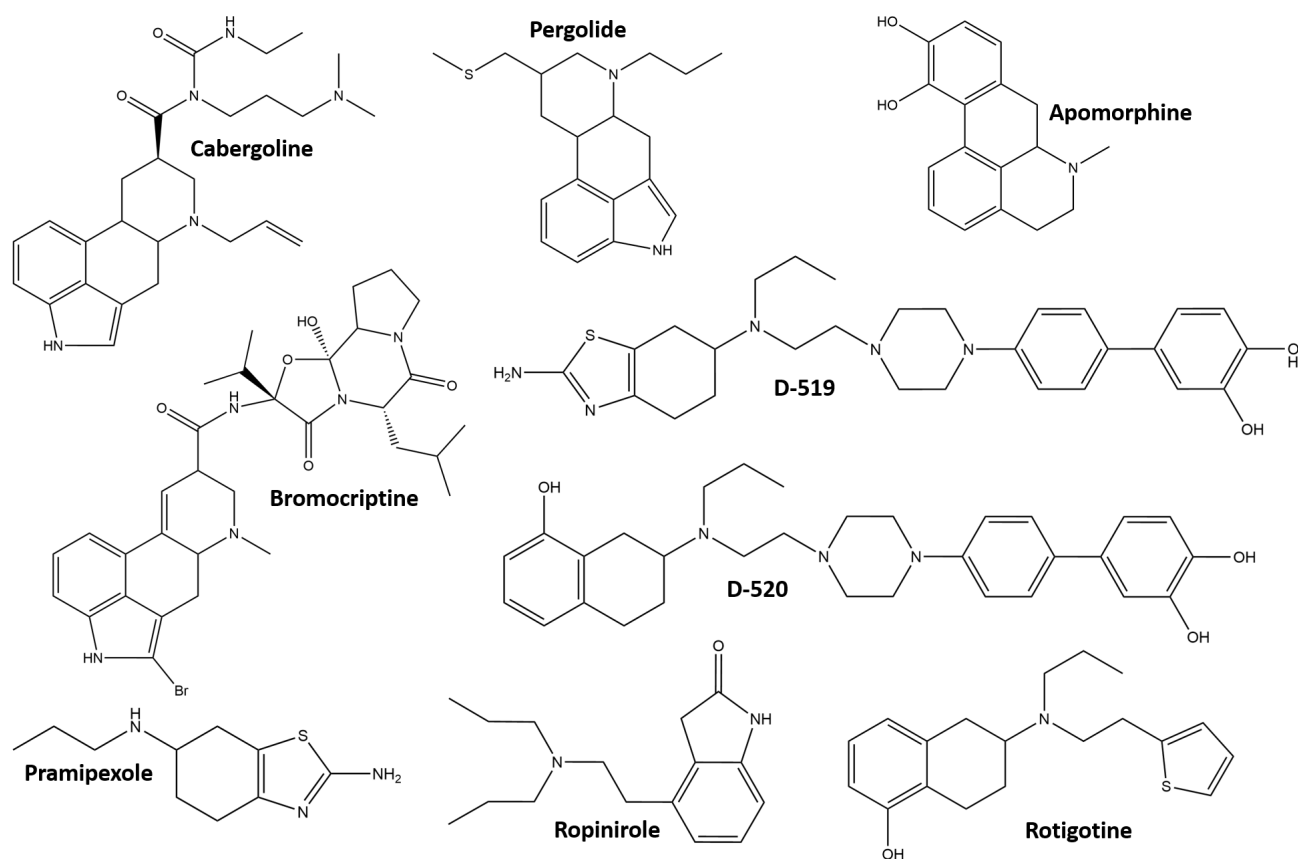


Fig. 7. Structural formulas of the DA agonists mentioned in the text.

valent adducts to lysine residues or to the N-terminus by Schiff base or Michael addition, or generating a dicatchol pyrrole lysine structure [161]. ThT fluorescence experiments have shown that DOPAL destabilizes preformed AS fibrils and inhibits aggregation, generating off-pathway oligomers [162]. According to near-infrared fluorescence spectroscopy and western-blotting experiments, DOPAL induces the formation of AS oligomers more effectively than DA [163]. DOPAL-induced AS quinonization and oligomerization are enhanced in the presence of Cu(II) ions [163]. Despite some contrasting evidence [164], a consensus is emerging in the literature that many DOPAL-induced oligomers are toxic and compromise several physiological processes in neurons [165]. Blocking the DOPAL aldehyde moiety by compounds containing primary (e.g., aminoindan) or secondary (e.g., rasagiline) amines abrogates its effects on protein aggregation and cytotoxicity [165].

AS oligomers generated by DOPAL have been investigated by SEC-HPLC [166]. Detected species have been classified, according to their size, in small (dimers and trimers) and large (tens of subunits) oligomers. The relative amounts of these species are finely tuned by several factors (mutations, PTMs, etc.). Among them, DOPAL-induced oxidation of methionine residues has been shown to favor the accumulation of small oligomers, hampering the formation of larger species [166,167]. In particular, the oxidation of the C-terminal methionine at position 127 improves the

Ability of AS to scavenge ROS derived from DOPAL and prevents the assembly of large neurotoxic oligomers [167].

DOPAL-mediated oligomerization is also influenced by familial PD point mutations of AS. In particular, the A53T, E46K and H50Q variants of AS have been shown to stimulate formation of large ($n > 3$) rather than small ($n = 2-3$) oligomers upon the incubation with DOPAL [163,168]. DOPAL-induced large oligomers have been associated with enhanced toxicity [166]. N-terminal acetylation and binding to phospholipid vesicles counteracts DOPAL-induced oligomerization, as shown by denaturing electrophoresis and SEC experiments [168].

5.4 Interactions with DOPAC

The DA metabolite DOPAC is the product of DOPAL detoxification by aldehyde dehydrogenase conversion, and it is usually rapidly removed by active transport from the cell *via* a sulfonylurea-sensitive transporter [169]. As its precursor DOPAL, it can undergo spontaneous oxidation to a quinone, thus producing H_2O_2 , a reactive molecule able to disturb the redox balance [170].

It has been observed *in vitro* that DOPAC at physiologically relevant concentrations binds non-covalently to AS and stabilizes the oligomeric state, hindering fibrillogenesis [170]. Because of the oxidative instability of the compound, the molecular mechanism could not be unveiled, but

this effect might be due to an alteration of the oligomer interface that plays a fundamental role in fibril formation. The interaction has been mapped by mutagenesis to the same N-terminal region that binds lipid vesicles [163]. At high molar excess (i.e., DOPAC:AS >3:1), DOPAC forms covalent adducts with AS, as also seen for DOPAL [170]. A following study demonstrated that DOPAC binding also decreases the ability of AS to interact with lipids, thus reducing its functionality [171]. Furthermore, AS/DOPAC oligomers showed a reduced propensity to conformational changes upon interaction with cellular membranes [172]. DOPAC-AS interaction has an influence also on the redox state of both molecules. Indeed, AS speeds up the spontaneous oxidation of DOPAC, whereas the oxidized form of DOPAC (the quinone derivative) leads to oxidation of AS methionine residues, probably due to H₂O₂ generation, as monitored by MS along an *in-vitro* kinetic assay [170]. AS oxidation has been reported to promote the formation of oligomers, rather than fibrils, *in vitro* [173]. The addition of catalase as an H₂O₂ scavenger prevents AS oxidation but does not affect AS aggregation properties, as assessed by ThT, TEM and SEC. These results suggest that the oxidation of AS methionines is not required for oligomerization and inhibition of fibrillogenesis by DOPAC [172].

5.5 Interactions with DOPET

As discussed in the previous section, DOPAL is further oxidized by mitochondrial aldehyde dehydrogenase to DOPAC, in the major oxidation pathway of DA (Fig. 2). In a minor pathway (under normal conditions), DOPAL can be, instead, reduced by alcohol dehydrogenase (ADH) to 3,4-dihydroxyphenylethanol (DOPET), also called hydroxytyrosol (Fig. 2). In addition to this endogenous source as DA metabolite, DOPET can have a dietary origin, being found in olive oil and wine. Moreover, DOPET can be produced from the hydrolysis of olive oil secoiridoids, such as oleuropein. Noteworthy, DOPET is able to cross the blood-brain barrier and exhibits antioxidant, anti-inflammatory, and anti-aggregation activities [174].

