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United Arab Emirates University

College of Medicine and Health Sciences

SCREENING FOR NOVEL INHIBITORS OF ALPHA-YNUCLEIN SEEDED NUCLEATION-DEPENDENT AGGREGATION AND TOXICITY AS A POTENTIAL THERAPEUTIC STRATEGY FOR PARKINSON'S DISEASE

Mustafa Taleb Ardah

This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Professor Omar El-Agnaf

December 2014

Declaration of Original Work

I, Mustafa Taleb Ardah, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Screening for novel inhibitors of alpha-synuclein seeded nucleation-dependent aggregation and toxicity as a potential therapeutic strategy for Parkinson's Disease*". Hereby, solemnly declare that this dissertation is an original research work that has been done and prepared by me and under the supervision of Professor Omar El-Agnaf, in the college of Medicine and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or similar title at this or any other university. The materials borrowed from other sources and included in my dissertation have been properly cited and acknowledged.

Student's Signature _____ Date _____

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Approval of the Doctorate Dissertation

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Abstract

α-Synuclein aggregation is the key pathogenic event in several important neurological disorders including Parkinson's Disease, dementia with Lewy bodies and multiple system atrophy, giving rise to a distinct group of neurodegenerative diseases known as synucleinopathies. Although the molecular basis of α-syn toxicity has not been precisely elucidated, recent studies indicate that α-syn toxicity is mediated by a nucleation-dependent aggregation process. To elucidate the structural basis of α-synuclein mediated toxicity, we developed various methods to prepare different α-synuclein species of a defined size and morphology distribution, and we investigated their toxicity in different human dopaminergic cell lines. We observed that crude α-synuclein oligomers preparations, containing both monomeric and heterogeneous mixtures of α-synuclein oligomers, were the most toxic species. The toxicity of α-synuclein aggregates was directly linked to the presence of the monomeric α-synuclein, and strongly dependent on its ability in seeded nucleation-dependent aggregation converting into amyloid fibrils. Therefore any effort to identify compounds that could inhibit or even reverse the aggregation process should assess the effect of the potential inhibitors on the seeded aggregation of α-synuclein, among others. We screened thirty Chinese herbal medicinal compounds for their effect on α-synuclein aggregation, seeded polymerization and toxicity by employing biophysical, biochemical and cell-culture-based techniques. Among the screened compounds, only ginsenoside Rb1, salvianolic acid B, dihydromyricetin and gallic acid were shown to be strong inhibitors of α-syn fibrillation, seeded aggregation and toxicity. Our results showed that gallic acid, ginsenoside Rb1 and salvianolic acid B inhibit α-synuclein fibrillation by binding and stabilizing the structure of

the soluble, non-toxic oligomers, which are devoid of β-sheet content. In contrast, dihydromyricetin was found to be able to bind to both oligomeric and monomeric species of α-synuclein. In the case of gallic acid, the inhibition of α-synuclein fibrillation is related to the compound's hydroxyl moieties whose number and position on the phenyl ring were proven to be significant for the process of inhibition, as indicated by the structure activity relationship data obtained from fourteen structurally similar benzoic acid derivatives. Overall, the compounds identified herein may represent the starting point for designing new molecules that could be utilized as drugs for the treatment of Parkinson's Disease and related disorders.

Keywords: α-Synuclein, Parkinson's Disease, aggregation, seeded-nucleation polymerization, amyloid fibrils, drug discovery

Title and Abstract (in Arabic)

تشخيص مركبات فريدة قادرة على منع تراكم بروتين ألفا سنيوكلين والسمية المرتبطة بهذا التراكم كعلاج محتمل لمرض باركنسون.

يعتبر تراكم البروتين ألفا سنيوكلين عاملا أساسيا في إمراضية عدد من الأمراض العصبية و على رأسها مرض باركنسون. بالرغم من أن دور هذا البروتين لا يزال قيد البحث، إلا أن الدراسات الحديثة تشير إلى أن سميته تبدأ بتشكيل نواة هي الركيزة لعملية تجمع و تراكم البروتين. لفهم الآلية الجزيئية المرتبطة بسمية ألفا سنيوكلين، طورنا تقنيات لتحضير أشكال مختلفة من البرويتن ذات أبعاد و تشكلات محددة، ثم قمنا بدراسة سميتها على الخلايا العصبية المنتجة للدوبامين ا(لدوبامنيرجية). لقد لاحظنا أن " أوليغمرات" البرويتن، الحاوية على خليط غير متجانس من " المونمرات" و " ألأوليغمرات" المتفاوتة الأبعاد، هي الأكثر سمية. كما ارتبطت سمية البروتين ألفا سنيوكلين بشكل مباشر بوجود " مونمرات" البروتين و اعتمدت بشكل أساسي على قدرة هذه "المونمرات" على تشكيل النواة الركيزة لعملية تجمع و تراكم البروتين لتؤدي بالمحصلة لتشكيل ألياف الأميلويد. بناء على ما سبق، فإن الجهود العلمية يجب أن تركز على تطوير مركبات قادرة على تثبيط تشكل هذه النواة.

تضمن البحث ثلاثين مركبا مستخلصا من أعشاب طبية صينية، حيث تم توظيف تقنيات بيوكيميائية وبيوفيزيائية و أخرى اعتمدت على الخلايا العصبية كوسيط البحث لدراسة قدرة هذه المركبات على منع تجمع أو تراكم البروتين ألفا سنيوكلين و تثبيط سميته.

من مجموعة المركبات التي تم دراستها, أربعة مركبات فقط أثبتت فعاليتها في تثبيط ومنع تراكم هذا البروتين والسمية الناتجة عن ذلك. هذه المركبات المكتشفة, قد تستخدم كبداية لتصنيع عقاقير قادرة على علاج مرض باركنسون.

الكلمات الدالة: بروتين ألفا سنيوكلين, مرض باركنسون, تراكم البروتين, تراكم البروتين المعتمد على التنوية, الآلياف االنشوية, أكتشاف الآدوية.

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Finally, I would like to thank my family (my mother, my sister and brothers) for their unconditional support throughout these years. Special credit has

to go to my wife, who has gone out of her way to accommodate my needs as a researcher. Words cannot describe her contribution.

Dedication

This work is dedicated to my late father, who has been a constant inspiration during my PhD study.

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List of Abbreviations/Nomenclature/Symbols

Aβ	β-Amyloid
AD	Alzheimer's Disease
ALS	Motor Neuron Disease
α-syn	α-Synuclein
APP	β-Amyloid Precursor Protein
β-syn	β-Synuclein
CK1	Casein Kinase 1
CK2	Casein Kinase 2
CR	Congo Red
DLB	Dementia with Lewy Bodies
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyri Bonucleic Acid
DA	Dopamine
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay
γ-syn	γ-Synuclein
G418	Geneticin Selective Antibiotic
GO	Glyoxa l
GRK2	G-protein-Coupled Kinase

Hsp70	Heat Shock Protein 70
HRP	Horseradish Peroxidase
LBs	Lewy Bodies
L-Dopa	Levodopa
LNs	Lewy Neurites
LRRK2	Leucine-rich Repeat Kinase 2 gene
MGO	Methylglyoxal
MSA	Multiple System Atrophy
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NAC	Non-amyloid Component
NMR	Nuclear Magnetic Resonance
PARK2	Parkin Gene
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween 20
PD	Parkinson's Disease
PINK1	PTEN-induced Kinase 1 Gene
PLK	Polo-like Kinases
ROS	Reactive Oxygen Species
rpm	Rounds Per Minute
RT	Room Temperature
s.d.	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis

SUMO	Small Ubiquitin-like Modifier
SN	Substantia Nigra
SNCA	α-Synuclein Gene
TEM	Transmission Electron Microscopy
Th-T	Thioflavin T
TG	Transglutaminase
Tween-20	Polyoxyethylenesorbitan Monolaurate
Ub	Ubiquitin
wt	Wild Type

1 Chapter 1: Introduction

1.1 Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder caused by the gradual loss of dopaminergic neurons (Obeso et al., 2008). PD affects about 1-2% of the current population over the age of 65, with an average duration of 8 years. Although the peak age of onset for PD is between 55 to 65 years, the age of onset for the disease ranges from 20 to 80 years, the incidence of which rises sharply after the fifth decade of life, affecting 1.4% and 3.4% of the population at 55 and 75 years of age, respectively (N. Wood, 1997). Cases of PD with an age of onset between 21 and 40 years have been called "young-onset Parkinson's Disease" and when it starts before the age of 21 it is called juvenile parkinsonism (Quinn, Critchley, & Marsden, 1987).

PD affects a region of the brain known as the substantia nigra, resulting in a dramatic loss of dopaminergic neurons and a concomitant plummeting in dopamine levels in the striatum. As a consequence, PD is manifested by a plethora of clinical symptoms, with the most prominent being impaired motor activity, muscle rigidity and a resting tremor. Neuropathologically, PD is characterized by the presence of intracellular inclusions known as Lewy bodies (LBs) and Lewy neuritis *(LNs)*, the main constituent of which are α-synuclein (α-syn) fibrils (El-Agnaf et al., 1998; El-Agnaf, Jakes, Curran, & Wallace, 1998; Jakes, Spillantini, & Goedert, 1994; Spillantini et al., 1997). Most cases of PD are idiopathic (85%), which are characterized by late-onset, non-inheritable movement disorder with neuronal loss in the brain (reviewed by (Irvine, El-Agnaf, Shankar, & Walsh,

2008). Dopamine replacement by L-3-4-dihydroxyphenylalanine (L-DOPA) has helped to increase dopamine in the neurotransmitter-depleted striatum. Although this is effective treatment in the early stages of PD, it will lose its therapeutic qualities with time. To date there is no treatment that can arrest or reverse the loss of dopaminergic neurons. The etiology of PD is poorly understood, but convergent pathological, biochemical, animal models and genetic evidence suggests that the formation of α-syn protein deposits is an important and, probably, seminal step in the development of PD and related disorders.

Although α-syn belongs to the family of natively unfolded proteins, which demonstrate little or no ordered structure, the protein has the intrinsic propensity to fibrillate, giving rise to insoluble fibrils similar to the ones detected in LBs (Conway, Harper, & Lansbury, 2000b; El-Agnaf, Jakes, Curran, Middleton, et al., 1998; Giasson, Uryu, Trojanowski, & Lee, 1999b; Hashimoto et al., 1998b). Additionally, five missense mutations in the α-syn gene (*SNCA*), namely A53T (M. H. Polymeropoulos et al., 1997), A30P (Krüger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Appel-Cresswell et al., 2013) and G51D (Kiely et al., 2013), have been linked to inherited forms of PD, while duplications and triplications of *SNCA* lead to autosomal dominant PD in a gene dosage-dependent fashion (Ibáñez et al., 2004; Singleton et al., 2003b). Moreover, transgenic animal and drosophila models expressing either wild-type or mutant human α-syn also develop fibrillar inclusions and a Parkinsonian phenotype (Cannon et al., 2013; Feany & Bender, 2000; Imai, Venderova, Park, Cai, & Schmidt, 2011; Kahle et al., 2001; Masliah et al., 2000). It is noteworthy that the fibrillation of α-syn is implicated in the development of a series of neurodegenerative diseases, including multiple system atrophy (MSA) and dementia with Lewy bodies (DLB), which

are collectively referred to as alpha-synucleinopathies (Spillantini & Goedert, 2000; Trojanowski & Lee, 2003). Taken together, these findings indicate a central role for α-syn aggregation in PD pathogenesis α-syn aggregation proceeds through several key intermediate stages, with monomeric α-syn first assembling into oligomeric forms that gradually generate insoluble amyloid fibrils. Because α-syn aggregation plays a crucial role in PD pathogenesis and related alpha-synucleinopathies, intensive effort has been put into identifying compounds that could block or even reverse the aggregation process. Over the years, polyphenols, a set of more than 8000 compounds that contain one or more phenolic rings, have emerged as potent amyloid inhibitors, interfering with the *in vitro* fibril assembly of many amyloidogenic proteins including α-syn, β-amyloid (Aβ), tau-protein and prions (reviewed by (Porat, Abramowitz, & Gazit, 2006).

