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REVIEW

Investigational α-synuclein aggregation inhibitors: hope for Parkinson's disease

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ABSTRACT

Introduction: The therapeutic management of Parkinson's disease (PD) is challenging and has not been fully resolved. The main challenges include motor fluctuations and levodopa-induced dyskinesia. Moreover, no disease-modifying or neuroprotective therapy is currently available.

Areas covered: This review focuses on α-synuclein aggregation inhibitors and their therapeutic role in PD, with special attention to heat shock proteins, immunotherapy (active and passive), the potential of targeting the Ser129 phosphorylation site, and the antibiotic possibilities.

Expert opinion: The induction of chaperones may provide beneficial strategy to target synucleinopathies, but further investigations are needed to find the best options. The promising preclinical results with immunotherapy suggest that it may be a valuable disease-modifying therapy in PD in the future. Clinical trials are currently in the initial phases, and future studies need to confirm the beneficial therapeutic effect in humans and clarify open questions as regards the exact mode of action and potential safety concerns. In case of covalent modifications, phosphorylation of α-synuclein is of outstanding importance; however, conflicting results and open questions exist which necessitate clarification. *In vitro* results suggest that several antibiotics may also influence α-synuclein aggregation, but these results are to be confirmed in the future.

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1. Introduction

The diseases that are characterized by an abnormal accumulation of α-synuclein (α-Syn) aggregates within neurons, nerve fibers, or glial cells are collectively referred to as α-synucleinopathies. The three main types of α-synucleinopathies are Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA). These conditions affect mainly the elderly population, thereby causing serious issues in the aging societies. Among them the most common condition is PD, which has both familial and sporadic forms. The prevalence of the disease is approximately 0.2% in the general population, a number gradually increasing with age [1]. PD is characterized by typical motor symptoms including tremor, rigidity, and hypokinesia, and non-motor symptoms, such as dementia, sleep disorders, emotional, cognitive, and behavioral disorders, and depression. The pathological hallmarks of PD are the degeneration of DA-ergic neurons in the substantia nigra pars compacta (SNpc, a brain region involved in the control of routine movements) and the presence of Lewy bodies (LBs).

In the pathogenesis of the sporadic form of the disease (accounting for approximately 80% of all cases), mitochondrial disturbances, oxidative stress, glutamate excitotoxicity, immunological mechanisms, and protein aggregation have all been heavily implicated; however, the exact etiology is still not fully understood.

Genome-wide association studies (GWAS) and other genetic studies have largely revealed the genetic background of the familial forms of the disease. The main genetic mutations, gene duplications, triplications, and susceptibility loci that are directly linked to PD are well summarized in a recent article [2]; in this review, we therefore focus only on the *SNCA* mutations (i.e. the coding gene of the α-Syn protein), which affect the protein aggregation potential.

The gold standard of PD therapy is based on long-term dopamine (DA) replacement with 3,4-dihydroxy-L-phenylalanine (L-DOPA), the precursor of DA; however, this therapy can unfortunately provoke side effects.

Because of these side effects and the lack of neuroprotective drugs available, the search for new therapeutic options in PD is still intense, and the pharmacological manipulations of α-Syn aggregation are in the limelight of these investigations.

This review summarizes the preclinical and phase I and II clinical studies in which α-Syn aggregation inhibitors are in the spotlight.

2. α-Syn and its role in the pathomechanism of PD

After Alzheimer's disease, PD and the DLB are the most common types of degenerative dementias in patients above

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Article highlights

- Manipulations related to heat shock proteins are promising approaches in preventing α-synuclein oligomerization and toxicity.
- Being successful at the preclinical level, immunotherapeutic approaches against α-synuclein oligomers have already entered the clinical phases of investigation.
- Results as regards Ser129 phosphorylation are controversial and necessitate clarification.
- Some well-known antibiotics showed promise as molecules interfering with α-synuclein aggregation, and are to be tested in clinical trials.
- Several natural polyphenols are implicated as potent inhibitors of α-synuclein aggregate formation, warranting further investigations at both preclinical and clinical levels.
- Peptide inhibitors are novel promising molecules in the field with currently limited data available, which necessitates further examinations at the preclinical level.

This box summarizes key points contained in the article.

65 years of age [3]. The common histological features in these conditions include the presence of LBs and Lewy neurites in the affected human brain tissues. Lewy neurites are abnormal cells, which contain granular material and abnormal α-Syn filaments.

Similarly, the main constituent protein of LBs is α-Syn; however, more than 70 additional types of proteins have been identified within LBs to date [4]. The most well-known LB proteins are ubiquitin, neurofilament, microtubule-associated protein (MAP) 1B, synphilin-1, tau, and parkin [4].

The assembly of LBs is summarized schematically in Figure 1. When α-Syn loses its native conformation, it can be aggregated to β-sheet-rich dimers/trimers and after that into oligomers. These oligomers form protofibrils, which are the basis of the fibrils. The increased number of these fibrils and other above-mentioned proteins eventually form LBs. Increasing evidence supports that the toxicity of α-Syn originates from the oligomers and not from the fibrils of α-Syn. For example Tanaka and his colleagues provided evidence that the mature fibrils may not be the most pathogenic α-Syn species and LBs may have a cytoprotective role by the reduction of the toxic soluble α-Syn forms [5]. Other results suggest that the accumulating α-Syn aggregates are located at the presynaptic terminals, which may have a pathological impact on synaptic function; moreover, this may result in the loss of dendritic spines at the postsynaptic area in DLB [6]. An important *in vivo* experiment proved the toxicity of the oligomers by testing α-Syn mutants which promote oligomer or fibril formations using a rat lentivirus system. In this way, it was possible to investigate the loss of the DA-ergic neurons in the substantia nigra. The most severe DA-ergic loss was observed in those animals that carried α-Syn variants that form oligomers (i.e. E57K and E35K), while those variants that form fibrils very quickly were less toxic [7]. But what causes the toxicity of these oligomers? There are several possible answers and theories. The first theory postulates that they might create pores in the cell membrane which finally cause cell death [8]. The other possibility is the accumulation of these oligomers near the endoplasmatic reticulum (ER), resulting in ER stress, contributing to neurodegeneration [9]. According to the third theory, the aggregated extracellular α-Syn activates microglia, which leads to inflammation and degeneration of the affected neurons [10,11]. In addition to this, the accumulation of these oligomers may also suppress long-term potentiation and overload the protein degradation systems (ubiquitin–proteasome system and autophagy–lysosomal pathway), which mechanisms may also be responsible for the cell death [12,13]. The above-mentioned evidence suggests that inhibition of α-Syn fibrillation or dissolving fibrils may be a dangerous strategy because toxic oligomers could be generated.