In a first *in-vitro* study [175], DOPET was found to inhibit AS aggregation and to destabilize preformed AS fibrils, as evaluated by ThT and TEM investigations. In particular, DOPET inhibits AS fibrillogenesis, leading to the formation of small amorphous aggregates. Moreover, DOPET was found to counteract AS-induced toxicity on PC12 cells [175].

The effects of DOPET on AS aggregation have been elucidated by employing complementary biochemical and biophysical approaches, including ThT assay, CD spectroscopy, TEM, native MS, limited proteolysis, intrinsic fluorescence, SEC and electrophoretic analyses [176]. ThT assay and TEM show that a 1:1 DOPET:AS ratio completely inhibits AS fibrillogenesis, leading to the formation of AS oligomers, which appear structurally disordered as indicated by CD spectroscopy. The CD analyses also

suggested that DOPET does not affect the secondary structures of monomeric AS. Chromatographic, TEM, and MS analyses of AS treated with DOPET showed the presence of spherical oligomers and that of protein species containing one-to-four oxidations of methionine residues, whose speed of formation and extent of oxidation were related to DOPET concentrations. Covalent adducts, likely involving the ϵ -amino group of lysine residues and the aromatic ring of DOPET, were also observed. Limited-proteolysis experiments have revealed similar fragment patterns at the beginning of the incubation with and without DOPET [176]. After longer incubation, AS alone displays a reduced susceptibility to proteolysis, in agreement with the formation of AS fibrils, while the protein species formed in the presence of DOPET are more susceptible to proteolysis. A slight protection to proteolysis of the C-terminal region was observed in the monomeric protein in the presence of DOPET, suggesting that this region could be involved in the AS-DOPET interaction [176]. The effects of DOPET on AS aggregation were also studied in the presence of catalase, to inhibit the methionine oxidation induced by DOPET. DOPET retained the anti-fibrillogenic activity also in the absence of AS oxidation [176]. The presence of DOPET during AS aggregation remarkably decreases AS cytotoxicity on SH-SY5Y human neuroblastoma cells, as measured by the MTT test. Since aggregated AS enhances intracellular ROS and binds the cell membranes at monosialotetrahexosylganglioside 1 (GM1)-enriched sites, both events were tested in the presence of DOPET. DOPET-induced AS aggregates displayed reduced ROS and membrane GM1 binding, in comparison to AS aggregates obtained in the absence of the compound. Overall, this study showed that DOPET interacts with AS by non-covalent and covalent interactions and redirects protein aggregation to the formation of not-toxic spherical oligomers and amorphous aggregates, which appear to be off-pathway of fibrils formation [176].

The interactions of DOPET and DOPAC with WT AS and the E46K pathological mutant have been compared [177]. Both compounds partially or completely inhibit fibril formation at 1:1 or 1:5 protein:catechol ratio, respectively. In the absence of catechol, WT and E46K formed fibrils with a different morphology, as observed by TEM. DOPAC at 1:5 protein:catechol ratio induced the formation of spherical and annular oligomers of 10–20 nm and 20–40 nm for E46K and WT AS, respectively. In the presence of DOPET, mainly amorphous structures were observed under the tested experimental conditions. The formation of oligomeric species was also investigated by SEC, which showed additional peaks due to protein dimers and trimers in the presence of each compound. A higher fraction of oligomeric species was observed in the early stages of incubation for E46K, both in the absence and the presence of catechols, compared to WT AS. CD spectroscopy showed that the off-pathway species induced by DOPET and DOPAC are characterized by a predominant random-

coil structure, similar to monomeric AS. In all the above investigations, DOPET appeared less active in affecting AS fibrillogenesis compared to DOPAC and the E46K mutant appeared less affected by catechols compared to the WT AS [177].

Important structural details of the catechol effects on AS fibrillogenesis were obtained by SEC, native-MS and hydrogen-deuterium isotope exchange (HDX)-MS. At the beginning of the incubation in the absence of catechols, E46K eluted at a slightly higher volume in SEC compared to WT AS suggesting increased compactness of the mutant. In the presence of DOPAC or DOPET, E46K eluted slightly earlier, suggesting a stabilization of a more extended conformer. On the contrary, catechols did not affect the retention time of the WT protein. Native-MS indicated the presence of multiple protein conformers with different compactness at the beginning of incubation, in agreement with other studies [177]. The addition of DOPAC shifted the equilibrium towards a more extended conformer, an effect that was more evident for the WT protein. Compared to DOPAC, the effect of DOPET was less evident on E46K and not significant in the case of WT AS. The most remarkable effects of both catechols were observed by native-MS at 48 hours of incubation for E46K and WT AS [177]. ThT assays and TEM indicated that DOPAC, and DOPET to a minor extent, were able to disaggregate intermediates and mature E46K aggregates obtained in the absence of the compounds. This effect was less evident in the case of mature WT AS aggregates.

On the basis of these results [176,177], the Authors proposed the following model for the effects of the two catechols on AS aggregation. Catechol binding affects the conformational equilibrium of monomeric AS, stabilizing a conformer that is less prone to convert into amyloid fibrils. These extended monomers assemble into not-toxic, off-pathway oligomers and amorphous aggregates. AS oxidation is not required for DOPET inhibition of AS fibril formation [176,177].

6. Concluding Remarks

DA exerts its toxicity through multiple mechanisms related to the products of its catabolism and to direct AS binding. DA binding to AS monomers is associated with conformational changes in the protein that redirect the aggregation pathway towards spherical, soluble, SDS-resistant, and toxic oligomers. DA also induces oxidation of methionine residues, although interaction with AS and conformational effects occur even in the absence of covalent protein modifications and DA oxidation. At the same time, DA is also capable of disassembling amyloid aggregates of AS, resulting in protein oligomers. Most of the DA related molecules discussed in this review are able to affect AS fibrillogenesis by non-covalent interactions -with the $_{125}YEMPS_{129}$ region in the majority of the cases- and by promoting methionine oxidation.

The study of DA analogs suggests a predominant role of π - π stacking and electrostatic interactions in disaggregating effects on preformed fibrils and anti-aggregating activity on soluble AS, hinting to the possibility of combining these two activities in more complex molecules. Among the DA metabolites, DOPAL is believed to play the most critical role in PD pathogenesis [165]. DOPAL can quinonize AS, induce oxidation of methionine residues, and promote the accumulation of small neurotoxic oligomers of AS more effectively than DA. On the other hand, DOPAC and DOPET redirect AS fibrillogenesis towards off-pathway, non-toxic aggregates. The microenvironment constituted by the presynaptic neuronal cytoplasm contains high concentrations of DA and its metabolites. This group of small molecules may play both a protective role by interacting directly with soluble AS and a cytotoxic role mediated by interaction with its oligomers. Structural studies on the complexes formed by AS with these ligands help us understand the determinants of molecular recognition and to rationally design new drugs active on specific protein variants, at specific stages of fibrillogenic progression.

The available treatments for PD are directed to symptoms without significant effects on the molecular mechanisms of disease progression. Strategies to directly target AS are under investigation, such as small molecules inhibiting the accumulation of toxic AS species or immunoglobulins promoting the clearance of AS aggregates. Relevant to this regard is the development of ligands that are selective for AS toxic species, such as nanobodies [178] and peptides [179]. Detailed characterization of the binding properties of several antibodies undergoing clinical trials for PD has been performed. Most of them bind to the AS residues 102-130, which are close to or overlapping with the $_{125}YEMPS_{129}$ binding site of DA and related molecules [180]. Clinical trials on two antibodies directed against AS aggregates (Cinpanemab and Prasinezumab) unfortunately have shown no significant difference in the clinical assessment of disease progression compared with placebo [181].