1.2 Synuclein family

Synucleins are small, soluble proteins highly expressed by neuronal cells (Jakes, et al., 1994) . The synuclein family has three distinct members α-, β- and γ-syn (reviewed by (Amer, Irvine, & El-Agnaf, 2006). Structurally, synucleins are characterized by the repetitive presence of the amino acid motif KTKEGV throughout the first 87 residues, while their C-terminal region is rich in acidic amino acids. Moreover synucleins are natively unfolded proteins meaning that they have no structure under physiological conditions and their structure is composed of random coils. Also, synucleins have high net charge and low hydropathy (reviewed by (Amer, et al., 2006). The synuclein family genes are mainly expressed in the CNS but their distribution in the brain is slightly varied.

However, the synuclein family is not so expressed in other tissues such as the heart, lung, skeletal muscle, ovaries, testes, colon and spleen.

1.2.1 α-Synuclein

α-Syn is a soluble protein with a sequence of 140 amino acid residues (Jakes, et al., 1994; Uéda et al., 1993). *SNCA* is the gene that α-syn is encoded by and it is located on the long arm of chromosome 4 (4q21-q23) (X. Chen et al., 1995; Xia et al., 2001). Some homologous protein for α-syn have been identified in electric ray (Maroteaux, Campanelli, & Scheller, 1988), rat (i.e. synuclein-1) (Maroteaux & Scheller, 1991), zebra finch (i.e. synelfin) (J. M. George, Jin, Woods, & Clayton, 1995) and other species. α-Syn was first discovered in the neuromuscular junction of the electric ray. Antibodies against α-syn labelled both synapses and nuclei lead to the name synuclein (Maroteaux, et al., 1988). Three isoforms of α-syn protein have been identified, 140 amino acids at full-length form while the other two are truncated at 3' end resulting in 126 and 112 amino acids proteins (Beyer et al., 2008; Campion et al., 1995). α-Syn can form amphipathic helices when it interacts with phospholipid layers (J. L. George et al., 2009), and the α-helical structure will intensify according to the binding to small synthetic, acidic, unimolecular vesicles (W. Sean Davidson, Ana Jonas, David F. Clayton, & Julia M. George, 1998). It was suggested that α-syn could be part of the fatty acid-binding protein family due to its biochemical properties and structural motif, hence α-syn could transport fatty acids between the aqueous and membrane phospholipid compartments of neuronal cytoplasm (Sharon et al., 2001). The abundance of α-syn in presynaptic terminal and early stages of development indicate that α-syn plays an important role on the maintenance of

synaptic vesicle pools and maintenance of presynaptic function (Cabin et al., 2002).

El-Agnaf and co-workers have demonstrated that α-syn can self-aggregate and form amyloid-like filaments similar to those that have been isolated from LBs, and that the mutants α-syn proteins associated with familial form of PD aggregate faster than the wild-type protein (El-Agnaf, Bodles, Guthrie, Harriott, & Irvine, 1998).

Animal models, genetics and biochemical studies support the hypothesis that accumulation of α-syn in the brain has a significant role in the pathogenesis of a number of neurodegenerative diseases like PD. α-Syn has little or no ordered structure under physiological conditions, and its secondary structure is a random coil (Weinreb, Zhen, Poon, Conway, & Lansbury, 1996), however, recent studies have indicated that α-syn occurs as a helically folded tetramer (Bartels, Choi, & Selkoe, 2011). The region covered by the first 100 amino acid residues of α-syn adopts an α-helical structure upon binding to phospholipid membranes (Chandra, Chen, Rizo, Jahn, & Südhof, 2003), lipid vesicles (Bussell & Eliezer, 2003b; W. S. Davidson, A. Jonas, D. F. Clayton, & J. M. George, 1998; Perrin, Woods, Clayton, & George, 2000) and detergent micelles (Bussell & Eliezer, 2003b). The consensus sequence KTKEGV is considered to play a key role in the α-helical structure assumed by α-syn (W. S. Davidson, et al., 1998; Weinreb, et al., 1996). The NAC region of α-syn is exceptionally hydrophobic and amyloidogenic, thus promoting the conformational change from random coil to β-structure (El-Agnaf & Irvine, 2000; Uéda, et al., 1993). In contrast, the acidic C-terminal region remains unfolded and associates with vesicles or micelles (Eliezer, Kutluay, Bussell, & Browne, 2001b). However, the C-terminus is responsible for many

transient long-range interactions with the N-terminus and the NAC region, thus conferring stability to the α-syn molecule (Sung & Eliezer, 2007; K. P. Wu, Kim, Fela, & Baum, 2008). As far as the physiological function of α-syn is concerned, it remains unclear. However, research indicates that α-syn may play a role in neural plasticity (J. M. George, et al., 1995) and in normal SNARE protein assembly, which is critical for neurotransmitter release and vesicle recycling (Burré et al., 2010).

1.2.2 β-Synuclein

The β-synuclein gene (*SNCB*) is located on chromosome 5q35 and codes for 134 amino acid protein named β-synuclein (β-syn). α- and β-Syn share 78% homology and, β-syn has a conserved C-terminal domain containing three identically placed tyrosine residues. Both α- and β-syn have similar subcellular localization and expression patterns in the brain. However, β-syn lacks 11 hydrophobic amino acid residues that correspond to the 72-84 residues of α-syn (Fig. 1.1). In a recent study, β-syn was shown to have a neuroprotective role by inhibiting α-syn aggregation. Cells over expressing β-syn are more resistant to α-syn accumulation (Hashimoto et al., 1998a). Moreover, cells that overexpress β-syn activate the serine threonine kinase AKT (also known as protein kinase B) signalling pathway and are more resistant to α-syn neurotoxicity (Hashimoto et al., 2004). In transgenic mice, β-syn reduces α-syn accumulation in the brain and improves survival rates, as well as motor dysfunction (Ahmad, Attoub, Singh, Martin, & El-Agnaf, 2007). Double transgenic mice expressing human *SNCA* and

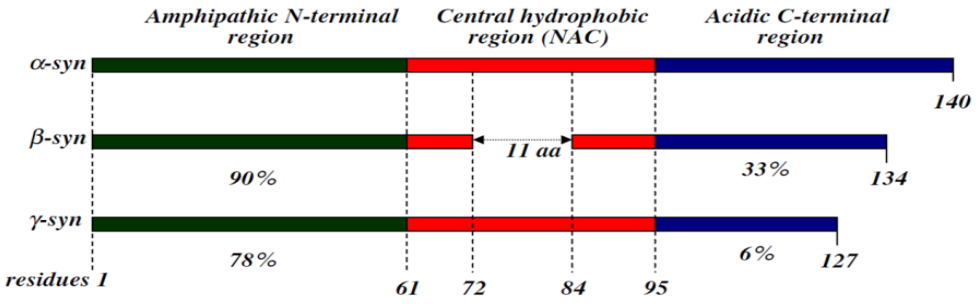


Figure 1.1: Sequence homology of synuclein family.

SNCB showed a reduced accumulation of α-syn in the brain (Hashimoto, et al., 2004). Additionally, it has been reported that β-syn exhibits a chaperone activity more efficiently than α-syn (D. Lee, Paik, & Choi, 2004). However, unlike α-syn, β-syn has much less a tendency to aggregate and form amyloid fibrils.

1.2.3 γ-Synuclein

The third member of the synuclein family, the γ-syn gene (*SNCG*) is located on chromosome 10q23 and encodes a 127 amino acid protein (reviewed by (Ahmad, et al., 2007). The γ-syn gene was first referred to as BCSG1 (breast cancer-specific gene 1), the levels of which increase dramatically in breast carcinoma (Ji et al., 1997). However, the name was later changed to *SNCG* due to its high homology to synuclein proteins (Lavedan et al., 1998), despite the fact that γ-syn is overexpressed in breast cancer tumours (Ninkina et al., 2003).

Similar to α- and β-syn, the N-terminus of γ-syn is conserved but its C-terminus shows reduced homology to other members of the family. γ-Syn colocalizes with neurofilaments in the axons (Ninkina, et al., 2003), and alters neurofilament network integrity by rendering neurofilament-H vulnerable to calcium-dependent

proteases (Buchman et al., 1998). Furthermore, γ-syn is expressed in the early stages of axonal outgrowth and maintained throughout its life, thus indicating that γ-syn has a role in the formation of the nervous system (Buchman, et al., 1998). Unlike α-syn, γ-syn has a weak tendency to form amyloid fibrils (Giasson, Murray, Trojanowski, & Lee, 2001).

1.3 Genes associated with PD

Genetic studies have linked several genes to PD. These genes can be categorized based on their mode of inheritance: autosomal dominant like α-syn (*SNCA*) and Leucine-rich repeats kinases 2 (*LRRK2*) or autosomal recessive like *Parkin* and

PINK1. However, this classification can be challenging due to the reduced penetration in the dominant form and the putative role of single heterozygous mutations in the recessive form (see Table. 1.1).

The study of PD genetics can be a complex task given the interaction of several genes that are encoded by nuclear or mitochondrial DNA both, or epigenetic (reversible, heritable change in gene expression without a change in the DNA sequence), not to mention the influence of environmental factors that alter the expression of relevant genes in relation to age-dependent effects.

Table 1.1**.** Loci and genes associated with PD adapted from Eschbach and Danzer,2013.