The six-exon-containing α-Syn gene (*SNCA*) is located on chromosome 4q21. The encoded α-Syn is a 140-residue protein, which is phylogenetically conserved and abundantly

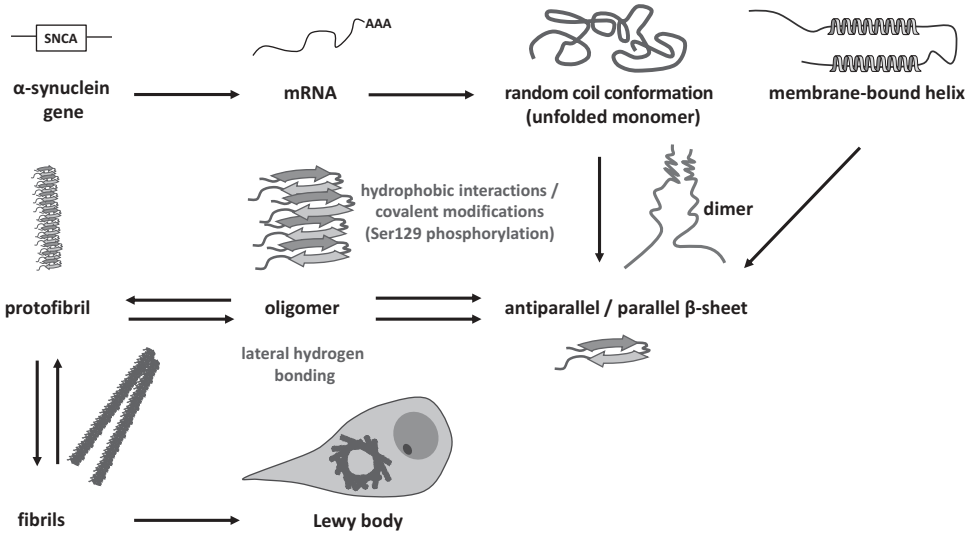

Figure 1. Oligomerization and Lewy body formation. The *SNCA* gene encoding α-synuclein is located on chromosome 4q21. The 140-residue α-synuclein protein can be present in two structural isoforms, including a native random coil monomer and a membrane-bound helical form. When the protein loses its native conformation, it can be aggregated to β-sheet-rich dimers/trimers and subsequently into oligomers. These oligomers can form protofibrils that are elemental components of α-synuclein fibrils. The accumulation of fibrils and other proteins (such as ubiquitin and Tau) eventually leads to the formation of Lewy bodies.

expressed throughout the central nervous system (CNS) [14]. Mutations in the *SNCA* gene are rare, but are usually highly penetrant and generally cause early onset autosomal dominant PD [15]. The most relevant mutations of this gene are p.A53T, p.A30P, p.E46K, p.H50Q, and p.G51D [15–19]. These mutations have been shown to alter fibrillization kinetics of the nascent protein. For example, A30P has a reduced ability to form amyloid-like fibrils, but it has an enhanced ability to form oligomers [20–22]. Moreover, there are evidences indicating that the A53T, E46K, and H50Q mutations accelerate fibril formation, whereas the G51D mutation attenuates it *in vitro* [23–26]. There are similar results with the recently identified A53E *SNCA* mutation, which also attenuates fibril formation [27–29].

The nascent α-Syn protein is 14 kDa and is mainly localized in the cell soma, nucleus, and the presynaptic terminal region of the neurons. Generally, α-Syn is assumed to be unfolded, forming a random coil, but some groups argue that α-Syn exists as a native tetramer in cells [30–32]. Its primary structure is divided into three domains: the N-terminal domain, a hydrophobic domain, and the C-terminal domain (Figures 2(a,b), and 3).

The N-terminal domain (residues 1–60) is highly conserved and normally unordered. However, this amphipathic domain contains four 11-amino-acid-long imperfect repeats (KTKEGV motif), which allow the domain to form a secondary structure resembling two α-helices separated by a break. The amphipatic nature of this domain and its ability to adopt an α-helical secondary structure indicate that α-Syn is a membrane-bound protein.

The other name of the hydrophobic domain (residues 61–95) is the non-Aβ component of plaque [non-amyloidogenic component (NAC)]. This domain is located in the second part of the second α-helix. It contains two imperfect repeats and is responsible for oligomerization, with residues 71–82 being essential in the process [33,34]. In addition, NAC mediates the conformational switch from the random-coil structure to a β-sheet structure, which is important for aggregation. This region has a phosphorylation site at Ser87.

The C-terminal domain (residues 96–140) has no characteristic secondary structure, but it is negatively charged due to the high number of acidic amino acids.

The amphipathic N-terminal and the hydrophobic NAC are highly conserved among species, whereas the C-terminal domain is variable in size and sequence [35].

The protein has a chaperone-like activity, with the proline-rich residues 125–140 being critical for this feature [36]. Besides Ser129, other phosphorylation sites exist in the C-terminal domain of the protein at Tyr125, Tyr133, and Tyr136 [37].

As mentioned above, α-Syn exists primarily in a random coil structure, covalent modifications (i.e. Ser129 phosphorylation)

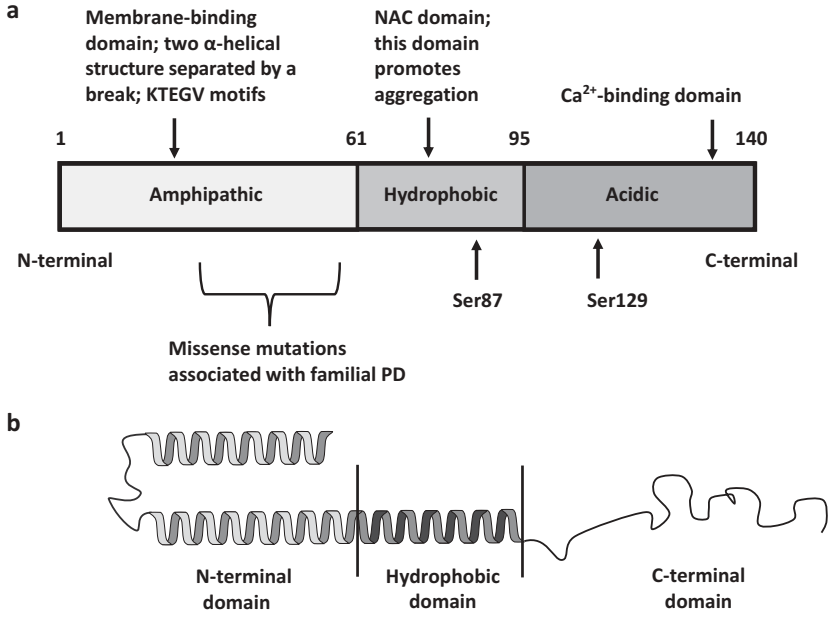

Figure 2. A schematic figure about the structure of α-synuclein. The protein can be divided into three distinct domains. The N-terminal amphipathic domain contains the evolutionary conserved KTEGV motifs and the main mutations associated with familial PD are located in this region. This region is responsible for the membrane binding as well. The hydrophobic NAC region is responsible for promoting aggregation. The C-terminal domain is negatively charged; it contains a Ca^{2+}-binding site and the main phosphorylation site at Ser129, which modulates α-synuclein aggregation.