In this scenario, information on the molecular mechanisms of AS aggregation and AS interaction with DA and related molecules may provide new therapeutic strategies. Indeed, it has become evident how these molecules lead to oligomers and end-point aggregates with diverse structure and toxicity. Molecules that counteract both fibrillogenesis and toxicity can be the starting point for designing new drugs. The comparison of toxic and non-toxic oligomers can provide the rationale for identifying new targets for immunotherapy.

AS existence as conformational ensembles and their variegated response to the environment represents a crucial aspect of the structure-function relationship of this protein and a major experimental challenge. Advances in experimental and computational methods are bringing us closer to the goal of modeling at atomic resolution such a difficult target in the presence or absence of ligands. Particularly

relevant to this regard is the contribution of cryo-EM to the characterization of AS fibril polymorphism in the presence or absence of heparin, providing a rationale for drug design [182]. Another central challenge is to translate structural information into *in-vivo* protein activity. Such an in-depth understanding is required for the design of new therapeutic strategies based on small molecules or antibodies, targeting the conformers with the most pronounced fibrillogenic potential.

Author Contributions

AN and RG designed the study. AN, SB, EP, CS, and RG contributed to writing the original draft and preparing the figures. AN, SB, EP, CS, and RG contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest. Given her role as Guest Editor of the journal, SB had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Fabio Moda.

References

- [1] Yang Y, Shi Y, Schweighauser M, Zhang X, Kotecha A, Murzin AG, *et al.* Structures of α -synuclein filaments from human brains with Lewy pathology. *Nature*. 2022; 610: 791–795.
- [2] Mahul-Mellier AL, Burtscher J, Maharjan N, Weerens L, Croisier M, Kuttler F, *et al.* The process of Lewy body formation, rather than simply α -synuclein fibrillization, is one of the major drivers of neurodegeneration. *Proceedings of the National Academy of Sciences of the United States of America*. 2020; 117: 4971–4982.
- [3] Sharma M, Burré J. α -Synuclein in synaptic function and dysfunction. *Trends in Neurosciences*. 2023; 46: 153–166.
- [4] Kulkarni A, Preeti K, Tryphena KP, Srivastava S, Singh SB, Khatri DK. Proteostasis in Parkinson's disease: Recent development and possible implication in diagnosis and therapeutics. *Ageing Research Reviews*. 2023; 84: 101816.
- [5] Martinez-Banaclocha M. Proteomic Complexity in Parkinson's Disease: A Redox Signaling Perspective of the Pathophysiology and Progression. *Neuroscience*. 2021; 453: 287–300.
- [6] Mor DE, Daniels MJ, Ischiropoulos H. The usual suspects, dopamine and alpha-synuclein, conspire to cause neurodegeneration. *Movement Disorders*. 2019; 34: 167–179.
- [7] Ma LY, Liu GL, Wang DX, Zhang MM, Kou WY, Feng T. Alpha-Synuclein in Peripheral Tissues in Parkinson's Disease. *ACS Chemical Neuroscience*. 2019; 10: 812–823.
- [8] Magalhães P, Lashuel HA. Opportunities and challenges of alpha-synuclein as a potential biomarker for Parkinson's disease and other synucleinopathies. *NPJ Parkinson's Disease*. 2022; 8: 93.
- [9] Wolgin M, Zobernig M, Dvornyk V, Braun RJ, Kielbassa AM. Systematic Review on Saliva Biomarkers in Patients Diagnosed with Morbus Alzheimer and Morbus Parkinson. *Biomedicines*. 2022; 10: 1702.
- [10] Muddaloor P, Farinango M, Ansary A, Dakka A, Nazir Z, Shamim H, *et al.* Prospect of Alpha-Synuclein (A-Syn) Isolation From Saliva as a Promising Diagnostic Biomarker Alternative in Parkinson's Disease (PD): A Systematic Review. *Cureus*. 2022; 14: e29880.
- [11] Ponzini E, Ami D, Duse A, Santambrogio C, De Palma A, Di Silvestre D, *et al.* Single-Tear Proteomics: A Feasible Approach to Precision Medicine. *International Journal of Molecular Sciences*. 2021; 22: 10750.
- [12] Ponzini E, Santambrogio C, De Palma A, Mauri P, Tavazzi S, Grandori R. Mass spectrometry-based tear proteomics for noninvasive biomarker discovery. *Mass Spectrometry Reviews*. 2022; 41: 842–860.
- [13] Koga S, Sekiya H, Kondru N, Ross OA, Dickson DW. Neuropathology and molecular diagnosis of Synucleinopathies. *Molecular Neurodegeneration*. 2021; 16: 83.
- [14] Giuliano C, Cerri S, Cesaroni V, Blandini F. Relevance of Biochemical Deep Phenotyping for a Personalised Approach to Parkinson's Disease. *Neuroscience*. 2023; 511: 100–109.
- [15] Cazzaniga FA, De Luca CMG, Bistaffa E, Consonni A, Legname G, Giaccone G, *et al.* Cell-free amplification of prions: Where do we stand? *Progress in Molecular Biology and Translational Science*. 2020; 175: 325–358.
- [16] Hawkes CH, Del Tredici K, Braak H. Parkinson's disease: the dual hit theory revisited. *Annals of the New York Academy of Sciences*. 2009; 1170: 615–622.
- [17] Hawkes CH, Del Tredici K, Braak H. Parkinson's disease: a dual-hit hypothesis. *Neuropathology and Applied Neurobiology*. 2007; 33: 599–614.
- [18] Braak H, Rüb U, Gai WP, Del Tredici K. Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *Journal of Neural Transmission*. 2003; 110: 517–536.
- [19] Wang RS, Oldham WM, Maron BA, Loscalzo J. Systems Biology Approaches to Redox Metabolism in Stress and Disease States. *Antioxidants & Redox Signaling*. 2018; 29: 953–972.
- [20] Araki K, Yagi N, Aoyama K, Choong CJ, Hayakawa H, Fujimura H, *et al.* Parkinson's disease is a type of amyloidosis featuring accumulation of amyloid fibrils of α -synuclein. *Proceedings of the National Academy of Sciences of the United States of America*. 2019; 116: 17963–17969.
- [21] Baba M, Nakajo S, Tu PH, Tomita T, Nakaya K, Lee VM, *et al.* Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *The American Journal of Pathology*. 1998; 152: 879–884.
- [22] den Jager WA. Sphingomyelin in Lewy inclusion bodies in Parkinson's disease. *Archives of Neurology*. 1969; 21: 615–619.
- [23] Gai WP, Yuan HX, Li XQ, Power JT, Blumbergs PC, Jensen PH. In situ and in vitro study of colocalization and segregation of alpha-synuclein, ubiquitin, and lipids in Lewy bodies. *Experimental Neurology*. 2000; 166: 324–333.
- [24] Shahmoradian SH, Lewis AJ, Genoud C, Hench J, Moors TE, Navarro PP, *et al.* Lewy pathology in Parkinson's disease consists of crowded organelles and lipid membranes. *Nature Neuroscience*. 2019; 22: 1099–1109.
- [25] Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Pro-*