Designation	Gene	Locus	Protein function	Inheritance	LBs
PARK 1	*SNCA* point mutation	4q21	among others: synaptic vesicle release and homeostasis	autosomal dominant (AD)	+
PARK 2	*Parkin*	6q25–q27	ubiquitin ligase	autosomal recessive (AR)	–/(+)
PARK 3	unknown	2p13	unknown	AD	+
PARK 4	*SNCA* genomic multiplication	4q21	see above	AD	+
PARK 5	*UCH-L1*	4p14 – 15	ubiquitin-hydrolase/ligase	AD	unknown
PARK 6	*PINK-1*	1q35 – 36	mitochondrial protein kinase	AR	+
PARK 7	*DJ-1*	1p36	oxidative stress response	AR	Unknown
PARK 8	*LRRK2*	12p11.2–q13.1	protein kinase	AD	+
PARK 9	*ATP13A2*	1p36	lysosomal type 5 P-ATPase	AR	unknown
PARK 10	unknown	1p32	unknown	not clear	unknown
PARK 11	*GIGYF2*	2q36–q37	unknown	AD	unknown
PARK 12	unknown	Xq21–q25	unknown	X linked	unknown
PARK 13	*HTRA2/Omi*	2p12	HtrA2/Omi stress-regulated serine endoprotease	AD	+
PARK 14	*PLA2G6*	22q13.1		AR	unknown
PARK 15	*FBXO7*	22q11.2-qter		AR	unknown
PARK 16	*SLC45A3* *NUCKS1* *RAB7L1* *SLC41A1* *PM20D1*	1q32	(magnesium transporter) RAB7L1 (small GTP-binding protein playing an important role in regulating exo- and endocytotic pathways) NUCKS1 (nuclear protein containing several consensus phosphor - ylation sites for casein kinase II and cyclin-dependent kinases)	not clear	unknown
PARK 17	*GAK/DGKQ*	chomosome 4p	GAK (cyclin G-associated kinase, a cell cycle regulator) DGKQ (diacylglycerol kinase, theta; involved in the phosphatidylinositol signalling system)	not clear	unknown
PARK 18	*HLA DRA*	6p21.3	class II HLA-DR antigens that are expressed by antigenpresenting cells	not clear	unknown

1.3.1 α-Synuclein gene (*SNCA*)

To date *PARK1* has been shown to play a significant role in PD as demonstrated in the genetic analysis of a large Italian family (Golbe, 1990). The clinical symptoms are similar to sporadic PD but have an early age of onset (mean

age 45.6 years), and slightly more rapid course towards death (9.2 years). The missense mutation G to A transition at position 209 in exon 4 of *SNCA* was identified as the disease locus resulted in replacement of alanine with threonine at position 53 (A53T) (Mihael H. Polymeropoulos et al., 1997). Kruger and co-workers have sequenced *SNCA* gene in a German family with familial PD, and they found a strong link between G for C substitution at nucleotide 88 in exon 3 of the *SNCA* gene. This substitute replaces alanine for proline at amino acid 30 (A30P) (Kruger et al., 1998). Patients with A30P mutation will have an onset of PD in their 50s, slightly later than the mean age in the A53T patients. In 2004, a third missense mutation in *SNCA* responsible for autosomal dominant PD and dementia was found in a Spanish family. A single base pair change at position 188 from G to A resulting in the replacement of acidic polar glutamate residue with the basic polar lysine residue at position 46 (E46K) (Zarranz, et al., 2004). Moreover, H50Q (Appel-Cresswell, et al., 2013) and G51D (Kiely, et al., 2013) mutations have been reported recently. Duplications (Chartier-Harlin et al., 2004) and triplication of *SNCA* (triplication involving the 1.5 Mb region) (Singleton et al., 2003a) have also been reported, and it is believed that the severity of PD depends on the dosage of *SNCA*.

1.4 Aggregation of α-Synuclein

The fact that aggregated α-syn is the major component of inclusions in α-synucleinopathy brains suggests that α-syn aggregation may be relevant to the pathogenesis of neurodegenerative disorders such as PD, DLB and MSA. The morphology of wild type (wt) or mutant α-syn fibrils generated *in vitro* is similar to that detected in LBs (Conway, Harper, & Lansbury, 1998; El-Agnaf, Jakes, Curran, Middleton, et al., 1998; Giasson, Uryu, Trojanowski, & Lee, 1999a;

Hashimoto, et al., 1998b; Serpell, Berriman, Jakes, Goedert, & Crowther, 2000), also implying a key role of α-syn aggregation in LB pathology.

The fibrillation of α-syn is a nucleation-dependent polymerization process (S. J. Wood et al., 1999), with an initial lag phase, an exponential phase and an equilibrium phase (Fig. 1.2)

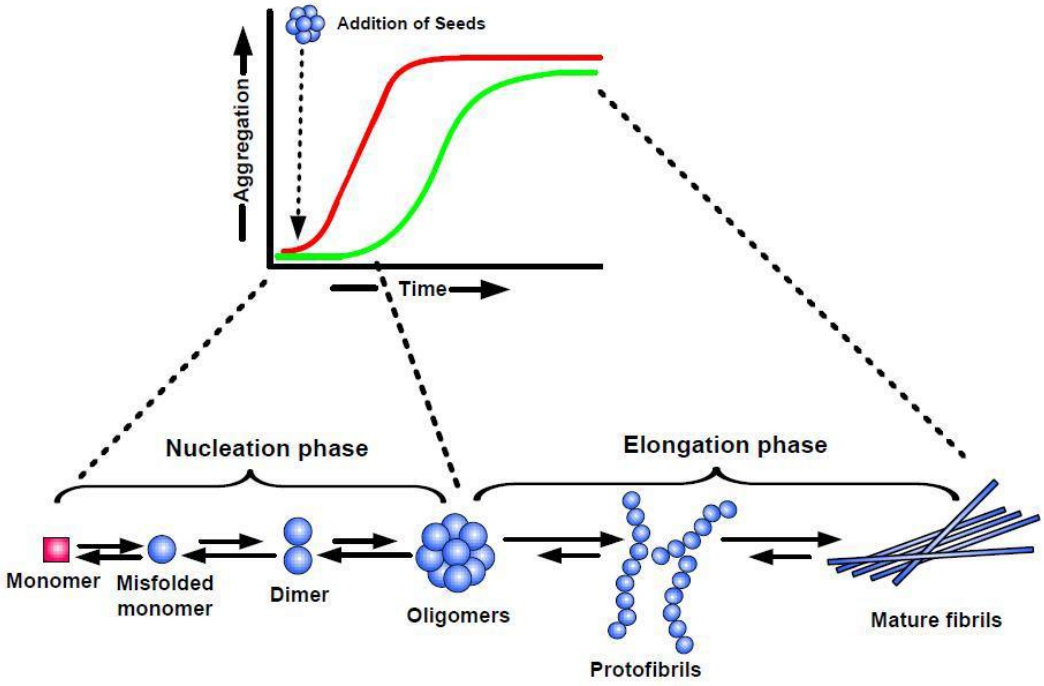


Figure 1.2: Nucleation-dependent polymerization model of amyloid aggregation (Adapted from (Kumar & Walter, 2011).

During the lag phase, the formation of nuclei takes place, followed by the exponential growth of the fibrils, which eventually reach a dynamic equilibrium state with protein in solution (Kaylor et al., 2005b; Serpell, et al., 2000; Uversky, M Cooper, Bower, Li, & Fink, 2002). α-Syn fibrillation proceeds through oligomerisation i.e. the formation of multiple non-fibrillar oligomer intermediates termed protofibrils, which subsequently convert into mature amyloid fibrils (Conway, Harper, et al., 2000b; Kaylor, et al., 2005b). The secondary structure content of the protofibrils varies as a β-sheet structure appears in larger aggregates

(El-Agnaf, Bodles, et al., 1998; Nath, Meuvis, Hendrix, Carl, & Engelborghs, 2010). It was shown that spheroidal oligomers assume both an α-helical and to a lesser degree, a β-sheet conformation, the extent of which decreases and increases, respectively, as aggregation proceeds (Apetri, Maiti, Zagorski, Carey, & Anderson, 2006).

It was suggested that fibrils develop from oligomers by further aggregation. The aggregation of monomeric α-syn into fibrils runs through different stages, where different intermediated forms protofibrils and insoluble fibrils, or remains as oligomers (Caughey & Lansbury, 2003; Hoyer et al., 2002; Munishkina, Phelan, Uversky, & Fink, 2003).

Various forms of protofibrils have been observed including spherical (Conway et al., 2000a; Ding, Lee, Rochet, & Lansbury, 2002; D. P. Hong, Han, Fink, & Uversky, 2011) and spheroidal (Apetri, et al., 2006), short chain-like (Conway, Lee, et al., 2000a), circular such as ring-like, annular (doughnut-like) and pore-like (Conway, et al., 1998; Ding, et al., 2002; Lashuel et al., 2002; Lowe, Pountney, Jensen, Gai, & Voelcker, 2004), tubular (Lashuel, et al., 2002), granular (Bhak, Lee, Hahn, & Paik, 2009) and globular (Apetri, et al., 2006). As far as the structure of α-syn fibrils is concerned, it is characterised by the presence of a central core composed of cross β-sheet structure, in which β-strands extend perpendicularly to the fibril axis (M. Chen, Margittai, Chen, & Langen, 2007; Der-Sarkissian, Jao, Chen, & Langen, 2003; Heise et al., 2005). Each β-strand comprises approximatelly the amino acid residues 31-110 of α-syn monomer (Del Mar, Greenbaum, Mayne, Englander, & Woods, 2005; Der-Sarkissian, et al., 2003; Heise, et al., 2005; Vilar et al., 2008).

The fibrillation of α-syn can be affected by various factors *in vitro*. The factors modulating fibrillation *in vivo* may differ from those operating *in vitro*; yet dissecting the *in vitro* aggregation of α-syn can lead to the identification of aggregation inhibitors and a better understanding of the underlying mechanisms.

1.5 Implications of α-syn oligomers and fibrils:

Even the LBs contain mature fibrils of α-syn, there is evidence suggesting that oligomeric and protofibrilar forms of α-syn are the pathogenic species in PD (El-Agnaf, Walsh, & Allsop, 2003; Winner et al., 2011). A number of mechanisms have been suggested as a potential consequences of α-syn aggregates or oligomers as endoplasmic reticulum stress (Smith et al., 2005), oxidative stress (Esteves, Arduíno, Swerdlow, Oliveira, & Cardoso, 2009), modified chaperon activity (Chandra, Gallardo, Fernández-Chacón, Schlüter, & Südhof, 2005), dysfunction of the ubiquitin proteasome system (Stefanis, Larsen, Rideout, Sulzer, & Greene, 2001; Y. Tanaka et al., 2001), defects in the mitochondria and Golgi apparatus (Gosavi, Lee, Lee, Patel, & Lee, 2002; Hsu et al., 2000; Song, Shults, Sisk, Rockenstein, & Masliah, 2004), defective axonal transport (A. A. Cooper et al., 2006; Dalfó, Barrachina, Rosa, Ambrosio, & Ferrer, 2004; Outeiro & Lindquist, 2003), vesicle permeabilisation (Volles & Lansbury, 2002; Volles et al., 2001) and lysosomal defects (Cuervo, Stefanis, Fredenburg, Lansbury, & Sulzer, 2004; Stefanis, et al., 2001). It has been suggested that α-syn pathology spread gradually throughout the neuraxis as PD progress (Braak et al., 2003). As the underlying mechanism of disease progression in PD is still unknown, some recent studies showed that grafted healthy neurons gradually develop the same

pathology as the host neurons in PD brains (Kordower, Chu, Hauser, Olanow, & Freeman, 2008; J. Y. Li et al., 2008) which raises the possibility that α-syn is released from neurons to the extracellular space and is taken up by other cells. This is consistant with the fact that α-syn can be detected in cerebrospinal fluid and plasma in measurable quantities (El-Agnaf et al., 2006; Mollenhauer et al., 2008). Also *in vitro* experiments have shown that fibrillar and oligomeric forms of α-syn can be taken up from extracellular space by neurons via the endocytic process (H. J. Lee et al., 2008). Other studies showed that recombinant α-syn oligomers are also taken up by cultured neurons and initiate cell death (Danzer et al., 2007; Danzer, Krebs, Wolff, Birk, & Hengerer, 2009). The prion-like mechanism, could represent a possible mechanism responsible for the host-to-graft transfer of PD pathology as suggested by Desplats and colleagues (Desplats et al., 2009) when they demonstrated that α-syn can be directly transmitted from neuronal cells overexpressing α-syn to transplanted embryonic stem cells, in tissue culture and transgenic animals. The increasing amount of data makes the possibility that a specific conformation of α-syn is transmitted from host cells that promote the aggregation of α-syn and trigger toxicity in adjacent neurons. The impact of seeding on amyloid aggregation has also been established by a number of studies indicating that α-syn aggregation can be induced and accelerated by the addition of exogenous oligomers and fibrils in cultured cells (Danzer, et al., 2007, Volpicelli-Daley, et al., 2011, Nonaka, et al., 2010) and in mouse brains (Luk, et al., 2012, Masuda-Suzukake, et al., 2013), supporting the hypothesis that the aggregation of the native protein is directly seeded by exogenous aggregates. Overall, these studies support the notion that amyloid toxicity is mediated by a nucleation-dependent polymerization process.