1- MDVFMKGLS**KAKEGV**VAAAE**KTKQGV**AEAAG**KTKEGV**LYVGS**KTKEGV**VHGVATVAE**KTKEQV**TNVGG**AVVTGV**TAVAQ**KTVEGA**G**S**IAAATGFVKK

DQLVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMP**S**EEGYQDYEPEA -140

Figure 3. The full sequence of the 140-residue α-synuclein protein. The protein has three domains: the N-terminal (1–60), the NAC (61–95), and the C-terminal domains (96–140). The NAC region is underlined. The amphipathic and the NAC regions contain seven imperfect lysine-rich, highly conserved motif repeats (KTKEGV), responsible for binding lipids (bold). The main mutations are shown in light grey background (Ala53Thr, Ala30Pro, Glu46Lys, His50Gln, Gly51Asp, and Ala53Glu). Chaperone-mediated autophagy recognition sites are marked with dark grey background. The main serine phosphorylation sites are shown in bigger font size (Ser87 and Ser129).

and hydrophobic interactions [33] facilitate the polymerization of various α-Syn proteins into an anti-parallel β-sheet conformation [38], but other evidences suggest that it may have a parallel, in-register arrangement of β-sheets too [39,40]. Moreover, lateral and linear hydrogen bonds further intensify the aggregation potential, which results in fibril formation (Figure 1). The aggregation of α-Syn is a critical step in the pathogenesis of PD, and the association between α-Syn and PD is supported by numerous facts.

The most compelling evidence is the observation that the overexpression of α-Syn (either by the duplication or triplication of the *SNCA* gene) results in early onset familial PD, similarly to point mutations which increase the aggregation potential of α-Syn [16,41,42]. Another serious evidence is that α-Syn is the primary constituent protein of LBs, which are pathological hallmarks of PD. The findings of studies knocking out the *SNCA* gene or taking advantage of the transgenic overexpression of the wild-type or mutant protein in rodents and flies have proved the *in vivo* relevance of α-Syn. Some of these transgenic animal models have exhibited neuronal cell death, dystrophic neurites, α-Syn aggregates, and alterations in DA metabolism and release [43–45]. Transgenic mice expressing A53T human α-Syn have been shown to develop severe motor impairment accompanied by motor neuron axonal degeneration and neuronal inclusions containing toxic α-Syn fibrils [46,47]. Besides transgenic models, several other α-Syn-based animal models have also been described. In lentiviral-based rat models of PD, a selective loss of nigral DA-ergic neurons has been described together with the development of α-Syn-containing inclusions [48,49]. In wild-type, non-transgenic mice, the intrastriatal injection of synthetic α-Syn fibrils led to the cell-to-cell transmission and intraneuronal accumulation of α-Syn, progressive loss of DA-ergic neurons in the SNpc, DA depletion, and motor coordination impairment [50]. Another α-Syn-based animal model of PD utilized adeno-associated viral vector to lead to overexpression of α-Syn in rodent midbrain DA-ergic neurons. In this model, striatal axonal degeneration, α-Syn-inclusions in dystrophic axons were followed by a selective and progressive loss of nigral DA-ergic neurons [51].

As a summary of the above, α-Syn has been implicated as a primary contributor to PD development; therefore, inhibiting its aggregation may serve as a therapeutic possibility.

3. Inhibitors of α-Syn aggregation

3.1. Heat shock proteins (Hsps)

The pathological hallmarks of PD include the progressive accumulation of pathogenic protein formations and intracellular inclusion bodies, resulting in the formation of LBs and Lewy neurites. This indicates the relevance of an altered protein metabolism in the pathogenesis of PD. The formation, quantity, folding, aggregation, and degradation of proteins are key aspects in appropriate protein homeostasis. The main roles of Hsps, molecular chaperones, and their molecular assistants, the co-chaperones, include the stabilization of the cytoskeleton, the maintenance of the cell cycle and other basic cell functions, the exertion of anti-apoptotic effects, the regulation of vesicle trafficking, and the maintenance of cellular homeostasis; however, focus is placed on protein folding, re-folding and degenerative functions of these molecules in this review. Hsps or molecular chaperones are constitutively expressed and are highly conserved. Their classification is based on their molecular weight. Accordingly, we can distinguish between small Hsps, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 protein families. The elimination of misfolded proteins is crucial to maintain intracellular homeostasis, and the chaperones control the turnover of these proteins. *In vivo*, the degradation of α-Syn protein can take place in the ubiquitin–proteasome and the autophagy–lysosomal pathways (macro-, micro- and chaperone-mediated autophagy) [52].

Following stress stimuli, the amount of unfolded proteins increase, which evokes the expression of chaperones. This mechanism is under the control of certain transcription factors that include heat shock factor 1 (HSF-1), which plays a role in the negative feed-back. In normal conditions, this factor exists in form of an inactive monomer within the cytosol due to its association with Hsp90 [53]. Stress stimuli can lead to the dissociation of these two molecules and the translocation of HSF-1 into the nucleus following phosphorylation and trimerization, where it can induce the expression of Hsp70 and other Hsps. After the accumulated chaperones reach a sufficient level, Hsp90 inactivates HSF-1 again.

The first evidence for the role of Hsps in PD was provided by pathological studies, in which Hsp90, 70, 60, 40, and 27 were identified within LBs [54–57]. After this observation, experiments performed in *in vitro* cell lines, yeasts, fruitflies, and mice provided further evidence indicating their role in PD.

In an early *in vitro* study, the PC12 cell line was used with 1-methyl-4-phenylpyridinium (MPP+) as a model of PD. The PC12 cells were heat shocked for 1 h at 41.5°C. When utilized 6 h before the addition of MPP+, this treatment significantly inhibited the induction of cell death evoked by the toxin [58]. In another cell line, the administration of MPP+ increased the expression of α-Syn mRNA, leading to protein accumulation and aggregation. Both the application of heat shock and the overexpression of HDJ-1 (a homolog of human Hsp40) inhibited this enhancement in α-Syn mRNA expression, facilitated the ubiquitination of α-Syn protein and increased the proteasome activity [59]. Furthermore, there is evidence indicating the protective role of Hsps in another *in vitro* toxin model of PD, that is rotenone toxicity in rat brain slices [60].

When human wild-type α-Syn or the inherited mutants (A53T or A30P) were expressed in *Saccharomyces cerevisiae*, a brief heat shock provided striking protection by inhibiting α-Syn-induced apoptosis [61].

In *Drosophila melanogaster*, directed expression of α-Syn causes degeneration of DA-ergic neurons in the SNpc. This DA-ergic neuronal loss can be ameliorated by directed expression of the molecular chaperone Hsp70 [55].