- ceedings of the National Academy of Sciences of the United States of America. 1998; 95: 6469–6473.
- [26] Ma L, Gholam Azad M, Dharmasivam M, Richardson V, Quinn RJ, Feng Y, *et al.* Parkinson's disease: Alterations in iron and redox biology as a key to unlock therapeutic strategies. *Redox Biology*. 2021; 41: 101896.
- [27] Wise RM, Wagener A, Fietzek UM, Klopstock T, Mosharov EV, Zucca FA, *et al.* Interactions of dopamine, iron, and alpha-synuclein linked to dopaminergic neuron vulnerability in Parkinson's disease and Neurodegeneration with Brain Iron Accumulation disorders. *Neurobiology of Disease*. 2022; 175: 105920.
- [28] Mor DE, Tsika E, Mazzulli JR, Gould NS, Kim H, Daniels MJ, *et al.* Dopamine induces soluble α -synuclein oligomers and nigrostriatal degeneration. *Nature Neuroscience*. 2017; 20: 1560–1568.
- [29] Srinivasan E, Chandrasekhar G, Chandrasekar P, Anbarasu K, Vickram AS, Karunakaran R, *et al.* Alpha-Synuclein Aggregation in Parkinson's Disease. *Frontiers in Medicine*. 2021; 8: 736978.
- [30] Sivakumar P, Nagashanmugam KB, Priyatharshni S, Lavanya R, Prabhu N, Ponnusamy S. Review on the interactions between dopamine metabolites and α -Synuclein in causing Parkinson's disease. *Neurochemistry International*. 2023; 162: 105461.
- [31] Panneton WM, Kumar VB, Gan Q, Burke WJ, Galvin JE. The neurotoxicity of DOPAL: behavioral and stereological evidence for its role in Parkinson disease pathogenesis. *PLoS ONE*. 2010; 5: e15251.
- [32] Goldstein DS. The catecholaldehyde hypothesis: where MAO fits in. *Journal of Neural Transmission*. 2020; 127: 169–177.
- [33] Umek N, Geršak B, Vintar N, Šoštarič M, Mavri J. Dopamine Autoxidation Is Controlled by Acidic pH. *Frontiers in Molecular Neuroscience*. 2018; 11: 467.
- [34] Sun Y, Pham AN, Hare DJ, Waite TD. Kinetic Modeling of pH-Dependent Oxidation of Dopamine by Iron and Its Relevance to Parkinson's Disease. *Frontiers in Neuroscience*. 2018; 12: 859.
- [35] Riederer P, Monoranu C, Strobel S, Iordache T, Sian-Hülsmann J. Iron as the concert master in the pathogenic orchestra playing in sporadic Parkinson's disease. *Journal of Neural Transmission*. 2021; 128: 1577–1598.
- [36] Mehta NJ, Marwah PK, Njus D. Are Proteinopathy and Oxidative Stress Two Sides of the Same Coin? *Cells*. 2019; 8: 59.
- [37] Latif S, Jahangeer M, Maknoon Razia D, Ashiq M, Ghaffar A, Akram M, *et al.* Dopamine in Parkinson's disease. *Clinica Chimica Acta; International Journal of Clinical Chemistry*. 2021; 522: 114–126.
- [38] Weng M, Xie X, Liu C, Lim KL, Zhang CW, Li L. The Sources of Reactive Oxygen Species and Its Possible Role in the Pathogenesis of Parkinson's Disease. *Parkinson's Disease*. 2018; 2018: 9163040.
- [39] Zucca FA, Vanna R, Cupaioli FA, Bellei C, De Palma A, Di Silvestre D, *et al.* Neuromelanin organelles are specialized autolysosomes that accumulate undegraded proteins and lipids in aging human brain and are likely involved in Parkinson's disease. *NPJ Parkinson's Disease*. 2018; 4: 17.
- [40] Minakaki G, Krainc D, Burbulla LF. The Convergence of Alpha-Synuclein, Mitochondrial, and Lysosomal Pathways in Vulnerability of Midbrain Dopaminergic Neurons in Parkinson's Disease. *Frontiers in Cell and Developmental Biology*. 2020; 8: 580634.
- [41] Zecca L, Bellei C, Costi P, Albertini A, Monzani E, Casella L, *et al.* New melanic pigments in the human brain that accumulate in aging and block environmental toxic metals. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105: 17567–17572.
- [42] Nagatsu T, Nakashima A, Watanabe H, Ito S, Wakamatsu K. Neuromelanin in Parkinson's Disease: Tyrosine Hydroxylase and Tyrosinase. *International Journal of Molecular Sciences*. 2022; 23: 4176.
- [43] Bussell R Jr, Eliezer D. A structural and functional role for 11-mer repeats in alpha-synuclein and other exchangeable lipid binding proteins. *Journal of Molecular Biology*. 2003; 329: 763–778.
- [44] Ueda K, Fukushima H, Masliah E, Xia Y, Iwai A, Yoshimoto M, *et al.* Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America*. 1993; 90: 11282–11286.
- [45] Sorrentino ZA, Xia Y, Gorion KM, Hass E, Giasson BI. Carboxy-terminal truncations of mouse α -synuclein alter aggregation and prion-like seeding. *FEBS Letters*. 2020; 594: 1271–1283.
- [46] Cattani J, Subramaniam V, Drescher M. Room-temperature in-cell EPR spectroscopy: alpha-Synuclein disease variants remain intrinsically disordered in the cell. *Physical Chemistry Chemical Physics*. 2017; 19: 18147–18151.
- [47] Theillet FX, Binolfi A, Bekei B, Martorana A, Rose HM, Stuver M, *et al.* Structural disorder of monomeric α -synuclein persists in mammalian cells. *Nature*. 2016; 530: 45–50.
- [48] Jao CC, Hegde BG, Chen J, Haworth IS, Langen R. Structure of membrane-bound alpha-synuclein from site-directed spin labeling and computational refinement. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105: 19666–19671.
- [49] Bartels T, Choi JG, Selkoe DJ. α -Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. *Nature*. 2011; 477: 107–110.
- [50] Musteikytė G, Jayaram AK, Xu CK, Vendruscolo M, Krainer G, Knowles TPJ. Interactions of α -synuclein oligomers with lipid membranes. *Biochimica et Biophysica Acta. Biomembranes*. 2021; 1863: 183536.
- [51] Li A, Rastegar C, Mao X. α -Synuclein Conformational Plasticity: Physiologic States, Pathologic Strains, and Biotechnological Applications. *Biomolecules*. 2022; 12: 994.
- [52] Dedmon MM, Lindorff-Larsen K, Christodoulou J, Vendruscolo M, Dobson CM. Mapping long-range interactions in alpha-synuclein using spin-label NMR and ensemble molecular dynamics simulations. *Journal of the American Chemical Society*. 2005; 127: 476–477.
- [53] Brodie NI, Popov KI, Petrotchenko EV, Dokholyan NV, Borchers CH. Conformational ensemble of native α -synuclein in solution as determined by short-distance crosslinking constraint-guided discrete molecular dynamics simulations. *PLoS Computational Biology*. 2019; 15: e1006859.
- [54] Ubbiali D, Fratini M, Piersimoni L, Ihling CH, Kipping M, Heilmann I, *et al.* Direct Observation of "Elongated" Conformational States in α -Synuclein upon Liquid-Liquid Phase Separation. *Angewandte Chemie*. 2022; 61: e202205726.
- [55] Grupi A, Haas E. Segmental conformational disorder and dynamics in the intrinsically disordered protein α -synuclein and its chain length dependence. *Journal of Molecular Biology*. 2011; 405: 1267–1283.
- [56] Dai X, Fu W, Chi H, Mesias VSD, Zhu H, Leung CW, *et al.* Optical tweezers-controlled hotspot for sensitive and reproducible surface-enhanced Raman spectroscopy characterization of native protein structures. *Nature Communications*. 2021; 12: 1292.
- [57] Brembati V, Faustini G, Longhena F, Bellucci A. Alpha synuclein post translational modifications: potential targets for Parkinson's disease therapy? *Frontiers in Molecular Neuroscience*. 2023; 16: 1197853.
- [58] Zhang S, Zhu R, Pan B, Xu H, Olufemi MF, Gathagan RJ, *et al.* Post-translational modifications of soluble α -synuclein regulate the amplification of pathological α -synuclein. *Nature Neuroscience*. 2023; 26: 213–225.