1.6 Mutations and truncations

1.6.1 Mutations

Five missense mutations in the α-syn gene (SNCA), namely A53T (Mihael H. Polymeropoulos, et al., 1997), A30P (Krüger, et al., 1998), E46K (Zarranz, et al., 2004), H50Q (Appel-Cresswell, et al., 2013) and G51D (Kiely, et al., 2013), have been linked to severe inherited forms of PD, while duplications and triplications of SNCA lead to autosomal dominant PD in a gene dosage-dependent fashion (Ibáñez, et al., 2004; Singleton, et al., 2003b). α-Syn mutations, A30P, A53T E46K and H50Q , have been reported to promote the aggregation of the protein (Chi, Armstrong, Jones, Eisenmesser, & Liu, 2014; Conway, Harper, et al., 2000b; El-Agnaf, Jakes, Curran, & Wallace, 1998; Giasson, et al., 1999a; Greenbaum et al., 2005; Khalaf et al., 2014; J. Li, Uversky, & Fink, 2001; Narhi et al., 1999). Whereas A30P mutation acts by stabilizing α-syn into oligomers (Conway, Harper, & Lansbury, 2000a), A53T and E46K increase the propensity of the protein to fibrillate (Conway, et al., 1998; Conway, Harper, et al., 2000b; Greenbaum, et al., 2005). It was recently reported that while E46K accelerates the oligomerization of α-syn it can seed fibril formation more efficiently compared to wt and A30P oligomers (Ono, Ikeda, Takasaki, & Yamada, 2011). Recently G51D mutation was found to significantly attenuate α-syn aggregation *in vitro* and to decrease the ability of α-syn to bind to lipid vesicles C-terminal to the site of mutation (Fares et al., 2014).

1.6.2 C-terminal truncations

Partially truncated α-syn has been detected in LBs from DLB brains (Anderson et al., 2006; Baba et al., 1998), glial cytoplasmic inclusions (GCIs) of

MSA (Gai, Power, Blumbergs, Culvenor, & Jensen, 1999) and abnormal neurites of Alzheimer's Disease without LBs (Lewis et al., 2010), suggesting that C-terminal truncations may play a role in the aggregation of α-syn. A number of *in vitro* studies showed that C-terminally truncated human α-syn, such as 1-87, 1-102, 1-110, 1-119 and 1-120 assembles at much faster rates compared to full length and mutant protein (Crowther, Jakes, Spillantini, & Goedert, 1998; Lewis, et al., 2010; C. W. Liu et al., 2005; Murray et al., 2003; Serpell, et al., 2000), and certain truncated fragments can also seed the aggregation of full length α-syn by nucleating full-length α-syn and then forming hybrid protofibrils, which develop into fibrils (J. Kim, Harada, Kobayashi, Kobayashi, & Sode, 2010; C. W. Liu, et al., 2005; Murray, et al., 2003). An i*n vivo* study showed that the expression of truncated human α-syn (1–120) on synuclein null background mouse, lead to the formation of pathological inclusions in the substantia nigra and olfactory bulb and to a reduction in striatal dopamine levels (Tofaris et al., 2006).

Although the pathological input of C-terminally truncated α-syn is still unclear, it is has been suggested that the negative charges of the C-terminal domain of α-syn exert counteracting influences on α-syn fibrillation at the initial steps of polymerization and seeding (Levitan et al., 2011; McLean & Hyman, 2002; Murray, et al., 2003).

1.7 Post-translational modifications

Several post-translational modifications have been described for α-syn (Fig. 1.3), including serine and tyrosine phosphorylation, ubiquitination, oxidation and tyrosine crosslinking, nitration and enzymatic cross-linking.

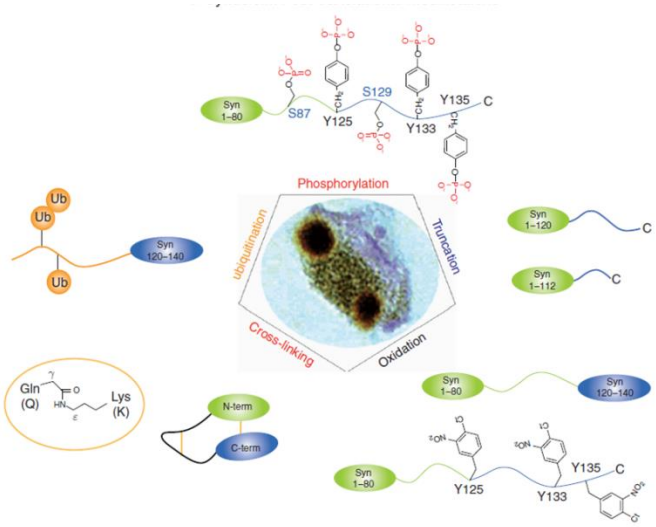


Figure 1.3: α-syn post-translational modifications reported on the basis of their identification in LB.

1.7.1 Phosphorylation

α-Syn accumulate in the LBs of DLB, PD, MSA and LB variant of Alzheimer's Disease has been shown to be phosphorylated at S129 (p-S129 α-syn). Furthermore, α-syn phosphorylated at S87 (p-S87 α-syn) has been detected in the LBs of DLB, while the levels of p-S87 α-syn were shown to be increased in the brains of AD, DLB and MSA patients, as well as the brains of synucleinopathic mice (Paleologou et al., 2010). Taken together these findings advocate a pathological role for phosphorylated α-syn, and much effort has been put into elucidating the kinases that regulate α-syn phosphorylation and the effect of phosphorylation on α-syn aggregation and toxicity.

Although, several kinases have been shown to phosphorylate α-syn *in vitro*, little is known about the kinases that phosphorylate α-syn in the brain. α-Syn can be constitutively phosphorylated *in vitro* and in cultured cells at S87 by casein kinase (CK) 1 (Okochi et al., 2000) and the dual specificity tyrosine-regulated kinase 1A (Dyrk1A) (E. J. Kim et al., 2006). Also, p-S87 α-syn colocalizes with CK1 in LBs of DLB brains (Paleologou, et al., 2010). As far as the S129

phosphorylation is concerned, several enzymes have been demonstrated to phosphorylate α-syn at this position: (a) CK1 and CK2 phosphorylate α-syn at S129 both *in vitro* and in cells (G. Lee et al., 2004; Okochi, et al., 2000; Zabrocki et al., 2008). P-S129 α-syn colocalizes with CK2 in the brain of PD transgenic mice (Wakamatsu et al., 2007), while CK2 was the major kinase isolated from rat brain homogenates that phosphorylated human α-syn at S129 (Ishii et al., 2007). Also, up regulation of CK2 by Fe(II) was shown to enhance p-S129 α-syn levels in cells (M. Takahashi et al., 2007), whereas inhibition of CK2 in SH-SY5Y cells only resulted in 30% reduction of p-S129 α-syn, suggesting that CK2 is not the only enzyme that phosphorylates α-syn, at least in cells (Chau, Ching, Schapira, & Cooper, 2009). (b) G protein-coupled receptor kinases (GRKs), such as GRK1, GRK2, GRK5 and GRK6 phosphorylate α-syn *in vitro* and in cells (Arawaka et al., 2006; Pronin, Morris, Surguchov, & Benovic, 2000). (c) Polo-like kinases (PLK 1-3) have also been reported to specifically phosphorylate α-syn at S129 *in vitro* and in cells (Inglis et al., 2009; Mbefo et al., 2010; Waxman & Giasson, 2011), and PLK2 was shown to increase in AD and DLB brains (Mbefo, et al., 2010). Furthermore, α-syn can also be phosphorylated at Y125 (L. Chen et al., 2009; Ellis, Schwartzberg, Grider, Fink, & Nussbaum, 2001; Nakamura, Yamashita, Takahashi, & Nakamura, 2001; Negro, Brunati, Donella-Deana, Massimino, & Pinna, 2002; T. Takahashi et al., 2003) *in vitro* and in cultured cells by c-Src, Fyn and Syk kinases (Ellis, et al., 2001; Nakamura, et al., 2001), at Y133 and Y136 (Ellis, et al., 2001; Negro, et al., 2002). Y125 phosphorylation of α-syn was detected in a PD *Drosophila* model (L. Chen, et al., 2009).

Given the lack of knowledge of the kinases that phosphorylate α-syn in the brain, the cost and the purification restrictions of *in vitro* phosphorylation, many

studies have employed phosphorylated mutant α-syn that either mimics phosphorylation by introducing a negative charge, i.e. replacement of serine in question by aspartic or glutamic acid, S→D or E, or renders phosphorylation impossible by introducing an amino acid that cannot be phosphorylated, *i.e.* alanine, S→A.

Comparison of the aggregation propensity of wt, S129A and S129D α-syn indicated that phosphorylation at S129 has no effect on the fibrillation of α-syn as all proteins produced fibrils with the same morphology and abundance (L. Chen & Feany, 2005), while another study comparing wt, S129A and S129E α-syn showed that it was the mutant that mimics the lack of phosphorylation S129A that promotes fibrillation, rather than the S129E mutant, which had a fibrillation propensity similar to wt (Paleologou, et al., 2010). An i*n vitro* phosphorylation of phosphomutant α-syn S87A and S87/S129A by CK1 or CK2 showed that phosphorylation at S129 inhibits fibrillation of α-syn (Paleologou, et al., 2010; Waxman & Giasson, 2008) in a dose dependent manner (Paleologou et al., 2008). However, *in vitro* phosphorylation of wt α-syn by CK2 promoted the fibrillation of α-syn (Fujiwara et al., 2002). S87 phosphorylation, *in vitro* aggregation studies demonstrated an inhibitory effect on fibrillation of α-syn (Paleologou, et al., 2010; Waxman & Giasson, 2008).

Expression of S129D α-syn in SH-SY5Y cells induced higher levels of cell death than wt and S129A α-syn (Chau, et al., 2009), expression of which has been associated with decreased toxicity in SH-SY5Y cells (Sugeno et al., 2008). In a drosophila model, S129A was shown to have a higher aggregation propensity, but to be less toxic, while the phosphomimic S129D or GRK2-phosphorylated α-syn resulted in fewer inclusions but accelerated the cell loss (L. Chen & Feany, 2005).