In mice, pretreatment with geldanamycin (a natural inhibitor of Hsp90) mitigated the DA-ergic neurotoxicity induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; a systemically applicable prodrug of MPP+). The molecular mechanisms underlying this neuroprotective effect are suggested to include a reduction of cytosolic Hsp90 and an

increase in Hsp70 levels, whereas the level of striatal nuclear HSF-1 was found to be significantly enhanced upon geldanamycin pretreatment. On the basis of these, pharmacological inhibition of Hsp90 may represent a potential therapeutic strategy in PD [62].

Based on these experiments, these proteins may appear in the future on the therapeutical palette of the neurodegenerative diseases including PD [63,64].

From pharmacological point of view, potential chaperone-mediated therapeutical strategies in PD can be divided into four different approaches (Table 1).

The first strategy takes advantage of Hsp90 inhibitors (Figure 4), an approach through which the level of HSF-1 increases, resulting in the expression of stress-induced proteins, including Hsp70. The first molecule that entered the clinical investigations was 17-AAG (tanespimycin) in 1999. This is a geldanamycin analog, which was developed to overcome the limitations of the original molecule: the poor blood–brain barrier permeability and the liver toxicity [63]. Many other inhibitors have subsequently been tested in cancer trials; however, they may have beneficial effect in PD as well [65]. Indeed, promising results have been published with geldanamycin (a natural Hsp90 inhibitor) in different *in vitro* and *in vivo* models of PD [62,66,67]. Geldanamycin and its analogs act by blocking the ATP-binding site on the N-terminal domain of Hsp90. The molecule has been shown to be protective in murine and fruitfly models of PD [62,66]. Notably, however, challenging results have also been published as regards the relationship between Hsp90 and PD. Indeed, a recent study revealed that Hsp90 prevented the aggregation of α-Syn in an ATP-independent manner. The Hsp90 chaperone can form a strong complex with toxic α-Syn oligomers, and these complexes are harmless and non-toxic to cells [114]. Along this line, the upregulation of chaperones by inhibiting Hsp90 might as well decrease the anti-cytotoxic effect of Hsp90, suggesting that it might be beneficial to target the heat shock response without altering the Hsp90 activity [114].

The second therapeutical option can be the modulation of HSF-1 transcription factor or other pathways activating Hsp70. For example, celastrol treatment significantly reduced the effect of MPTP in a murine model of PD. Celastrol is an Hsp70 inducer, which evokes the hyperphosphorylation of HSF-1 and thereby promotes its binding to the regulated gene promoters. Furthermore, treatment with FLZ was protective against MPP+-induced neurotoxicity in several PD models [68–70]. The FLZ compound (*N*-[2-(4-hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxy-phenyl)-acrylamide) is a novel squamosamide derivative, originating from a Chinese herb. A recent study reported that FLZ treatment could alleviate motor dysfunction and ameliorate DA-ergic neuronal dysfunction in an α-Syn transgenic mouse model by enhancing Hsp70 protein expression and downstream transcriptional activity [68]. The study revealed that FLZ increased the expression of Hip (a co-chaperone of Hsp70), augmenting

Table 1. Experimental and pharmacological manipulations with potential to target α-Syn aggregation.

Chaperone-related manipulations	
[58,59,61]	Heat shock
[62–67]	Hsp90 inhibitors (e.g. geldanamycin, tanespimycin (17-AAG), alvespimycin (17-DMAG), SNX compounds (lead: SNX-0723)
[68–71]	HSF-1 activators (e.g. arimoclomol, HSF-1A, celastrol, valproate, geranylgeranylacetone, squamosamide derivative FLZ)
[72,73]	Virus-mediated overexpression of Hsps (i.e. Hsp70, Hsp40 (?), or HSF-1)
[74–77]	CPP-mediated overexpression of Hsps (e.g. TAT-Hsp70, TAT-Hsp40, or TAT-HSF-1)
Immunotherapy	
[78–84]	Passive immunization (C-terminal-targeted As (e.g. 9E4, 1H7, 5C1, ab274, PRX002); N-terminal-targeted As (e.g. Syn303))
[85,86]	Active immunization (e.g. PD01A, PD03A)
Modulating Ser129 phosphorylation state	
[87–93]	Targeted mutations (S129A) inhibiting phosphorylation (contradictory results)
[91–94]	Targeted mutations (S129D or S129E) mimicking phosphorylation (contradictory results)
[95,96]	Pharmacological modulation of involved kinases and phosphatases (contradictory results)
Small molecules interfering with α-Syn oligomerization and accumulation	
[97–113]	Inhibitors of α-Syn polymerization (e.g. rifampicin, ceftriaxone, various polyphenols, peptide inhibitors)
[104,110]	α-Syn fibril destabilizers (e.g. various polyphenols)
[102]	Activators of α-Syn clearance (e.g. rapamycin)

Abs: antibodies; α-Syn: alpha-synuclein; CPP: cell-penetrating peptide; HSF-1: heat shock factor 1; Hsp: heat shock protein; 17-AAG: 17-allylamino-17-demethoxygeldanamycin; 17-DMAG: 17-dimethylaminoethylamino-17-demethoxygeldanamycin; (?): questionable.

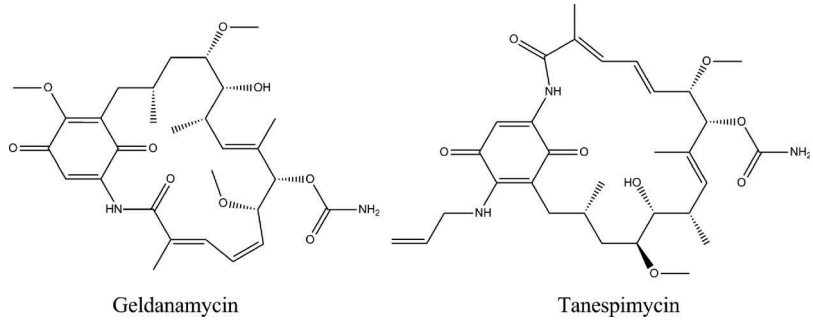

Figure 4. Schematic representation of the Hsp90 inhibitors galdenamycin and its derivative, tanespimycin.

the activity of Hsp70, a phenomenon which may underlie the observed beneficial effect. In conclusion, pharmacological induction of Hsp70 chaperone may represent a potential therapy for α-Syn-related diseases, including PD. The most relevant molecules which influence the function of Hsp70 have recently been comprehensively reviewed [71].

The third potential therapeutical strategy is the gene therapy, in which both the DA-related strategies and the disease-modifying possibilities deserve attention [115]. Among the latter, virus-mediated upregulation of Hsp70 may represent a novel possibility for neuroprotection in PD. There are promising results with Hsp70 gene transfer by a recombinant adeno-associated virus in mice [72]. The virus-mediated Hsp70 upregulation significantly reduced the MPTP-mediated apoptosis in the substantia nigra and the associated decline in striatal DA levels and the number of tyrosine hydroxylase-positive fibers. Another study investigating the effect of adeno-associated virus vector-mediated overexpression of different Hsps in a CDCrel-1 (a toxic parkin substrate)-overexpressing transgenic rat model of PD revealed the protective role of the overexpression of Hsp70 and H-BH, a constitutively active form of HSF-1, but interestingly not of Hsp40 [73].