- [59] Rossetti G, Musiani F, Abad E, Dibenedetto D, Mouhib H, Fernandez CO, *et al.* Conformational ensemble of human α -synuclein physiological form predicted by molecular simulations. *Physical Chemistry Chemical Physics*. 2016; 18: 5702–5706.
- [60] Yang X, Wang B, Hoop CL, Williams JK, Baum J. NMR unveils an N-terminal interaction interface on acetylated- α -synuclein monomers for recruitment to fibrils. *Proceedings of the National Academy of Sciences of the United States of America*. 2021; 118: e2017452118.
- [61] Hwang CS, Shemorry A, Varshavsky A. N-terminal acetylation of cellular proteins creates specific degradation signals. *Science*. 2010; 327: 973–977.
- [62] Dikiy I, Eliezer D. N-terminal acetylation stabilizes N-terminal helicity in lipid- and micelle-bound α -synuclein and increases its affinity for physiological membranes. *The Journal of Biological Chemistry*. 2014; 289: 3652–3665.
- [63] McFarland MA, Ellis CE, Markey SP, Nussbaum RL. Proteomics analysis identifies phosphorylation-dependent alpha-synuclein protein interactions. *Molecular & Cellular Proteomics*. 2008; 7: 2123–2137.
- [64] Burmann BM, Gerez JA, Matečko-Burmann I, Campioni S, Kumari P, Ghosh D, *et al.* Regulation of α -synuclein by chaperones in mammalian cells. *Nature*. 2020; 577: 127–132.
- [65] Pujols J, Peña-Díaz S, Lázaro DF, Peccati F, Pinheiro F, González D, *et al.* Small molecule inhibits α -synuclein aggregation, disrupts amyloid fibrils, and prevents degeneration of dopaminergic neurons. *Proceedings of the National Academy of Sciences of the United States of America*. 2018; 115: 10481–10486.
- [66] Byrd EJ, Wilkinson M, Radford SE, Sobott F. Taking Charge: Metal Ions Accelerate Amyloid Aggregation in Sequence Variants of α -Synuclein. *Journal of the American Society for Mass Spectrometry*. 2023; 34: 493–504.
- [67] Barrett PJ, Timothy Greenamyre J. Post-translational modification of α -synuclein in Parkinson's disease. *Brain Research*. 2015; 1628: 247–253.
- [68] Schmid AW, Fauvet B, Moniatte M, Lashuel HA. Alpha-synuclein post-translational modifications as potential biomarkers for Parkinson disease and other synucleinopathies. *Molecular & Cellular Proteomics*. 2013; 12: 3543–3558.
- [69] Paleologou KE, Oueslati A, Shakked G, Rospigliosi CC, Kim HY, Lamberto GR, *et al.* Phosphorylation at S87 is enhanced in synucleinopathies, inhibits alpha-synuclein oligomerization, and influences synuclein-membrane interactions. *The Journal of Neuroscience*. 2010; 30: 3184–3198.
- [70] Negro A, Brunati AM, Donella-Deana A, Massimino ML, Pinna LA. Multiple phosphorylation of alpha-synuclein by protein tyrosine kinase Syk prevents eosin-induced aggregation. *FASEB Journal*. 2002; 16: 210–212.
- [71] Chen L, Periquet M, Wang X, Negro A, McLean PJ, Hyman BT, *et al.* Tyrosine and serine phosphorylation of alpha-synuclein have opposing effects on neurotoxicity and soluble oligomer formation. *The Journal of Clinical Investigation*. 2009; 119: 3257–3265.
- [72] Chen J, Zaer S, Drori P, Zamel J, Joron K, Kalisman N, *et al.* The structural heterogeneity of α -synuclein is governed by several distinct subpopulations with interconversion times slower than milliseconds. *Structure*. 2021; 29: 1048–1064.e6.
- [73] Pálmadóttir T, Waudby CA, Bernfur K, Christodoulou J, Linse S, Malmendal A. Morphology-Dependent Interactions between α -Synuclein Monomers and Fibrils. *International Journal of Molecular Sciences*. 2023; 24: 5191.
- [74] Galvagnion C, Buell AK, Meisl G, Michaels TCT, Vendruscolo M, Knowles TPJ, *et al.* Lipid vesicles trigger α -synuclein aggregation by stimulating primary nucleation. *Nature Chemical Biology*. 2015; 11: 229–234.
- [75] Mukherjee S, Sakunthala A, Gadhe L, Poudyal M, Sawner AS, Kadu P, *et al.* Liquid-liquid Phase Separation of α -Synuclein: A New Mechanistic Insight for α -Synuclein Aggregation Associated with Parkinson's Disease Pathogenesis. *Journal of Molecular Biology*. 2023; 435: 167713.
- [76] Wood SJ, Wypych J, Steavenson S, Louis JC, Citron M, Biere AL. alpha-synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease. *The Journal of Biological Chemistry*. 1999; 274: 19509–19512.
- [77] Uversky VN, Li J, Fink AL. Evidence for a partially folded intermediate in alpha-synuclein fibril formation. *The Journal of Biological Chemistry*. 2001; 276: 10737–10744.
- [78] Ghosh D, Singh PK, Sahay S, Jha NN, Jacob RS, Sen S, *et al.* Structure based aggregation studies reveal the presence of helix-rich intermediate during α -Synuclein aggregation. *Scientific Reports*. 2015; 5: 9228.
- [79] Alam P, Bousset L, Melki R, Otzen DE. α -synuclein oligomers and fibrils: a spectrum of species, a spectrum of toxicities. *Journal of Neurochemistry*. 2019; 150: 522–534.
- [80] Fusco G, Chen SW, Williamson PTF, Cascella R, Perni M, Jarvis JA, *et al.* Structural basis of membrane disruption and cellular toxicity by α -synuclein oligomers. *Science*. 2017; 358: 1440–1443.
- [81] Mannini B, Mulvihill E, Sgromo C, Cascella R, Khodarahmi R, Ramazzotti M, *et al.* Toxicity of protein oligomers is rationalized by a function combining size and surface hydrophobicity. *ACS Chemical Biology*. 2014; 9: 2309–2317.
- [82] Xu CK, Castellana-Cruz M, Chen SW, Du Z, Meisl G, Levin A, *et al.* The Pathological G51D Mutation in Alpha-Synuclein Oligomers Confers Distinct Structural Attributes and Cellular Toxicity. *Molecules*. 2022; 27: 1293.
- [83] Schwarz TC, Beier A, Ledolter K, Gossenreiter T, Höfurtherner T, Hartl M, *et al.* High-resolution structural information of membrane-bound α -synuclein provides insight into the MoA of the anti-Parkinson drug UCB0599. *Proceedings of the National Academy of Sciences of the United States of America*. 2023; 120: e2201910120.
- [84] Buell AK, Galvagnion C, Gaspar R, Sparr E, Vendruscolo M, Knowles TPJ, *et al.* Solution conditions determine the relative importance of nucleation and growth processes in α -synuclein aggregation. *Proceedings of the National Academy of Sciences of the United States of America*. 2014; 111: 7671–7676.
- [85] Cremades N, Cohen SIA, Deas E, Abramov AY, Chen AY, Orte A, *et al.* Direct observation of the interconversion of normal and toxic forms of α -synuclein. *Cell*. 2012; 149: 1048–1059.
- [86] Ray S, Singh N, Kumar R, Patel K, Pandey S, Datta D, *et al.* α -Synuclein aggregation nucleates through liquid-liquid phase separation. *Nature Chemistry*. 2020; 12: 705–716.
- [87] Sawner AS, Ray S, Yadav P, Mukherjee S, Panigrahi R, Poudyal M, *et al.* Modulating α -Synuclein Liquid-Liquid Phase Separation. *Biochemistry*. 2021; 60: 3676–3696.
- [88] Neupane S, De Cecco E, Aguzzi A. The Hidden Cell-to-Cell Trail of α -Synuclein Aggregates. *Journal of Molecular Biology*. 2023; 435: 167930.
- [89] Braak H, Del Tredici K, Rüb U, de Vos RAI, Jansen Steur ENH, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging*. 2003; 24: 197–211.
- [90] Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of α -synuclein: from structure and toxicity to therapeutic target. *Nature Reviews. Neuroscience*. 2013; 14: 38–48.
- [91] Mehra S, Gadhe L, Bera R, Sawner AS, Maji SK. Structural and Functional Insights into α -Synuclein Fibril Polymorphism. *Biomolecules*. 2021; 11: 1419.
- [92] Holec SAM, Liu SL, Woerman AL. Consequences of variability in α -synuclein fibril structure on strain biology. *Acta Neuropathologica*. 2022; 143: 311–330.