In transgenic mice, however, S129D was associated with increased α-syn deposition but decreased toxicity, unlike S129A α-syn, which promoted dopaminergic cell loss (Gorbatyuk et al., 2008). Studies on a rat model showed that S129A α-syn enhances α-syn toxicity and promotes the formation, proteinase K-resistant and β-sheet-rich aggregates (Azeredo da Silveira et al., 2009), whereas S129D α-syn is not toxic but leads to the formation of fewer, larger aggregates (Azeredo da Silveira, et al., 2009). Subsequent studies on a transgenic rat model revealed that wt, S129A and S129D α-syn resulted in comparable degrees of toxicity and inclusion formation, and it was proposed that the differences seen in the results with rodents may be due to differences in the experimental design, such as an incubation or dose of the α-syn delivering virus (McFarland et al., 2009). Variations in the results may also lie in the fact that S129D/E α-syn do not reproduce all structural and fibrillation aspects of native phosphorylation; phosphorylated α-syn has an extended conformation and does not fibrillate, while S129D/E mutations have a localized effect and they possess a fibrillation property similar to wt α-syn (Paleologou, et al., 2008).

Interestingly, Y125 phosphorylation was shown to reduce the soluble oligomeric forms of α-syn induced by expression of S129D or the GRK2-phosphorylated α-syn in *Drosophila* (L. Chen, et al., 2009).

There are also certain findings suggesting that α-syn aggregation is an event occurs prior to phosphorylation. Indeed, fibrillar α-syn has been shown to be a good substrate for CK1, CK2 and PLKs *in vitro* (Mbefo, et al., 2010; Paleologou, et al., 2010; Waxman & Giasson, 2008), while in transgenic *Drosophila*, the appearance of p-S129 α-syn followed the deposition of un phosphorylated α-syn (T. Takahashi, et al., 2003). Additionally, expression of PLKs in cells increased

the phosphorylation of soluble α-syn, but it did not promote its aggregation (Waxman & Giasson, 2011).

Finally, given that phosphorylation is an important post-translational modification that regulates protein function and subcellular localization, as well as a wide range of cellular functions, it can not be excluded that α-syn phosphorylation may have a non-pathogenic function. A proteomics-based study identified phosphorylation-dependent α-syn interactions (McFarland, et al., 2009), while there is increasing evidence that phosphorylated α-syn has a nuclear localization (Mbefo, et al., 2010; Wakamatsu, et al., 2007).

1.7.2 Oxidation and tyrosine crosslinking

Oxidation is another post translational modification that has been shown to affect α-syn aggregation. Although all amino acids can be potentially oxidized, methionine is the most readily oxidized amino acid (reviewed by (Vogt, 1995). α-Syn contains 4 Met residues (at positions 1, 5, 116 and 127), and it was recently reported that Met5 is more prone to oxidation compared to the others (W. Zhou et al., 2010). An *in vitro* oxidation of which (induced by hydrogen peroxide) has been shown to inhibit the aggregation of the protein at normal pH (Uversky et al., 2002), and promotes the formation of stable, non-toxic oligomers (W. Zhou, et al., 2010). The degree of inhibition of fibrillation of oxidized α-syn was shown to be proportional to the number of oxidized Met that the protein contains, i.e. the more oxidized Met the slower the fibril formation, suggesting that the oxidation of individual Met has a cumulative effect on α-syn fibrillation (Hokenson et al., 2004). Interestingly, Met-oxidized α-syn could also inhibit the fibrillation of non-oxidized α-syn in a concentration dependent fashion (Uversky, Yamin, et al., 2002).

Furthermore, direct oxidation of α-syn by hydrogen peroxide in the presence of cytochrome c or hemin was shown to induce aggregation of the protein possibly due to Tyr cross-linking (Hashimoto, Takeda, Hsu, Takenouchi, & Masliah, 1999; Olteanu & Pielak, 2004; Souza, Giasson, Chen, Lee, & Ischiropoulos, 2000). When exposed to hydrogen peroxide, cytochrome c forms tyrosyl radicals, which in turn can be transferred to Tyr residues on other proteins (Deterding, Barr, Mason, & Tomer, 1998), such as α-syn, which contains Tyr residues at positions 39, 125, 132 and 135.

1.7.3 Nitration

Nitrated α-syn has been detected in brain tissues of patients with PD and other α-synucleinopathies (Dalfó, Martinez, Muntané, & Ferrer, 2006; Giasson et al., 2000). α-Syn can be nitrated at Tyr residues through the action of oxygen and nitric oxide, as well as their products, such as peroxynitrite. Whereas it was initially believed that the exposure of α-syn to nitrating agents, such as peroxynitrite, induces aggregation of the protein (Paxinou et al., 2001; Souza, et al., 2000), it was later shown that *in vitro* Tyr nitration of α-syn inhibits the fibrillogenesis of protein (Norris, Giasson, Ischiropoulos, & Lee, 2003; Yamin, Uversky, & Fink, 2003), and it has been suggested that it is the formation of covalent dityrosine crosslinks rather than nitration *per se* that promotes the fibrillogenesis of the protein under oxidative conditions (Norris, et al., 2003; Yamin, et al., 2003).

1.7.4 Ubiquitination

Ubiquitin (Ub) is a small protein, the role of which is closely related to the function of proteasome and the regulation of protein turnover in cells. Ub is

conjugated to the protein to be degraded by the proteasome via a three step process, which requires the action of certain enzymes, that are collectively known as E1, E2 and E3 ligases. α-Syn can be ubiquitinated, and so far three proteins, namely parkin, Siah-1 (seven in absentia homologue-1) and Siah-2, have been identified to play the role of E3 enzymes. Siah-2 ubiquitinates the unmodified form of α-syn (Liani et al., 2004), while Siah-1 ubiquitinates both the glycosylated (Franck et al., 2006) and the unmodified α-syn (J. T. Lee, Wheeler, Li, & Chin, 2008). Whereas the lysines at positions 21, 23, 32 and 34 are liable for *in vitro* ubiquitination, only lysines 6, 10 and 12 are used for *in vivo* ubiquitination, which resembles the ubiquitination of filaments (Nonaka, Iwatsubo, & Hasegawa, 2005). In LBs, α-syn has been show to be mono-, bi- and tri-ubiquitinated (Tofaris, Razzaq, Ghetti, Lilley, & Spillantini, 2003), while in cells, UCHL1 ubiquitinates α-syn at Lys-63 (Y. Liu, Fallon, Lashuel, Liu, & Lansbury, 2002), linked ubiquitination of which is usually not associated with proteosomal degradation, but promotes LB formation (Lim et al., 2005; Lim, Dawson, & Dawson, 2006). Similarly, the Siah-1 mono- and di-ubiquitination of α-syn does not target the protein for degradation in HeLa cells, but instead it promotes α-syn insolubility and apoptotic cell death in HeLa and PC12 cells (J. T. Lee, et al., 2008). Interestingly, the Siah-1 mediated ubiquitination of α-syn was shown to be abolished by the A30P, but not the A53T mutation, possibly due to the inhibition of the ubiquitination of the lysines near alanine 30 (i.e. K21, K23, K32 and K34) resulting from the A30P-induced conformational change in α-syn (J. T. Lee, et al., 2008).

1.7.5 Glycosylation and glycation (non-enzymatic glycosylation)

Glycosylation is a post-translational modification during which glycans, i.e. oligosaccharides or polysaccharides are enzymatically attached to proteins or other macromolecules. Glycosylation is a site-specific modification and the major sites of glycosylation in proteins are the Asn and Arg (*N*-glycosylation), and Ser, Tyr and Thr (*O*-glycosylation). Although *O*-glycosylated α-syn, αSp22, has been identified in normal human brain (Shimura et al., 2001), it is still unknown whether glycosylation affects the aggregation of the protein. Nevertheless, in LBs from PD patients, α-syn co-localises with Advanced Glycation Endproducts (AGEs), a heterogeneous group of molecules formed through glycation (also known as non-enzymatic glycosylation, which as the name implies is, unlike glycosylation, non enzymatic and occurs randomly) of proteins via the Maillard reaction (i.e. chemical reaction between amino acids and reducing sugars) and Schiff's base formation (i.e. the formation of imine products from the reaction between amino acids and a reactive carbonyl group of reducing sugars) (Castellani, Smith, Richey, & Perry, 1996).

In brain, glycation of proteins is attributed to sugars like glucose, fructose, and reactive dicarbonyls, such as methylglyoxal (MGO), glyoxal (GO) and 3-deoxy glucosone (Negre-Salvayre, Salvayre, Augé, Pamplona, & Portero-Otín, 2009). Dicarbonyl molecular species have also been implicated in the production of AGEs, which can modify prion proteins and Aβ (Williams, Weinberg, & Smith, 2011). Similarly, *in vitro* glycation of α-syn by D-ribose (ribosylation) induced the formation of molten globules, which were toxic to SH-SY5Y cells (L. Chen, Wei, Wang, & He, 2010).

1.7.6 SUMOylation

SUMOylation is a reversible post-translational modification that covalently attaches small ubiquitin-like modifier (SUMO) polypeptides, which are similar to Ub, to lysines of proteins. The process is similar to ubiquitination, with the difference that SUMOylation does not mark proteins for degradation) (reviewed by (Dorval & Fraser, 2007). It is well documented that SUMOylation is implicated in the pathogenesis of several neurodegenerative diseases, including polyglutamine diseases (Ueda et al., 2002), neuronal intranuclear inclusion disease and MSA (Pountney et al., 2003), while a number of proteins involved in neurodegeneration e.g. amyloid precursor protein (reviewed by (Sarge & Park-Sarge, 2011), huntingtin (Steffan et al., 2004), tau (Dorval & Fraser, 2006), DJ-1 (Shinbo et al., 2006) also undergo SUMOylation.

α-Syn has also been shown to be SUMOylated in cells co-expressing α-syn and SUMO proteins (SUMO 1-3) (Dorval & Fraser, 2006) and it was recently reported that SUMO1 co-localises with α-syn in LBs of PD and DLB brains (Y. M. Kim et al., 2011).

1.2 Cross-linking and Tissue Tranglutaminase

Transglutaminases (TG) constitute a family of Ca^{2+}-dependent enzymes that catalyse the intermolecular and/or, more rarely, the intramolecular cross-linking of intracellular proteins by forming isopeptide bonds between lysines and glutamines (Folk & Chung, 1985; Konno et al., 2005). Tissue transglutaminase or transglutaminase 2, that possesses GTPase, ATPase and transamidating activities (Achyuthan & Greenberg, 1987), has been reported to play a role in a number of diseases, including neurodegenerative diseases (A. J. Cooper, Jeitner, & Blass, 2002). Aβ (Jensen, Sørensen, Petersen, Gliemann, & Rasmussen, 1995) and APP

protein (G. J. Ho, Gregory, Smirnova, Zoubine, & Festoff, 1994), the NAC fragment of α-syn (Jensen, et al., 1995) and α-syn (Junn, Ronchetti, Quezado, Kim, & Mouradian, 2003) are all substrates of tTG *in vitro* and/or *in vivo*.