The fourth potential strategy is based on the cell-penetrating peptide (CPP) technology, a method which has an important advantage as compared with the virus-mediated approach: it does not require stereotaxic surgery [71]. These small basic protein domains are able to transport the Hsps or other compounds through the cell membranes and across the blood–brain barrier. The most widely utilized basic domain is derived from the trans-activator of transcription (TAT) peptide of the human immunodeficiency virus [74]. After a fusion to TAT, Hsp70 (in form of TAT-Hsp70) can penetrate into the cells, and the administration of this construct was able to protect DA-ergic neurons in midbrain cultures and in the substantia nigra in models of PD [75]. In addition to the CPP-mediated delivery of Hsp70 *per se*, beneficial results have also been reported with that of Hsp40 and HSF-1 [76,77]. The CPP technology may be of outstanding relevance in future studies.

3.2. Immunotherapy

In recent years, immunotherapeutical approaches have been in the spotlight of research aiming at the development of effective disease-modifying therapies in PD. Immunotherapy may be used to target different aspects of α-Syn-mediated toxicity, which include the prevention of the propagation of α-Syn aggregation, and the facilitation of the clearance of already existing toxic aggregates by promoting autophagy or macrophage activation. The two main forms of immunotherapy are active and passive immunization, with both having advantages and disadvantages. Active immunization, also known as vaccination, refers to the administration of a special antigen which in turn evokes the activation of the patient's immune system. Vaccination requires a well-functioning immune system capable of producing an efficient amount of antibodies; active immunization is, therefore, not suitable for immunodeficient patients. On the other hand, vaccination is cheap and does not require frequent administration. Passive immunization refers to the administration of the preproduced antibodies. This method is more expensive and requires regular administration; however, it is also more controlled [78,85].

The majority of *in vivo* passive immunization studies published so far have been conducted in different transgenic murine models. In some studies, acute models were used, in which either preformed fibrils of α-Syn were injected in wild-type mice or an adeno-associated virus-mediated α-Syn overexpression was achieved [78]. The first efficient passive immunization study was reported in 2011, in which PDGF-hu-wt-aSyn mice (line D) were injected with anti-C-terminal mouse monoclonal antibody, 9E4 (epitope aa 118–126, mIgG1). The histological examination confirmed a significant reduction of C-terminally truncated α-Syn aggregates in parallel with behavioral and cognitive improvements [78,79]. In the following years, several studies targeting the C-terminal part of α-Syn in transgenic models reported similar motor and behavioral improvements together with histologically confirmed reduction of α-Syn [78,80–82]. Furthermore, there are data already available with passive immunization targeting the N-terminal of α-Syn. Syn303, an antibody targeting the N-terminal of α-Syn was investigated in an acute model, where preformed fibrils of α-Syn were injected in wild-type mice. In this model, Syn303 not only reduced motor deficits, but also ameliorated DA-ergic neuronal degeneration [78,83]. In another study, a virus-mediated α-Syn overexpression was used in rats. In this case, two different pools of goat polyclonal antibodies were applied, raised either against the N-terminal or the midpart of α-Syn. The first pool directed against the N-terminal was more effective and led to a decrease of α-Syn accumulation as well as reduced neuroinflammation [78,84]. Based on the promising preclinical studies, passive immunization therapies entered the clinical phases of investigation. To date, only one phase I trial has been completed, which involved 41 healthy volunteers and tested PRX002 (hIgG1), a humanized form of an antibody previously tested in animal models (NCT02095171). PRX002 was administered intravenously in ascending doses, and the results confirmed the ability of the candidate to significantly lower the free α-Syn level in the plasma [78]. No serious treatment-related adverse events were reported [78].

Among the active immunization approaches, short peptides called AFFITOPEs by Affiris AG reached the clinical phase of investigation. These peptides mimic the C-terminal region of α-Syn, but their sequence is not completely identical to the original peptide [85]. AFFITOPE vaccines, PD01A and PD03A, were previously tested in transgenic murine models, where both vaccines provided remarkable improvements. In these animal models, vaccines resulted in a reduction of aggregated α-Syn level in the neurons, ameliorated the neurodegenerative processes, and improved motor and cognitive functions as well [85,86]. The first clinical trial with one of the AFFITOPE vaccines, PD01A was completed and demonstrated a favorable safety profile (NCT01568099) [85]. Long-term follow-up and application of a booster vaccine were included in two recently completed clinical trials, but the results are not yet available (NCT01885494 and NCT02216188).

3.3. Targeting Ser129 phosphorylation

Investigations on the possible post-translational modifications of α-Syn revealed the presence of ubiquitination, sumoylation,

oxidation, nitration, C-terminal truncation, and phosphorylation sites in this protein [116–120]. There is increasing evidence indicating the relevance of phosphorylation of α-Syn in oligomerization, fibrillogenesis, LB formation, and eventually neurotoxicity. The majority of α-Syn is phosphorylated at Ser129 in the LBs in PD and other synucleinopathies [120–123]. To date the most relevant identified kinases, which phosphorylate α-Syn at Ser129 are: casein kinases (CK1 and CK2), the G protein-coupled receptor kinases (GRKs 1, 2, 5, and 6), human rhodopsin kinase-5, dual-specificity-Yak1-related kinase-1 (DYRK1), leucine rich-repeat kinase 2 (LRRK2), and polo-like kinases (PLKs 1, 2, and 3) [87,122,124–129]. The most important kinase in case of tyrosine phosphorylation is Abl kinase [130]. Protein phosphatase 2A has been shown to play important role in dephosphorylation of α-Syn [131].

The importance of phosphorylation was strengthened by *in vitro*, fruitfly, and murine models of PD (with human wild-type or mutant protein expressed) [87–90,95]. In these models, Ser129 phosphorylation was a key event in α-Syn-mediated neurotoxicity [87].

In the first *in vitro* study, site-directed mutagenesis was used to change Ser129 of α-Syn to alanine (S129A), which abolished phosphorylation at this site in a human neuroblastoma cell line (H-SY5Ycells). The co-expression of wild-type α-Syn and another LB component, synphilin-1, in this cell line resulted in the formation of cytoplasmic eosinophilic inclusions with features reminiscent of LBs, whereas that of S129A α-Syn and synphilin-1 resulted in the development of only few or no inclusions. Moreover, administration of 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole, a casein kinase 2 inhibitor, diminished the number of the inclusions, whereas H_2O_2, a molecule which increases α-Syn phosphorylation, augmented the number of inclusions formed as results of the co-expression of α-Syn, synphilin-1, and parkin [88].