- [93] Schweighauser M, Shi Y, Tarutani A, Kametani F, Murzin AG, Ghetti B, *et al.* Structures of α -synuclein filaments from multiple system atrophy. *Nature*. 2020; 585: 464–469.
- [94] Frieg B, Antonschmidt L, Dienemann C, Geraets JA, Najbauer EE, Matthes D, *et al.* The 3D structure of lipidic fibrils of α -synuclein. *Nature Communications*. 2022; 13: 6810.
- [95] Sohrabi T, Mirzaei-Behbahani B, Zadali R, Pirhaghi M, Morozova-Roche LA, Meratan AA. Common Mechanisms Underlying α -Synuclein-Induced Mitochondrial Dysfunction in Parkinson's Disease. *Journal of Molecular Biology*. 2023; 435: 167992.
- [96] Li J, Zhu M, Manning-Bog AB, Di Monte DA, Fink AL. Dopamine and L-dopa disaggregate amyloid fibrils: implications for Parkinson's and Alzheimer's disease. *FASEB Journal*. 2004; 18: 962–964.
- [97] Norris EH, Giasson BI, Hodara R, Xu S, Trojanowski JQ, Ischiropoulos H, *et al.* Reversible inhibition of alpha-synuclein fibrillization by dopaminochrome-mediated conformational alterations. *The Journal of Biological Chemistry*. 2005; 280: 21212–21219.
- [98] Galkin M, Topcheva O, Priss A, Borisova T, Shvadchak VV. Dopamine-Induced Oligomers of α -Synuclein Inhibit Amyloid Fibril Growth and Show No Toxicity. *ACS Chemical Neuroscience*. 2023; 14: 2027–2034.
- [99] Pham CLL, Leong SL, Ali FE, Kenche VB, Hill AF, Gras SL, *et al.* Dopamine and the dopamine oxidation product 5,6-dihydroxyindole promote distinct on-pathway and off-pathway aggregation of alpha-synuclein in a pH-dependent manner. *Journal of Molecular Biology*. 2009; 387: 771–785.
- [100] Mazzulli JR, Armakola M, Dumoulin M, Parastatidis I, Ischiropoulos H. Cellular oligomerization of alpha-synuclein is determined by the interaction of oxidized catechols with a C-terminal sequence. *The Journal of Biological Chemistry*. 2007; 282: 31621–31630.
- [101] Herrera FE, Chesi A, Paleologou KE, Schmid A, Munoz A, Vendruscolo M, *et al.* Inhibition of alpha-synuclein fibrillization by dopamine is mediated by interactions with five C-terminal residues and with E83 in the NAC region. *PLoS ONE*. 2008; 3: e3394.
- [102] Dibenedetto D, Rossetti G, Caliandro R, Carloni P. A molecular dynamics simulation-based interpretation of nuclear magnetic resonance multidimensional heteronuclear spectra of α -synuclein-dopamine adducts. *Biochemistry*. 2013; 52: 6672–6683.
- [103] Zou Y, Qian Z, Gong Y, Tang Y, Wei G, Zhang Q. Critical nucleus of Greek-key-like core of α -synuclein protofibril and its disruption by dopamine and norepinephrine. *Physical Chemistry Chemical Physics*. 2019; 22: 203–211.
- [104] Outeiro TF, Klucken J, Bercury K, Tetzlaff J, Pucha P, Oliveira LMA, *et al.* Dopamine-induced conformational changes in alpha-synuclein. *PLoS ONE*. 2009; 4: e6906.
- [105] Corti R, Marrano CA, Salerno D, Brocca S, Natalello A, Santambrogio C, *et al.* Depicting Conformational Ensembles of α -Synuclein by Single Molecule Force Spectroscopy and Native Mass Spectroscopy. *International Journal of Molecular Sciences*. 2019; 20: 5181.
- [106] Tavassoly O, Nokhrin S, Dmitriev OY, Lee JS. Cu(II) and dopamine bind to α -synuclein and cause large conformational changes. *The FEBS Journal*. 2014; 281: 2738–2753.
- [107] Cappai R, Leck SL, Tew DJ, Williamson NA, Smith DP, Galatis D, *et al.* Dopamine promotes alpha-synuclein aggregation into SDS-resistant soluble oligomers via a distinct folding pathway. *FASEB Journal*. 2005; 19: 1377–1379.
- [108] Saha S, Khan MAI, Mudhara D, Deep S. Tuning the Balance between Fibrillation and Oligomerization of α -Synuclein in the Presence of Dopamine. *ACS Omega*. 2018; 3: 14213–14224.
- [109] Mor DE, Ischiropoulos H. The Convergence of Dopamine and α -Synuclein: Implications for Parkinson's Disease. *Journal of Experimental Neuroscience*. 2018; 12: 1179069518761360.
- [110] Choi BK, Choi MG, Kim JY, Yang Y, Lai Y, Kweon DH, *et al.* Large α -synuclein oligomers inhibit neuronal SNARE-mediated vesicle docking. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110: 4087–4092.
- [111] Martinez-Vicente M, Tallozy Z, Kaushik S, Massey AC, Mazzulli J, Mosharov EV, *et al.* Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. *The Journal of Clinical Investigation*. 2008; 118: 777–788.
- [112] Ganguly U, Ganguly A, Sen O, Ganguly G, Cappai R, Sahoo A, *et al.* Dopamine Cytotoxicity on SH-SY5Y Cells: Involvement of α -Synuclein and Relevance in the Neurodegeneration of Sporadic Parkinson's Disease. *Neurotoxicity Research*. 2019; 35: 898–907.
- [113] Abdelkarim H, Marshall MS, Scesa G, Smith RA, Rue E, Marshall J, *et al.* α -Synuclein interacts directly but reversibly with psychosine: implications for α -synucleinopathies. *Scientific Reports*. 2018; 8: 12462.
- [114] Pandey AK, Buchholz CR, Nathan Kochen N, Pomerantz WCK, Braun AR, Sachs JN. pH Effects Can Dominate Chemical Shift Perturbations in ^1H , ^{15}N -HSQC NMR Spectroscopy for Studies of Small Molecule/ α -Synuclein Interactions. *ACS Chemical Neuroscience*. 2023; 14: 800–808.
- [115] Santambrogio C, Ponzini E, Grandori R. Native mass spectrometry for the investigation of protein structural (dis)order. *Biochimica et Biophysica Acta. Proteins and Proteomics*. 2022; 1870: 140828.
- [116] Kaltashov IA, Ivanov DG, Yang Y. Mass spectrometry-based methods to characterize highly heterogeneous biopharmaceuticals, vaccines, and nonbiological complex drugs at the intact-mass level. *Mass Spectrometry Reviews*. 2022; e21829.
- [117] Li J, Santambrogio C, Brocca S, Rossetti G, Carloni P, Grandori R. Conformational effects in protein electrospray-ionization mass spectrometry. *Mass Spectrometry Reviews*. 2016; 35: 111–122.
- [118] Santambrogio C, Natalello A, Brocca S, Ponzini E, Grandori R. Conformational Characterization and Classification of Intrinsically Disordered Proteins by Native Mass Spectrometry and Charge-State Distribution Analysis. *Proteomics*. 2019; 19: e180060.
- [119] Kaltashov IA, Bobst CE, Pawlowski J, Wang G. Mass spectrometry-based methods in characterization of the higher order structure of protein therapeutics. *Journal of Pharmaceutical and Biomedical Analysis*. 2020; 184: 113169.
- [120] Liu R, Xia S, Li H. Native top-down mass spectrometry for higher-order structural characterization of proteins and complexes. *Mass Spectrometry Reviews*. 2023; 42: 1876–1926.
- [121] Frimpong AK, Abzalimov RR, Uversky VN, Kaltashov IA. Characterization of intrinsically disordered proteins with electrospray ionization mass spectrometry: conformational heterogeneity of alpha-synuclein. *Proteins*. 2010; 78: 714–722.
- [122] Konijnenberg A, Ranica S, Narkiewicz J, Legname G, Grandori R, Sobott F, *et al.* Opposite Structural Effects of Epigallocatechin-3-gallate and Dopamine Binding to α -Synuclein. *Analytical Chemistry*. 2016; 88: 8468–8475.
- [123] Grandori R. Detecting equilibrium cytochrome c folding intermediates by electrospray ionisation mass spectrometry: two partially folded forms populate the molten-globule state. *Protein Science*. 2002; 11: 453–458.
- [124] Natalello A, Santambrogio C, Grandori R. Are Charge-State Distributions a Reliable Tool Describing Molecular Ensembles of Intrinsically Disordered Proteins by Native MS? *Journal of the American Society for Mass Spectrometry*. 2017; 28: 21–28.
- [125] Cheirdaris DG. Force Spectroscopy in Mechanical Protein Domains Unfolding. *Advances in Experimental Medicine and Bi-*