1.8 α-Synuclein as a target for PD treatment

Conventional treatment of PD can only treat the symptoms; therefore, it can only be beneficial if the disease is diagnosed at an early stage. Unfortunately, with the progression of the disease, the treatment can gradually lose its effectiveness, as it is believed that treatment fails to prevent the ongoing cell death of dopaminergic neurons in SN. As discussed above dopaminergic neurons death is believed to be due to the development of LBs, which is composed mainly of α-syn aggregates. α-Syn changes from random coil to β-sheet conformation to form prefilbrillar oligomers and then transient protofibrils before maturing into amyloid fibrils and finally LBs (Park, Lasiene, Chou, Horner, & Pun, 2007). Several strategies have now focused on the prevention of α-syn aggregation and toxicity as a novel treatment strategy for PD (Amer, et al., 2006). As we learn more about the role of α-syn in PD, it becomes clear that preventing the aggregation or may be silencing *SNCA* can be a possible therapeutic strategy.

1.9 Inhibiting α-synuclein aggregation and toxicity as a novel therapeutic approach for PD

1.9.1 Small peptides

El-Agnaf and colleagues were the first to design peptide inhibitors for α-syn aggregation and toxicity (Hosia et al., 2004). A peptide library was synthesized from overlapping 7-mer peptides spanning the whole α-syn sequence. By means of a novel ELISA, the sequence responsible for α-syn self-aggregation was

identified (residues 69-72 of α-syn). This sequence was used as the basis for the design of various peptide inhibitors of α-syn aggregation and toxicity (El-Agnaf et al., 2004). Prompting a focus on developing peptidomimetics and identifying small molecule inhibitors for α-syn aggregation (Amer, et al., 2006), Hosia et al followed it up by designing several hairpin peptides and investigating their effect on amyloid formation including α-syn aggregation (Hosia, et al., 2004). A group of hairpin peptides showed to induce non amyloidogenic α-syn aggregates (non-toxic) suggested an inhibitory effect (Hosia, et al., 2004).

1.9.2 Dopamine and catecholamines

Many studies of dopamine (DA), DA metabolites, DA analogous and catecholamine in general have shown an effect on α-syn aggregation. In a recent study, Lee et al showed that DA can promote the production and secretion of α-syn oligomers (H. J. Lee et al., 2011). An increased formation of α-syn oligomers was found in the vesicles in the presence of DA. The oligomers were shown to be SDS resistant, non-fibrillar yet β-sheets-rich. Similar findings were seen with DA metabolites, where cell death is induced (Shaltiel-Karyo et al., 2010). However, it was shown that catechol o-metheltransferase inhibitors could prevent the toxicity of α-syn fibrils (Shaltiel-Karyo, et al., 2010).

1.9.3 Heat shock proteins

Heat shock proteins (HSP) are a class of functionally related proteins that act as molecular chaperones up-regulated by the cell under stress (Di Giovanni et al., 2010) . Hsp70 confers thermotolerance and protects against apoptosis, endotoxins, reactive oxygen species, radiations and ischemia (Norris, et al., 2003). It has been reported that Hsp70 is reduced in PD brains (Norris, et al., 2003). Both

Hsp70 and Hsp40 can bind and inhibit α-syn aggregation *in vitro* (Latawiec et al., 2010). A recent study showed that Hsp104 can reduce the phosphorylated form of α-syn and the nigrostriatal dopaminergic loss in a PD animal model (Arawaka, Machiya, & Kato, 2010). More recently, a compound termed SNX-0723 (2-fluoro-6-[(3S)-tetrahydrofuran-3-ylamino]-4-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1H-indol-1-yl)benzamide) a Hsp90 inhibitor, significantly increased striatal dopamine content in rats, suggesting a positive neuromodulatory effect on striatal terminals (McFarland et al., 2014). Taken together all these findings regarding Hsp inhibitors also represents an exciting new therapeutic strategy for PD.

1.9.4 Anti-Parkinsonian agents and other drugs

One class of drugs that was identified to ameliorate α-syn fibril formation in PD was sirtuin inhibitors (SIRT2) (Grünblatt et al., 2004). It was shown that SIRT2 inhibitors rescue α-syn mediated toxicity in a drosophila model of PD (Auluck, Chan, Trojanowski, Lee, & Bonini, 2002). Li et al., demonstrated the inhibitory effect of the antibiotic rifampicin on α-syn fibrillation that disaggregated α-syn amyloid fibrils in a concentration-dependent manner (J. Li, Zhu, Rajamani, Uversky, & Fink, 2004). Furthermore, rifampicin was also shown to reduce α-syn deposition in a transgenic mouse model of MSA (Putcha et al., 2010). Gangliosides (lipids that contain saccharides) have shown to interact with the helical structure of α-syn thus preventing its aggregation (Putcha, et al., 2010). Similar molecules like glycosphingolipids have been shown to behave as gangliosides, which indicate the structural importance of sialic acid and carbohydrate moieties in these molecules (Alcaín & Villalba, 2009). Selegiline is another drug which is anti-Parkinsonian and was also reported to delay the

nucleation stage of the wild type and mutant forms of α-syn oligomers (Braga et al., 2011). Interestingly, selegiline is a monoamino oxidase B (MAO-B) inhibitor, which increases the dopamine levels. The combine effect of selegiline by inhibiting MAO-B and delaying the nucleation of α-syn aggregation seems to favour the formation of non-toxic aggregates of α-syn (J. Li, et al., 2004).

1.9.5 Herbal and plant extracts

Herbal and plant extracts have been used as remedies for thousands of years for a variety of diseases including neurodegenerative diseases such as PD. Recent work has highlighted a few herbal and plant extract as potential drug such as Anemopaegma mirandum (Catuaba), Fructus Psoraleae, Turmeric, Pueraria Thomsonii and Bacopa Monnieri. However, only few of these extracts have been investigated for their effect on α-syn aggregation and toxicity.

Research showed that Baicalein (an herbal extract) has the two anti-oligomeric and anti-amyloidogenic properties that make it a potential drug for PD (Frydman-Marom, Shaltiel-Karyo, Moshe, & Gazit, 2011). Moreover, entacapone and tolcapone were also shown to prevent α-syn oligomerization (Di Giovanni, et al., 2010). Poly-acromatic scaffolds (polyphenols, flavonoids and phthalocyanines) have also shown anti-amyloidogenic qualities, this quality can be enhanced by an addition of metals into the heterocycle centre (Lu et al., 2011). Studies screened natural polyphenols have shown that (−)-epigallocatechin-3-gallate (EGCG) from green tea can also prevent α-syn aggregation. It was shown that EGCG could bind non-covalently to unfolded α-syn resulting in non-toxic SDS-stable oligomers. Thus, preventing the formation of the toxic amyloid fibrils. *In vivo* work has yet to determine and test its effectiveness in PD animal models, but nevertheless EGCG shows great promise as a potential drug for PD

(Ehrnhoefer, 2006; Dagmar E. Ehrnhoefer et al., 2008; D. E. Ehrnhoefer et al., 2008; Lamberto et al., 2011).

1.10 Immunotherapy for neurodegenerative diseases

Immunotherapy is considered the best disease-modifying treatment for neurodegenerative diseases. AD has been the focus of many immunotherapeutic studies, while less attention has been paid to PD and other neurodegenerative disorders. The reason for this difference is that the (Aβ) protein in AD is an extracellular molecule (Glenner & Wong, 1984) that circulates in the blood and is reading recognized by antibodies. In contrast, deposits of α-syn have been considered to be exclusively intracellular in nature (Spillantini, et al., 1997) . However, the recent discovery that toxic oligomeric versions of α-syn accumulate in the membrane and can be excreted to the extracellular environment (Desplats, et al., 2009; El-Agnaf, et al., 2006; Mollenhauer, et al., 2008) has provided a rationale for the development of immunotherapeutic approaches for PD, dementia with Lewy bodies, frontotemporal dementia, and other neurodegenerative disorders characterized by the abnormal accumulation of these proteins. Active immunization, passive immunization, and T cell-mediated cellular immunotherapeutic approaches have been developed targeting Aβ, α-syn and tau.

1.10.1 α-Synuclein as immunotherapeutic target

Several neurodegenerative diseases are characterized by the accumulation of misfolded proteins and different approaches using active or passive immunization are being explored as potential therapies. Recently, immunotherapy has been evaluated as an approach to treat α-syn pathology. Animals vaccinated with recombinant α-syn which results in a high-titre anti-α-syn antibody response on α-

syn, showed fewer pathologic aggregates in the striatum versus control animals that received a mock vaccine. This shows that a protective vaccination strategy results in induction of regulatory T cells and activated microglia. This can induce immune tolerance against α-syn (Romero-Ramos, von Euler Chelpin, & Sanchez-Guajardo, 2014). While antibodies against α-syn specifically target and aid in clearance of extracellular α-syn by microglia, thereby preventing their actions on neighbouring cells (Bae et al., 2012). It was reported that passive immunization with α-syn antibody reduced neuronal and glial accumulation of α-syn and ameliorated neurodegeneration and behavioral deficits associated with α-syn overexpression (Bae, et al., 2012). Targeting the C-terminal of α-syn with specific antibodies, found to attenuate synaptic and axonal pathology, reduced the accumulation of C-terminal truncated α-syn in axon, and improved motor and memory deficit in mouse PD model (Games et al., 2014). When transgenic α-syn mice were treated with anti-human α-syn antibodies, an improvement in behavioural deficits as well as decreased accumulation of α-syn aggregates, was observed and in addition the antibodies appear to enhance the phagocytosis and autophagy of α-syn (Masliah et al., 2011). In another study by the same group (Masliah et al., 2005), the active immunization of transgenic α-syn mice with human α-syn lead to the production of relatively high-affinity antibodies and reduced the accumulation of aggregated human α-syn in neuronal bodies and synapses. It was also proposed that immunization was found to degrade α-syn via the lysosomal pathway (Masliah, et al., 2005). It is possible that antibodies may recognize abnormal α-syn accumulation in the neuronal surface or even secreted forms of the protein.

Using intracellular antibody fragments (intrabodies) is another immunotherapy approach that could potentially find application in the treatment of PD, that can recognize α-syn and alter its folding. For that, human single-chain antibody fragments (scFv) bind to oligomeric α-syn and block extracellular α-syn toxicity in human neuroblastoma cells (Emadi, Barkhordarian, Wang, Schulz, & Sierks, 2007; Emadi, Kasturirangan, Wang, Schulz, & Sierks, 2009). In a similar study, intrabodies transfected into a cell line that overexpresses wild-type α-syn, stabilized detergent-soluble monomeric α-syn and inhibited the formation of detergent-insoluble high-molecular-weight α-syn species (C. Zhou, Emadi, Sierks, & Messer, 2004).

1.11 Aim of project

There are numerous studies suggesting a seminal role for α-syn aggregation in neurodegenerative diseases. Although the molecular basis of α-syn toxicity has not been precisely elucidated, recent studies indicate that α-syn toxicity is mediated by a nucleation-dependent polymerization process. Screening small compounds that can block, slow down or reverse α-syn aggregation and inhibit the seeded nucleation-dependent polymerization process, therefore, provides an attractive approach for targeting the progression of the PD and related disorders. The aim of the project was to elucidate the mechanism and structural basis of α-syn mediated toxicity, and to screen small compounds that are capable of inhibiting the aggregation and the mediated α-syn cytotoxicity. The idea behind screening compounds extracted from Chinese medicinal herbs and plants (CMH), that have been used for the treatment of a wide range of diseases for over a millennium including dementia and neurodegenerative disorders.