In a subsequent *in vitro* experiment, rat oligodendroglial cell line was utilized (OLN-93) to model MSA disease with the co-expression of α-Syn and p25α, an oligodendroglial protein which can stimulate α-Syn aggregation. The treatment of these cells with the kinase inhibitor, 2-dimethylamino-4,5,6,7-tetrabromo-1H benzimidazole, a molecule which targets kinases such as casein kinase 2 and polo-like kinases, abrogated the toxicity. Ser129 phosphorylation was associated with the formation of phosphorylated oligomers detectable by immunoblotting, a process blocked by this inhibitor [89].

In a *Drosophila* PD model, S129A mutation interfered with phosphorylation and suppressed the DA-ergic neuronal cell death evoked by the expression of human α-Syn [87]. However, when Ser129 was replaced with the negatively charged residue, aspartate (S129D, a phosphorylation mimic), an enhanced α-Syn toxicity was detected.

Studies in mice using phosphoprotein phosphatase 2A (PP2A), an enzyme which dephosphorylates α-Syn at Ser129, revealed multiple alterations in the animals, including enhanced neuronal activity, increased dendritic arborizations, reduced astroglial, microglial activation, improved motor performance, and reduced α-Syn aggregation [95].

However, there are conflicting results regarding this issue [132]. Indeed, in a rat genetic PD model overexpressing human α-Syn, PLK2 overexpression could reduce the accumulation of the protein, suppress the dopaminergic neurodegeneration, and moderate hemiparkinsonian motor impairments too [96]. This beneficial effect was dependent on the activity of PLK2 and α-Syn phosphorylation; therefore, modulation of its kinase activity may be a viable target for the treatment of PD and other synucleinopathies [96]. In another rat model of PD, recombinant adeno-associated virus was unilaterally injected into the SNpc to overexpress human wild-type α-Syn or one of the two human α-Syn mutants (S129A or S129D). With this technique, the levels of human wild-type or mutant α-Syn was about four times higher as compared with the endogenous rat α-Syn. An increased rate of DA-ergic neuronal cell death and a reduction of DA and tyrosine hydroxylase levels were measured in the S129A-transfected group compared to that transfected by wild-type α-Syn. Furthermore, no pathological changes were apparent in S129D-treated rats. These results, therefore, suggest that the non-phosphorylated form (S129A) could enhance the α-Syn-induced nigral pathology, whereas Ser129 phosphorylation (S129D) could abolish the α-Syn-induced nigrostriatal degeneration [91]. The investigation of biochemical, structural, and membrane binding properties of wild-type α-Syn and its phosphorylation mimic forms (i.e. S129E, S129D) revealed that phosphorylation of the wild-type protein at S129 augments its conformational flexibility and inhibits fibrillogenesis *in vitro*, whereas it does not perturb its membrane-bound conformation [92]. Moreover, Paleologou and her colleagues showed that these phosphorylation mimics using acidic amino acid residues are insufficient to reproduce the effect of phosphorylation on the structural and aggregation properties of the protein *in vitro* [92]. These results were supported by findings of a study in a rat model of PD, investigating the effects of S129A and S129D. In this study, S129A significantly increased α-Syn toxicity, induced the formation of β-sheet-rich aggregates, and increased its affinity for intracellular membranes. On the other hand, S129D did not provoke DA-ergic cell death, but intriguingly, larger aggregates were formed, and apoptotic signals were also found to be activated. Accordingly, this study concluded that phosphorylation is not important in the accumulation of cytotoxic pre-inclusion aggregates [93].

Eventually, there are results indicating that the phosphorylation status of α-Syn at Ser129 does not influence DA-ergic neuronal cell death [93,94].

The phosphorylation sites are highly conserved among species in case of α-Syn, and interestingly, only the Ser87 site is located within the NAC domain of the protein, a region essential for aggregation and fibrillogenesis. Similarly to Ser129 phosphorylation, these sites show enhanced phosphorylation in synucleinopathies as well as their transgenic models [133]. In this study, Ser87 phosphorylation inhibited the oligomerization and reduced the synuclein-membrane interactions [133]. In the work of Oueslati et al., mimicking phosphorylation at Ser87 inhibited α-Syn aggregation, resulting in a reduced toxicity and alleviated motor impairment in rats. These results suggest that Ser87 phosphorylation plays a regulatory role in α-Syn induced neuropathology and may represent a valuable therapeutic strategy for the treatment

of PD [134]. However, contradictory results also exist in this field, as Ser87-phosphorylated α-Syn has a distinct morphology and was found to be more neurotoxic as compared with the wild-type protein [126].

Phosphorylation sites can be found in the C-terminal region as well (i.e. Tyr125, Tyr133, and Tyr136), their role, however, has not yet been clarified. It may be of importance, however, that the degree of tyrosine phosphorylation of α-Syn was shown to gradually decrease with age in both flies and humans, with simultaneous development of DA-ergic neurodegeneration and inclusion body formation [37,90,92,135]. These results indicated that aging-related alterations in post-translational modifications that influence protein aggregation may be important in the development of PD and other neurodegenerative disorders [37].

Summarizing these results, it is questionable that the Ser129 phosphorylation promotes or inhibits α-Syn aggregation and neurotoxicity. However, this knowledge would be essential to define the role of α-Syn in the pathogenesis of PD and for the development of therapeutic strategies in this degenerative disease.

3.4. The role of small organic molecules in α-Syn aggregation

Antibiotics are widely used as antimicrobial agents to fight infectious diseases. Since their appearance, an enhanced control of infectious diseases led to a significantly better outcome and decreased mortality. In recent years, several antibiotics again have become the focus of interest due to their newly explored properties besides antimicrobial activity, such as anti-inflammatory, antitumor, and possibly neuroprotective activity (Figure 5) [97]. In this respect, rifampicin was first suggested to have neuroprotective properties after the observation that its use is associated with a decreased amyloid-β deposition and a reduced incidence of dementia in leprosy patients [97,136]. Rifampicin was able to inhibit amyloid-β 1–4 peptide aggregation and fibril formation, and prevented amyloid-β induced neurotoxicity [137]. A clinical study involving patients with mild-to-moderate Alzheimer's disease showed that rifampicin was able to slow cognitive impairment measured by the Standardized Alzheimer's Disease Assessment Scale cognitive subscale (SADAScog) [138]. However, a more recent multicenter trial did not confirm these promising results [139]. Nevertheless, the inhibitory effect of rifampicin on amyloid-β aggregation suggested that it may have a similar effect on α-Syn as well. Accordingly, an *in vitro* study demonstrated that rifampicin is able to stabilize α-Syn as a monomer and block the fibrillation process; moreover, it was also able to disaggregate existing fibrils [98]. Another *in vitro* study confirmed the ability of rifampicin to prevent MPP+-induced toxicity in PC12 cells, increase their survival and reduce α-Syn oligomer formation [99]. Rifampicin has not yet been investigated in clinical trials of PD, only in MSA, where a clinical trial of rifampicin failed to demonstrate efficacy for slowing progression [100].