- ology. 2021; 1339: 187–193.
- [126] Sandal M, Valle F, Tessari I, Mammi S, Bergantino E, Musiani F, *et al.* Conformational equilibria in monomeric alpha-synuclein at the single-molecule level. *PLoS Biology*. 2008; 6: e6.
- [127] Brucale M, Sandal M, Di Maio S, Rampioni A, Tessari I, Tosatto L, *et al.* Pathogenic mutations shift the equilibria of alpha-synuclein single molecules towards structured conformers. *Chembiochem*. 2009; 10: 176–183.
- [128] Palomino-Hernandez O, Santambrogio C, Rossetti G, Fernandez CO, Grandori R, Carloni P. Molecular Dynamics-Assisted Interpretation of Experimentally Determined Intrinsically Disordered Protein Conformational Components: The Case of Human α -Synuclein. *The Journal of Physical Chemistry. B*. 2022; 126: 3632–3639.
- [129] Robustelli P, Piana S, Shaw DE. Developing a molecular dynamics force field for both folded and disordered protein states. *Proceedings of the National Academy of Sciences of the United States of America*. 2018; 115: E4758–E4766.
- [130] Jain MK, Bhat R. Modulation of human α -synuclein aggregation by a combined effect of calcium and dopamine. *Neurobiology of Disease*. 2014; 63: 115–128.
- [131] Tavassoly O, Lee JS. Methamphetamine binds to α -synuclein and causes a conformational change which can be detected by nanopore analysis. *FEBS Letters*. 2012; 586: 3222–3228.
- [132] Madampage C, Tavassoly O, Christensen C, Kumari M, Lee JS. Nanopore analysis: An emerging technique for studying the folding and misfolding of proteins. *Prion*. 2012; 6: 116–123.
- [133] Latawiec D, Herrera F, Bek A, Losasso V, Candotti M, Benetti F, *et al.* Modulation of alpha-synuclein aggregation by dopamine analogs. *PLoS ONE*. 2010; 5: e9234.
- [134] Roskoski R Jr. Small molecule protein kinase inhibitors approved by regulatory agencies outside of the United States. *Pharmacological Research*. 2023; 194: 106847.
- [135] Tatenhorst L, Eckermann K, Dambeck V, Fonseca-Ornelas L, Walle H, Lopes da Fonseca T, *et al.* Fasudil attenuates aggregation of α -synuclein in models of Parkinson's disease. *Acta Neuropathologica Communications*. 2016; 4: 39.
- [136] Robustelli P, Ibanez-de-Opakua A, Campbell-Bezat C, Giordanetto F, Becker S, Zweckstetter M, *et al.* Molecular Basis of Small-Molecule Binding to α -Synuclein. *Journal of the American Chemical Society*. 2022; 144: 2501–2510.
- [137] Pujols J, Peña-Díaz S, Conde-Giménez M, Pinheiro F, Navarro S, Sancho J, *et al.* High-Throughput Screening Methodology to Identify Alpha-Synuclein Aggregation Inhibitors. *International Journal of Molecular Sciences*. 2017; 18: 478.
- [138] Peña-Díaz S, Pujols J, Vasili E, Pinheiro F, Santos J, Manglano-Artuñedo Z, *et al.* The small aromatic compound SynuClean-D inhibits the aggregation and seeded polymerization of multiple α -synuclein strains. *The Journal of Biological Chemistry*. 2022; 298: 101902.
- [139] Pena-Díaz S, Pujols J, Ventura S. Small molecules to prevent the neurodegeneration caused by α -synuclein aggregation. *Neural Regeneration Research*. 2020; 15: 2260–2261.
- [140] Dominguez-Mejide A, Vasili E, König A, Cima-Omori MS, Ibáñez de Opakua A, Leonov A, *et al.* Effects of pharmacological modulators of α -synuclein and tau aggregation and internalization. *Scientific Reports*. 2020; 10: 12827.
- [141] Richter F, Subramaniam SR, Magen I, Lee P, Hayes J, Attar A, *et al.* A Molecular Tweezer Ameliorates Motor Deficits in Mice Overexpressing α -Synuclein. *Neurotherapeutics*. 2017; 14: 1107–1119.
- [142] Bengoa-Vergniory N, Faggiani E, Ramos-Gonzalez P, Kirkiz E, Connor-Robson N, Brown LV, *et al.* CLR01 protects dopaminergic neurons in vitro and in mouse models of Parkinson's disease. *Nature Communications*. 2020; 11: 4885.
- [143] Pena-Díaz S, Ventura S. One ring is sufficient to inhibit α -synuclein aggregation. *Neural Regeneration Research*. 2022; 17: 508–511.
- [144] Bieschke J, Russ J, Friedrich RP, Ehrnhoefer DE, Wobst H, Neugebauer K, *et al.* EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107: 7710–7715.
- [145] Caruana M, Högen T, Levin J, Hillmer A, Giese A, Vassallo N. Inhibition and disaggregation of α -synuclein oligomers by natural polyphenolic compounds. *FEBS Letters*. 2011; 585: 1113–1120.
- [146] Wang Y, Wu S, Li Q, Lang W, Li W, Jiang X, *et al.* Epigallocatechin-3-gallate: A phytochemical as a promising drug candidate for the treatment of Parkinson's disease. *Frontiers in Pharmacology*. 2022; 13: 977521.
- [147] Herrera-Vaquero M, Bouquio D, Kallab M, Biggs K, Nair G, Ochoa J, *et al.* The molecular tweezer CLR01 reduces aggregated, pathologic, and seeding-competent α -synuclein in experimental multiple system atrophy. *Biochimica et Biophysica Acta. Molecular Basis of Disease*. 2019; 1865: 165513.
- [148] Jha NN, Kumar R, Panigrahi R, Navalkar A, Ghosh D, Sahay S, *et al.* Comparison of α -Synuclein Fibril Inhibition by Four Different Amyloid Inhibitors. *ACS Chemical Neuroscience*. 2017; 8: 2722–2733.
- [149] Mandel S, Maor G, Youdim MBH. Iron and alpha-synuclein in the substantia nigra of MPTP-treated mice: effect of neuroprotective drugs R-apomorphine and green tea polyphenol (-)-epigallocatechin-3-gallate. *Journal of Molecular Neuroscience*. 2004; 24: 401–416.
- [150] Xu Q, Langley M, Kanthasamy AG, Reddy MB. Epigallocatechin Gallate Has a Neurorescue Effect in a Mouse Model of Parkinson Disease. *The Journal of Nutrition*. 2017; 147: 1926–1931.
- [151] Cacabelos R. Parkinson's Disease: From Pathogenesis to Pharmacogenomics. *International Journal of Molecular Sciences*. 2017; 18: 551.
- [152] Choi J, Horner KA. Dopamine Agonists. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. 2023. Available at: <http://www.ncbi.nlm.nih.gov/books/NBK551686/> (Accessed: 13 June 2023).
- [153] Siddique YH, Sachdev R, Idrisi M, Shahid M. Effect of Cabergoline on Cognitive Impairments in Transgenic Drosophila Model of Parkinson's Disease. *Letters in Drug Design & Discovery*. 2020; 17: 1261–1269.
- [154] Ono K, Hirohata M, Yamada M. Anti-fibrillogenic and fibrildestabilizing activities of anti-Parkinsonian agents for alpha-synuclein fibrils in vitro. *Journal of Neuroscience Research*. 2007; 85: 1547–1557.
- [155] Ono K, Takasaki JI, Takahashi R, Ikeda T, Yamada M. Effects of antiparkinsonian agents on β -amyloid and α -synuclein oligomer formation in vitro. *Journal of Neuroscience Research*. 2013; 91: 1371–1381.
- [156] García-Beltrán O, Urrutia PJ, Núñez MT. On the Chemical and Biological Characteristics of Multifunctional Compounds for the Treatment of Parkinson's Disease. *Antioxidants*. 2023; 12: 214.
- [157] Elmabruk A, Das B, Yedlapudi D, Xu L, Antonio T, Reith MEA, *et al.* Design, Synthesis, and Pharmacological Characterization of Carbazole Based Dopamine Agonists as Potential Symptomatic and Neuroprotective Therapeutic Agents for Parkinson's Disease. *ACS Chemical Neuroscience*. 2019; 10: 396–411.
- [158] Modi G, Voshavar C, Gogoi S, Shah M, Antonio T, Reith MEA, *et al.* Multifunctional D2/D3 agonist D-520 with high in vivo efficacy: modulator of toxicity of alpha-synuclein aggregates. *ACS Chemical Neuroscience*. 2014; 5: 700–717.