In the first results chapter (Chapter 3) various aggregated α-syn preparations are prepared. The effect of these species on α-syn mediated cytotoxicity and the addition of the monomeric wt or p-S129 α-syn effect on the mediated toxicity was investigated by cell viability assay (MTT), Thioflavin S (Th-S), congo red bunding assay (CR) and immunoblotting assays. In the second results chapter (Chapter 4) the effect of the phenolic compound, GA and its derivatives on α-syn fibril formation and α-syn mediated toxicity is investigated. The methods used in this chapter include ELISA, Th-S assay, TEM, MTT, CR and NMR. In the third and fourth chapter of results (Chapter 5 and 6), CMH compounds were examined for their effect on α-syn oligomerization and aggregation and mediated cell cytotoxicity by means of Th-S, TEM, MTT, immunoblotting and NMR.

2 Chapter 2: Materials and Methods

2.1 Suppliers

Details of the suppliers for chemicals and equipment are summarized in

Table 2.1. Name and location of Suppliers of Chemicals and Equipment

Short Name	Full name and Location
Aldrich/Sigma	Sigma-Aldrich Co. Ltd., Dorset, UK.
BD Biosciencese	Becton, Dickinson and Company, Franklin Lakes, USA.
Invitrogen	Invitrogen Corporation, 5791 Van Allen Way Carlsbad, CA 92008, USA
New England Biolabs	New England Biolabs, Ipswich, MA, USA
Nunc	Nalgene, Nunc International, Life Technologies Ltd., Paisley, UK.
Santa Cruz Biotechnology	Santa Cruz Biotechnology, Delaware Avenue, Santa Cruz, CA, U.S.A.
Gilson	Gilson, Inc.3000 Parmenter Street, Middleton, WI, USA.
Sterilin	Sterilin Limited, Aberbargoed, Bargoed, UK.
Panreac	Panreac. Ltd. Barcelona, Spain.
Amersham	GE Healthcare ,Uppsala Sweden
Bio-Rad	Bio-Rad Laboratories, Alfred Nobel Drive Hercules, CA, USA.
Pierce	Thermo Scientific Life Science Research Products, Rockford, IL, USA.
Hyclone	Thermo Scientific Life Science Research Products, Rockford, IL, USA
Abcam	Abcam, Cambridge, MA, USA

2.2 Materials and equipment

Table 2.2. Summary of Materials and Suppliers.

Materials/Equipment	Company
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Dulbsecco's Modified Eagle Medium	Invitrogen
Tissue Culture Flask 75CM X75CM	Nunc
Ethanol	Panreac
Ethidium bromide	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal bovine serum (heat activated)	Invitrogen
Hydrogen chloride (HCl)	Sigma-Aldrich
IPTG	Sigma
Methanol	Panreac
Mouse monoclonal IgG antibody α-synuclein (211)	Santa Cruz Biotechnologies
Nitrocellulose membrane filter paper sandwich, 0.45μm pore size	Invitrogen
OPTI-MEM Medium	Invitrogen
Penicilin-Streptomycin solution (100 ml solution/ 10,000 units penicillin, 10,000 units streptomycin)	Invitrogen
Nitrocellulose membranes	Whattman
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich
Sodium Azide (NaN_3)	Sigma-Aldrich
Sodium dodecylsulphate (SDS)	Sigma-Aldrich
Tris	Sigma-Aldrich
NP-40	Sigma
Triton X-100	Sigma-Aldrich
BCA Protein assay reagent kit	Pierce
Cell dissociation solution, non-enzymatic	Sigma-Aldrich
Benzamidine sepaharose beads	Amersham

Thioflavine T	Sigma
Thioflavin S	Sigma
SuperSignal ELISA Femto Maximum Sensitivity Substrate	Thermo Scientific
Microcentrifuge tube 1.5 ml	Rose Scientic
Tissue Culture Plates 96 Well	Nunc
Agarose Ultra Pure Electrophoesis Grade	Invitrogen
Bovine Serum Albumin	Sigma-Aldrich
TEMED	Sigma
Ammonium persulphate	Bio-Rad
Protein MW marker	Invitrogen
Loading buffer	Sigma-Aldrich
Glutathion sepharose beads 4B slurry	Amersham
Thrombin	Sigma
Tissue Culture Flask 25 cm	Nunc
Tissue Culture Flask 75 cm	Nunc
SuperSignal West Pico Substrate	Thermo Scientific
SuperSignal West Femto Substrate	Thermo Scientific
Paraformaldehyde	Sigma-Aldrich
Geneticin	Sigma-Aldrich
FL-140 rabbit polyclonal antibody	Santa Cruz
211 mouse monoclonal antibody	Santa Cruz
HRP-conjugated goat anti-rabbit	DAKO
Protease inhibitor	Pierce
HRP-conjugated goat anti-mouse	DAKO
Uranyl acetate	Agar
Sulfo-NHS-LC-Biotin	Pierce
Zeba desalting columns	Pierce
Formvar coated 400 mesh copper grids	Agar

2.3 Cell lines

Table 2.3. List of Cell lines used in this study

Cell line	Origin	Culture Media
wt BE(2)-M-17	Human Neuroblastoma	Dulbecco's MEM/Nutrient Mix F-12 (1:1) containing 10% fetal bovine serum and 1% penicillin-streptomycin.
BE(2)-M-17-wt-α-syn	Human Neuroblastoma	Dulbecco's MEM/High Glucose containing 10% fetal bovine serum, 1% penicillin-streptomycin and 250 µg/ml of G418 antibiotic.
wt-SHSY-5Y	Human Neuroblastoma	Dulbecco's MEM/Nutrient Mix F-12 (1:1) containing 15% fetal bovine serum, 1% penicillin-streptomycin, and supplemented with 1% non-essential MEM amino acid supplement and 2 mM freshly prepared glutamine..
SHSY-5Y-wt-α-syn	Human Neuroblastoma	Dulbecco's MEM/Nutrient Mix F-12 (1:1) containing 15% fetal bovine serum, 1% penicillin-streptomycin, and supplemented with 1% non-essential MEM amino acid supplement and 2 mM freshly prepared glutamine..

2.4 Compounds

Table 2.4. List of compounds tested in this study as inhibitors of α-syn aggregation and toxicity.

Compound	Name	M.W
CMC1	Salvianolic acid B	718
CMC2	Ginsenoside Rg1	800
CMC3	Ginsenoside Rg3	785
CMC4	Ginsenoside Rb1	1109
CMC5	AmmoniµM Glycyrrhizinate	840
CMC6	Puerarin	416.4
CMC7	Sinomenine	329.4
CMC8	Paeoniflorin	480.45
CMC9	Gamma-schisandrin	400.46
CMC10	Dihydromyricetin	320
CMC11	Magnolol	266.32
CMC12	Honokiol	266.34
CMC13	Geniposide	388.37
CMC14	Bilobalide	326.3
CMC15	Evodin	470.52

CMC16	Tetrahydropalmatine	355.42
CMC17	Protopine	353.37
CMC18	Sipeimine	429.64
CMC19	Synephrine	167.21
CMC20	Peinine	429.64
CMC21	Rhynchophylline	384.47
CMC22	Isorhynchophylline	384.47
CMC23	Berberine	336.1
CMC24	Gallic acid	170.12
CMC25	Praeruptorin	386.4
CMC26	Icariin	677.66
CMC27	Schisandrol B	416.464
CMC28	(+)-Tetrandrine	622.76
CMC29	Neohesperidin	610.56
PA1	3,4,5-trihydroxybenzoic acid	170.12
PA2	2,4,6-trihydroxybenzoic acid	188.13
PA3	2,3-dihydroxybenzoic acid	154.12
PA4	2,4-dihydroxybenzoic acid	154.12
PA5	2,5-dihydroxybenzoic acid	154.12
PA6	2,6-dihydroxybenzoic acid	154.12
PA7	3,4-dihydroxybenzoic acid	154.12
PA8	3,5-dihydroxybenzoic acid	154.12
PA9	2-hydroxybenzoic acid	138.12
PA10	3-Hydroxybenzoic acid	138.12
PA11	4-Hydroxybenzoic acid	138.12
PA12	Benzoic acid	122.12
PA13	4-methoxybenzoic acid	212.20
PA14	3,4,5-trimethoxybenzoic acid	152.15
PA15	3,4,5-trifluorobenzoic acid	176.09

2.5 Solutions and buffers

2.5.1 Phosphate Buffered Saline (PBS), pH 7.4

One PBS tablet was dissolved in 200 ml d.H_2O. The buffer was filtered through a 0.2 μm filter and stored at 4°C.

2.5.2 Phosphate Buffered Saline with 0.05% Tween-20 (PBST)

PBS with 0.05% Tween-20.

2.5.3 Blocking buffer for western blotting

5% of dried skimmed milk dissolved in PBST.

2.5.4 Stacking gel buffer

0.5M Tris-HCL: 6g of Tris-base were dissolved in 75ml d.H_2O and the pH was adjusted to 6.8. The volume was then made up to 100ml with d.H_2O.

2.5.5 30% acrylamide

29.2g acrylamide and 0.8g of bis-acrylamide was dissolved in 100ml of d.H_2O.

2.5.6 20% sodium dodecyl sulfate (SDS)

20g lauryl sodium dodecyl sulphate was dissolved in 100ml d.H_2O.

2.5.7 10% ammonium persulfate (APS)

0.1g APS was dissolved in 1 ml of d.H_2O.

2.5.8 Transfer buffer

3g of Tris-base and 14.4g Glycine were dissolved in 800ml d.H_2O to which 200ml of methanol was added.

2.5.9 Ethlenediamin Tetra Acetic acid (EDTA)

93.05g EDTA disodium salt was dissolved in 400 ml d.H_2O and pH was adjusted to 7 then the solution was made up to a final volume of 500 ml.

2.5.10 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution

60 g of MTT was dissolved in 10ml of PBS.

2.5.11 Lysis solution for MTT assay

20% (v/v) SDS dissolved in warm water containing 50% (v/v) dimethylformamide. The pH was then adjusted to 4.7 using a solution of 80% acetic acid and 2.5% 1 M HCI.

2.5.11 Column washing buffer

50mM Tris-HCL, 150mM NaCl, 10mM EDTA, 1% Triton X-100, the pH adjusted to 8.0

2.5.12 Thioflavin-T solution

20μM Th-T in PBS

2.5.13 Thioflavin-S solution

20μM Th-T in PBS

2.5.14 SDS-PAGE sample loading buffer

250 mM TrisHCl pH6.8, 10% SDS, 30% Glycerol, 5% β-mercapitalethanol, 0.02% bromophenol blue

3 Chapter 3: Alpha-synuclein neurotoxicity is mediated by ongoing seeded polymerization process and is inhibited by α-syn S129 phosphorylation.