Ceftriaxone, a β-lactam antibiotic has also been suggested to have neuroprotective capabilities. Ceftriaxone is well-tolerated and is able to cross the blood–brain barrier. A recent study by Ruzza et al. revealed that ceftriaxone is capable of specifically binding to α-Syn and diminishing its polymerization *in vitro* [101]. In a murine model of PD, rapamycin was described to improve α-Syn oligomer clearance by increasing autophagy, as measured by an elevated immunoreactivity of MAP light chain 3 (LC3) [102].

The promising results of *in vitro* and *in vivo* models suggest that several antibiotics may be of therapeutic value in PD via counteracting α-Syn aggregation. Importantly, rapamycin and

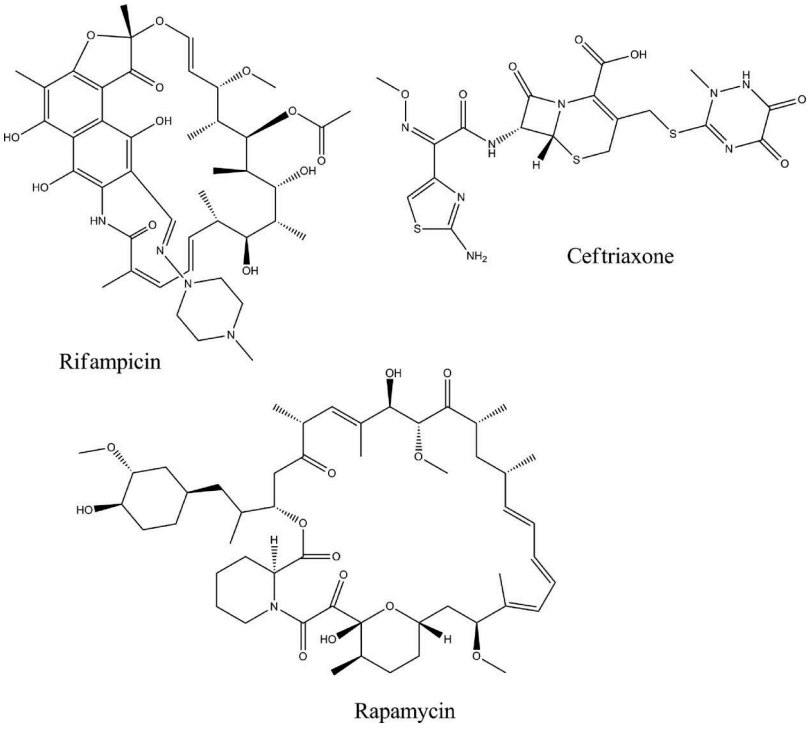

Figure 5. Schematic representation of three antibiotics with the potential to interfere with α-synuclein oligomerization and accumulation.

ceftriaxone are both blood–brain barrier permeable; therefore, they may reach the target areas and act directly in the affected brain regions.

Polyphenols are natural compounds widely present in several medicinal plants, vegetables and fruits, for example green tea, grapes, red wine, apples, or strawberries. Several polyphenols have been described to have antioxidant and anti-inflammatory properties, and have been suggested to have neuroprotective capacities as well [97]. Several different polyphenolic compounds have been identified to have anti-fibrillogenic or fibril-destabilizing effects and are therefore widely studied in models of PD and Alzheimer's disease as well. The most well-known among these molecules is epigallocatechin gallate (EGCG), which has been described to be able to prevent fibrillogenesis of both α-Syn and amyloid-beta [103]. EGCG was also able to convert already existing α-Syn fibrils into smaller, non-toxic aggregates [104]. In an MPTP-induced model of PD, EGCG prevented α-Syn accumulation in the SNpc [105]. In another study, a mixture of tea polyphenols inhibited α-Syn oligomer accumulation in the striatum of MPTP-treated monkeys and also reduced DA-ergic neuronal injury and motor impairment. The main compound of this mixture was also EGCG [106]. Besides EGCG, several other polyphenolic compounds have been demonstrated to counteract α-Syn fibrillation, such as quercetin, baicalein, or curcumin [97,107–109]. Polyphenols have been confirmed to not only prevent α-Syn aggregation but also to disaggregate existing α-Syn oligomers [110]. The promising results with polyphenolic compounds suggest that they warrant further investigations to confirm their potential therapeutic value in *in vivo* models to identify the possibility of future drug development.

Another interesting approach to counteract α-Syn aggregation is the designing of peptide inhibitors. Identifying residues 64–100 as a binding region of α-Syn led to the development of short peptides containing this region, which were able to inhibit α-Syn aggregation [111]. Another group identified residues 77–82 as a key region for protein aggregation and designed an *N*-methylated peptide containing this region, which was able to inhibit α-Syn aggregation [112]. Based on these results, several small peptide inhibitors have been synthesized using a multiplexed intracellular protein-fragment complementation assay library screening system. Among these, a peptide inhibitor was created which successfully abolished α-Syn aggregation and prevented its toxicity [113]. The results of these investigations point to the possibility of using peptide inhibitors as novel drug candidates to develop disease-modifying therapies for PD.

4. Conclusions

Results from genetic, neuropathological, and biochemical studies indicate that α-Syn represents an important therapeutic target for synucleinopathies, including PD. Moreover genetic studies with duplication or triplication drew the attention on the importance of the quantity of α-Syn. According to this hypothesis, the reduction of the total α-Syn level may be of therapeutic benefit. This reduction can be achieved either by downregulating the expression or enhancing the degradation of the protein. Chaperones play essential roles in protein folding and degradation, and their pharmacological manipulation could provide therapeutic possibilities for synucleinopathies. Altering post-translational modifications that influence aggregation (e.g. phosphorylation) may represent a beneficial target as well; however, the results are at present too contradictory to permit final conclusion. Decreasing the toxic soluble oligomeric species is of high importance.

Passive and active immunization targeting α-Syn have both been tested in preclinical studies with promising results, and initial-phase clinical trials are already underway. Immunotherapeutical approaches may offer valuable future candidates for drug development.

Several antibiotics have been suggested to be able to prevent α-Syn aggregation *in vitro*; however, these results are only initial and need to be confirmed by both *in vitro* and *in vivo* investigations. Polyphenolic compounds exhibited promising anti-aggregation properties in *in vitro* and preclinical studies, but further investigations are needed to confirm their beneficial effects. In recent years, several peptide inhibitors have been designed to counteract α-Syn aggregation, but these investigations are only in very preliminary phase.