- [159] Yedlapudi D, Joshi GS, Luo D, Todi SV, Dutta AK. Inhibition of alpha-synuclein aggregation by multifunctional dopamine agonists assessed by a novel in vitro assay and an in vivo *Drosophila* synucleinopathy model. *Scientific Reports*. 2016; 6: 38510.
- [160] Lima VDA, Esquinelato R, Carmo-Gonçalves P, Nascimento LAD, Lee H, Eliezer D, *et al.* The dopamine receptor agonist apomorphine stabilizes neurotoxic α -synuclein oligomers. *FEBS Letters*. 2022; 596: 309–322.
- [161] Werner-Allen JW, DuMond JF, Levine RL, Bax A. Toxic Dopamine Metabolite DOPAL Forms an Unexpected Dicatechol Pyrrole Adduct with Lysines of α -Synuclein. *Angewandte Chemie*. 2016; 55: 7374–7378.
- [162] Gallardo-Fernández M, Hornedo-Ortega R, Cerezo AB, Troncoso AM, Garcia-Parrilla MC. Hydroxytyrosol and dopamine metabolites: Anti-aggregative effect and neuroprotective activity against α -synuclein-induced toxicity. *Food and Chemical Toxicology*. 2023; 171: 113542.
- [163] Jinsmaa Y, Isonaka R, Sharabi Y, Goldstein DS. 3,4-Dihydroxyphenylacetaldehyde Is More Efficient than Dopamine in Oligomerizing and Quinonizing α -Synuclein. *The Journal of Pharmacology and Experimental Therapeutics*. 2020; 372: 157–165.
- [164] Masato A, Plotegher N, Terrin F, Sandre M, Faustini G, Thor A, *et al.* DOPAL initiates α Synuclein-dependent impaired proteostasis and degeneration of neuronal projections in Parkinson's disease. *NPJ Parkinson's Disease*. 2023; 9: 42.
- [165] Kumar VB, Hsu FF, Lakshmi VM, Gillespie KN, Burke WJ. Aldehyde adducts inhibit 3,4-dihydroxyphenylacetaldehyde-induced α -synuclein aggregation and toxicity: Implication for Parkinson neuroprotective therapy. *European Journal of Pharmacology*. 2019; 845: 65–73.
- [166] Carmo-Gonçalves P, do Nascimento LA, Cortines JR, Eliezer D, Romão L, Follmer C. Exploring the role of methionine residues on the oligomerization and neurotoxic properties of DOPAL-modified α -synuclein. *Biochemical and Biophysical Research Communications*. 2018; 505: 295–301.
- [167] Coelho-Cerqueira E, de Araújo Correia Campos C, Follmer C. Formation of large oligomers of DOPAL-modified α -synuclein is modulated by the oxidation of methionine residues located at C-terminal domain. *Biochemical and Biophysical Research Communications*. 2019; 509: 367–372.
- [168] Lima VDA, do Nascimento LA, Eliezer D, Follmer C. Role of Parkinson's Disease-Linked Mutations and N-Terminal Acetylation on the Oligomerization of α -Synuclein Induced by 3,4-Dihydroxyphenylacetaldehyde. *ACS Chemical Neuroscience* 2019; 10: 690–703.
- [169] Lamensdorf I, Hrycyna C, He LP, Nechushtan A, Tjurmina O, Harvey-White J, *et al.* Acidic dopamine metabolites are actively extruded from PC12 cells by a novel sulfonyleurea-sensitive transporter. *Naunyn-Schmiedeberg's Archives of Pharmacology*. 2000; 361: 654–664.
- [170] Zhou W, Gallagher A, Hong DP, Long C, Fink AL, Uversky VN. At low concentrations, 3,4-dihydroxyphenylacetic acid (DOPAC) binds non-covalently to alpha-synuclein and prevents its fibrillation. *Journal of Molecular Biology*. 2009; 388: 597–610.
- [171] Zhou W, Long C, Fink AL, Uversky VN. 3, 4-Dihydroxyphenylacetic Acid (DOPAC) Impairs α -Synuclein Interaction with Lipids. *The Open Proteomics Journal*. 2010; 3: 1–7.
- [172] Palazzi L, Fongaro B, Leri M, Acquasaliente L, Stefani M, Bucciantini M, *et al.* Structural Features and Toxicity of α -Synuclein Oligomers Grown in the Presence of DOPAC. *International Journal of Molecular Sciences*. 2021; 22: 6008.
- [173] Glaser CB, Yamin G, Uversky VN, Fink AL. Methionine oxidation, alpha-synuclein and Parkinson's disease. *Biochimica et Biophysica Acta*. 2005; 1703: 157–169.
- [174] Leri M, Scuto M, Ontario ML, Calabrese V, Calabrese EJ, Bucciantini M, *et al.* Healthy Effects of Plant Polyphenols: Molecular Mechanisms. *International Journal of Molecular Sciences*. 2020; 21: 1250.
- [175] Hornedo-Ortega R, Cerezo AB, Troncoso AM, Garcia-Parrilla MC. Protective effects of hydroxytyrosol against α -synuclein toxicity on PC12 cells and fibril formation. *Food and Chemical Toxicology*. 2018; 120: 41–49.
- [176] Palazzi L, Leri M, Cesaro S, Stefani M, Bucciantini M, Polverino de Laureto P. Insight into the molecular mechanism underlying the inhibition of α -synuclein aggregation by hydroxytyrosol. *Biochemical Pharmacology*. 2020; 173: 113722.
- [177] Fongaro B, Cappelletto E, Sosic A, Spolaore B, Polverino de Laureto P. 3,4-Dihydroxyphenylethanol and 3,4-dihydroxyphenylacetic acid affect the aggregation process of E46K variant of α -synuclein at different extent: Insights into the interplay between protein dynamics and catechol effect. *Protein Science*. 2022; 31: e4356.
- [178] Butler YR, Liu Y, Kumbhar R, Zhao P, Gadhav K, Wang N, *et al.* α -Synuclein fibril-specific nanobody reduces prion-like α -synuclein spreading in mice. *Nature Communications*. 2022; 13: 4060.
- [179] Santos J, Gracia P, Navarro S, Peña-Díaz S, Pujols J, Cremades N, *et al.* α -Helical peptidic scaffolds to target α -synuclein toxic species with nanomolar affinity. *Nature Communications*. 2021; 12: 3752.
- [180] Brás IC, Dominguez-Meijide A, Gerhardt E, Koss D, Lázaro DF, Santos PI, *et al.* Synucleinopathies: Where we are and where we need to go. *Journal of Neurochemistry*. 2020; 153: 433–454.
- [181] Whone A. Monoclonal Antibody Therapy in Parkinson's Disease - The End? *The New England Journal of Medicine*. 2022; 387: 466–467.
- [182] Tao Y, Sun Y, Lv S, Xia W, Zhao K, Xu Q, *et al.* Heparin induces α -synuclein to form new fibril polymorphs with attenuated neuropathology. *Nature Communications*. 2022; 13: 4226.