5. Expert opinion

After the promising results obtained from *in vitro*, yeast, and *Drosophila* studies, intensive research has begun with chaperones in synucleinopathies. The first of the four discussed therapeutical possibilities takes advantage of Hsp90 inhibition, which leads to enhanced Hsp70 formation. At the same time, however, Hsp90 was also found to prevent α-Syn from aggregating in an ATP-independent manner, resulting in the development of harmless and non-toxic complexes [114]. Accordingly, Hsp90 inhibition may not be the most suitable approach to enhance the expression of chaperones due to its potential protective role. The second therapeutic option is the modulation of HSF-1 or other mechanisms that elevate the expression of Hsp70. This strategy is promising and can be linked to the third option, gene therapy, an approach that can be used to increase the expression of chaperones. Gene therapy has both advantages and disadvantages. The most beneficial advantage is its potential disease-modifying effect, and the potential long-term alteration of gene expression by the use of genome-integrated lentiviral vectors. The two main disadvantages are craniotomy, an unavoidable potential source of adverse events, and insertion mutagenesis, a phenomenon which may appear when the viral vector integrates into the host genome. In this respect, potential disease-modifying and neuroprotective approaches have come into the spotlight, including the virus-mediated upregulation of chaperones. The main disadvantages of the recently applied viral vectors include the low penetration through the blood–brain barrier, the poor specificity to the target cells, the limitation of the size of the transferred gene, the potential risks of immunogenicity and carcinogenicity, and because of their low penetration through the blood–brain barrier reaching their target requires stereotaxic surgery [115]. Therefore, non-viral vector-mediated alternative strategies are in the limelight of

research. One of them is the CPP technology, which has an important benefit as compared with the virus-mediated approaches, as CPPs are able to transport the Hsps through the blood–brain barrier and, therefore, craniotomy is not necessary [71].

Although immunotherapy targeting α-Syn is in the early phase of development, promising results have already been published. However, there are a number of open questions which still remain to be answered. Most data came from animal models or *in vitro* studies. As regards animal experiments, the use of different animal models in studies applying passive immunotherapy and the fact that the different antibodies were not compared in the same model represent an important limitation [78]. These animal models differ in respect of the presence of α-Syn, the extent of neurodegeneration, and the manifestation of behavioral and motor symptoms as well, which makes it difficult to draw general conclusions. Furthermore, the applied antibodies were administered in different dosing regimens. In the future investigations, it would be necessary to compare the different antibodies targeting different regions of α-Syn in the same animal model. It would also be interesting to compare the antibodies in both the transgenic and acute animal models, as it would promote the better understanding of the role of α-Syn in the different stages of PD.

Active immunization has a number of advantages over passive immunization techniques. It is cheaper and requires less frequent administration. However, its therapeutic effect strongly depends on the immune system of the patient, and it is therefore less controlled. The delivery of antibodies into brain is an important issue that can also be more efficiently overcome by passive immunotherapy, as it is possible to synthesize antibodies capable of penetrating the blood–brain barrier [85].

Another important concern regarding immunotherapy is the safety and the risk of potential vascular or autoimmune adverse events. Animal studies are needed to investigate these safety concerns, and the promising *in vitro* and *in vivo* results need to be confirmed by clinical trials. To date, only the first clinical trials have been initiated, and long-term follow up studies need to clarify the long-term risks of immunotherapeutical approaches [85]. Although a number of questions need to be clarified, immunotherapy may be an effective disease-modifying and neuroprotective therapy as it targets underlying molecular mechanism and not only the symptoms.

Phosphorylation of proteins plays essential roles in their biochemical and biological functions and may affect their intracellular localization. Phosphorylation induces a conformational switch relevant in the regulation of protein–protein and protein–ligand interactions. In this respect, the C-terminal region deserves a special attention, as phosphorylation in this region is likely to influence the affinity of α-Syn for other proteins [37,140–143]. This protein is localized in different parts of the cell, including the nucleus, mitochondria, and lysosomal vesicles [14,37,144,145]; however, the physiological role of α-Syn has not yet been clearly established. It is likely that it has an important function in controlling synaptic neurotransmission, possibly via the regulation of SNARE complex integrity [37,146–149]. In this review, we summarized the results of studies investigating the roles of the three main phosphorylation sites of the protein, and concluded that the main question, i.e. whether phosphorylation enhances or protects against α-Syn toxicity, still remains unresolved.

As regards Ser129 phosphorylation, the results are largely based on gene overexpression studies using S129E/D and S129A directed mutations. The most important criticism of these works is that these substitutions do not reproduce all aspects of phosphorylation, which may in part explain the contradictory results. Besides the Ser129 phoshorylation site, the Ser87 site has also been investigated in a few studies, and the results are likewise contradictory. It would be necessary to investigate the physiological role of Ser87 and its effect on the protein function in wild-type and mutant α-Syn proteins both *in vitro* and *in vivo*.

The main risk factor of neurodegenerative diseases such as PD is aging, which gives a special importance to the observation indicating that tyrosine phosphorylation gradually decreases with age and is further diminished in PD [90]. As tyrosine phosphorylation of α-Syn may have neuroprotective effects, its gradual decrease during aging may contribute to the increased incidence of PD among the elderly; however, this hypothesis needs confirmation.

In future studies, the further identification of kinases and phosphatases that are involved in regulating α-Syn phosphorylation and dephosphorylation, respectively, should facilitate the understanding of the role of phosphorylation of α-Syn in PD pathogenesis.

In recent years, several antibiotics have been proposed to have neuroprotective properties besides their well-known antimicrobial activity. To date, only limited data are available from *in vitro* experiments and animal models [97]. The currently available data are promising, suggesting that rifampicin, ceftriaxone, and rapamycin are able to diminish α-Syn aggregation and may result in neuroprotection. However, these data are at present insufficient to permit final conclusion, and further confirmation is necessary by *in vitro* and *in vivo* experiments, with the latter being essential to shed light on crucial questions such as the optimal dose and duration of therapy sufficient to achieve neuroprotection, as well as the possible adverse effects.

Several polyphenolic compounds have been described to prevent α-Syn aggregation in *in vitro* studies. EGCG has already been tested in animal models as well, where it also exerted beneficial effects not only by counteracting α-Syn accumulation but also by preventing neuronal damage and motor impairment. A growing number of evidence is available suggesting the neuroprotective effects of polyphenols. However, most data came from *in vitro* investigations or to a lesser extent, from animal models. Human data are up to date lacking, thereby future investigations are needed to assess whether the preclinical results may be replicated in clinical studies as well. In addition, further investigations are needed to assess the safety, the necessary doses, and the duration of the therapy to achieve neuroprotection.

Peptide inhibitors are a novel field in the development of drugs which may prevent the toxic effects of α-Syn aggregation. So far only a limited number of peptide inhibitors have been designed, which on the other hand, demonstrated

promising effects against α-Syn aggregation and toxicity. Further investigations are warranted to develop potent peptide inhibitors, and to assess their possible adverse effects, toxicity, degradation, and efficacy in animal models.

A growing number of investigations aim to target α-Syn aggregation and thereby result in neuroprotection. However, most data are currently only from *in vitro* studies or animal experiments, and despite promising results there are also conflicting data available, which raise more questions regarding the specificity of these therapeutic attempts. Targeting α-Syn aggregation may be a valuable future therapeutic option, but for most therapeutic options, the exact mode of action, the therapy duration and dosage and potential side effects need to be investigated.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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