3.1 Introduction

Parkinson's Disease (PD) is a progressive neurodegenerative disorder, the clinical features of which include bradykinesia, rigidity, resting tremor and postural instability (Dunnett & Björklund, 1999). These motor symptoms are linked to the degeneration of the dopaminergic neurons in an area of the brain known as substantia nigra, which appears to be selectively affected in PD (Dauer & Przedborski, 2003; Fearnley & Lees, 1991). Similarly to several other neurodegenerative diseases, including Alzheimer's disease (AD) and Prion diseases. PD is referred to as a proteinopathy indicating that the disease is associated with the accumulation and deposition of a misfolded protein (Chiti & Dobson, 2006; Forman, Trojanowski, & Lee, 2004; Ross & Poirier, 2004). Indeed, the neuropathological hallmarks of PD are neuronal inclusions either in neuronal perikarya or in cell processes, known as Lewy bodies (LBs) and Lewy neurites (LNs), respectively (Dickson, 2012), the main component of which is fibrillar α-synuclein (α-syn) (Arai et al., 1999; V. M. Lee & Trojanowski, 2006; Spillantini, et al., 1997).

α-Syn is a small, heat-resistant, acidic protein composed of 140 amino acid residues (Jakes, et al., 1994), expressed predominantly in certain regions of the brain, such as neocortex, hippocampus, olfactory bulb, striatum, thalamus, and cerebellum (Iwai et al., 1995). α-Syn can be detected in great abundance at presynaptic nerve terminals (Iwai, et al., 1995; Kahle et al., 2000; Murphy,

Rueter, Trojanowski, & Lee, 2000) and is found in both soluble and membrane-associated fractions of the brain (Iwai, et al., 1995). In terms of structure, monomeric α-syn is soluble, possessing an extended structure of random coils (Eliezer, et al., 2001b). However, this protein is characterized by the intrinsic propensity to aggregate, forming fibrils both *in vitro* (Conway, Harper, et al., 2000b; El-Agnaf, Jakes, Curran, & Wallace, 1998; Hashimoto, et al., 1998b) and *in vivo* (Spillantini, et al., 1997).

Although the precise mechanism of α-syn aggregation in the brain remains to be elucidated, *in vitro* studies have shown that α-syn fibrillation is a nucleation-dependent process (S. J. Wood, et al., 1999), which proceeds through a nucleation (lag-phase), an elongation (growth-phase) and a steady-state phase (Conway, Lee, et al., 2000a; S. J. Wood, et al., 1999). In agreement with this nucleated assembly model of fibrillation, it has been shown that the addition of exogenous, preformed nuclei, referred to as 'seeds', results in the reduction of the lag phase and the immediate aggregation of α-syn (Conway, Lee, et al., 2000a; S. J. Wood, et al., 1999) in a dose dependent manner (S. J. Wood, et al., 1999). Similar results have also been reported for β-amyloid (Aβ), which is implicated in the pathogenesis of AD. More specifically, it has been shown that the absence of the pre-aggregated seeds increases the lag time needed for the formation of Aβ fibrils (Jarrett & Lansbury, 1993), while seeding of monomeric Aβ aggregation with fibrillar Aβ induces neuronal cell death (Wogulis et al., 2005). Furthermore, it has been reported that the toxicity of Aβ42, is not linked to specific prefibrillar aggregate(s), but to the ability of these species to grow and form fibrils which can be achieved by the presence of Aβ42 monomers (Jan et al., 2011). The impact of seeding on amyloid aggregation has also been established by a number of studies

indicating that α-syn aggregation can be induced and accelerated by the addition of exogenous oligomers and fibrils both in cultured cells (Danzer, et al., 2007; Nonaka, Watanabe, Iwatsubo, & Hasegawa, 2010; Volpicelli-Daley et al., 2011) and in mouse brains (Luk et al., 2012; Masuda-Suzukake et al., 2013), supporting the hypothesis that the aggregation of the native protein is directly seeded by exogenous aggregates. Overall, these studies support the notion that amyloid toxicity is mediated by a nucleation-dependent polymerization process.

Over the years numerous factors have emerged as potent modulators of α-syn aggregation *in vitro* and/or *in vivo*, with α-syn phosphorylation at S129 being the most controversial. Given that the α-syn deposited in the LBs of many α-synucleinopathies is phosphorylated at S129 and that this finding is also recapitulated in many animal models (reviewed by (Paleologou & El-Agnaf, 2012), it can be argued that this post-translational modification of α-syn has a pathological role. However, despite the systematic effort put into elucidating the impact of phosphorylation on α-syn aggregation and toxicity, it remains unclear whether α-syn phosphorylation promotes or inhibits the aggregation and toxicity of the protein, as the results of these studies are rather contradictory and inconclusive. Indeed, while there are many studies indicating that phosphorylation at S129 (either by employing certain kinases and/or the phosphorylation mimics S129D/E and the non-phosphorylatable mutant S129A) promotes α-syn aggregation and inclusion formation (Arawaka, et al., 2006; Fujiwara, et al., 2002; Gorbatyuk, et al., 2008; Kragh et al., 2009; M. Takahashi, et al., 2007; B. Wu et al., 2011; Zabrocki, et al., 2008). There are equally as many studies showing that phosphorylation at S129 inhibits α-syn accumulation (Oueslati, Schneider, Aebischer, & Lashuel, 2013) and aggregation (Paleologou, et al., 2008; Waxman

& Giasson, 2008), with the phosphorylation-blocking S129A α-syn actually being more prone to aggregation and inclusion formation (Azeredo da Silveira, et al., 2009; L. Chen, et al., 2009; Gonçalves & Outeiro, 2013; Paleologou, et al., 2008; Sancenon et al., 2012; Tenreiro et al., 2014). There are also reports indicating that phosphorylation either has no effect on inclusion formation (L. Chen, et al., 2009; Schreurs et al., 2014) or that aggregation of α-syn is independent of phosphorylation (Basso et al., 2013; Waxman & Giasson, 2011). As far as the p-S129 α-syn-related toxicity is concerned, the results of the various studies are equally contradictory and inconclusive. Whereas studies on yeast, oligodendrial cells and a drosophila model of PD have show that α-syn phosphorylated at S129 (p-S129 α-syn) by various kinases (casein kinase 1 (CK1), CK1, Polo-like kinases (PLKs), G-protein-coupled kinases (GRKs)) correlates with α-syn toxicity (L. Chen, et al., 2009; Kragh, et al., 2009; Zabrocki, et al., 2008), studies employing the phosphomutants S129D and S129A are particularly contradictory. In studies, S129D α-syn appears to be toxic (Chau, et al., 2009), while there are also reports supporting that the phosphomimic S129D α-syn plays a rather protective role (Gorbatyuk, et al., 2008; Kuwahara, Tonegawa, Ito, Mitani, & Iwatsubo, 2012; B. Wu, et al., 2011) or has no effect on the toxicity of the protein (Azeredo da Silveira, et al., 2009; Fischer et al., 2009). Similarly, blocking of α-syn phosphorylation at S129 by employing the mutant S129A α-syn has been shown to be toxic by some studies (Azeredo da Silveira, et al., 2009; Gorbatyuk, et al., 2008; Kuwahara, et al., 2012; Sancenon, et al., 2012; Tenreiro, et al., 2014) and protective (L. Chen, et al., 2009; Kragh, et al., 2009) or without any effect (Chau, et al., 2009; Fischer, et al., 2009; M. Tanaka et al., 2004) by others.

The discrepancies observed by the aforementioned studies may be explained by the fact that the kinases phosphorylating α-syn in the brain remain unknown, and by the findings that phosphomimics do not recapitulate all aspects of phosphorylation (Paleologou, et al., 2008) and that α-syn phosphorylation at S129 may occur after the accumulation and deposition of fibrillar α-syn, given that fibrillar α-syn has been shown to be a good substrate for many kinases (Mbefo, et al., 2010; Paleologou, et al., 2010; Waxman & Giasson, 2008, 2011). However, there is an immediate need to investigate further the correlation between the aggregation and toxicity of α-syn and the phosphorylation at S129, especially in the light of the recent findings supporting the idea that α-syn mediated toxicity is caused the nucleation-dependent polymerization process.

3.2 Experimental procedure

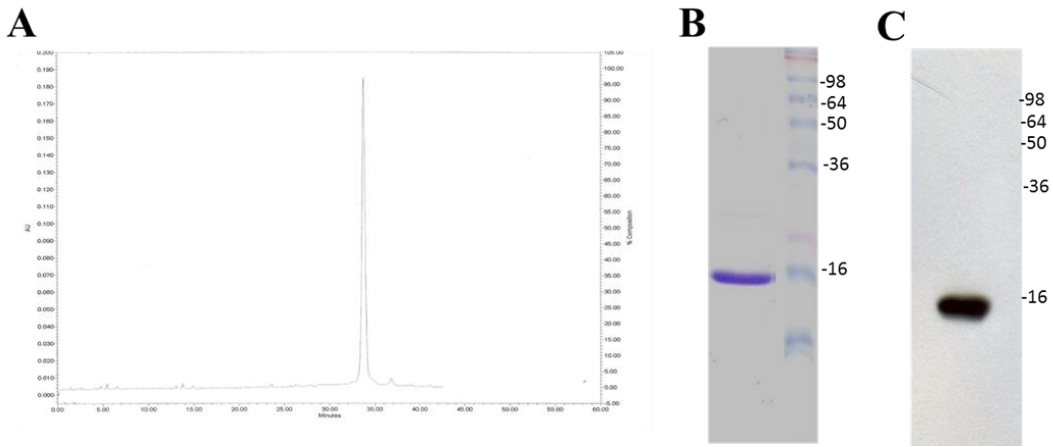
3.2.1 Expression and purification of recombinant human α-syn.

A GST-α-syn fusion construct in the pGEX-4T1 vector (kindly provided by Dr. Hyangshuk Rhim of the Catholic University College of Medicine, Seoul, Korea) was inserted into BL21 *E.coli* bacteria by heat shock. The transformed bacteria were grown in an LB medium supplemented with 0.1 mg/ml ampicillin at 37°C in an orbital shaker to an OD_{600} of 0.5. Expression was then induced by adding 0.5 mM IPTG (Sigma-Aldrich Chemie GmbH, Germany), and the culture was incubated for 2 hours at 37°C. The cells were harvested by a 15 minute centrifugation at 9000 x g, and the resulting pellet was resuspended in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% DTT) and shaken for 10 mins at room temperature. To improve the efficiency of cell lysis, the resuspended pellet was subjected to 6 freeze-thaw cycles in liquid nitrogen and a 37°C water bath. The lysate was then centrifuged at 27,000 x g for 15 mins, and the resulting supernatant was retained for purification by affinity chromatography using sepharose beads conjugated to glutathione, which has a high affinity for the GST tag. The cell lysate was mixed with glutathione sepharose beads and incubated for 1 hour at room temperature, followed by centrifugation at 500 x g at 4°C for 8 mins. The beads were then washed twice with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, pH 8.0), twice with 50 mM Tris-HCl, pH 8.0, and once with 1X PBS. The washed beads were resuspended in 5 ml of 1X PBS, and the GST tag was cleaved with human plasma thrombin (1 unit/µl), (Sigma-Aldrich, USA). The thrombin-catalyzed cleavage reaction was incubated overnight at room temperature with continuous mixing followed by a 5 minute incubation at 37°C. The reaction

mixture was then centrifuged for 8 mins at 500 x g at 4°C, and benzamidine sepharose beads (Amersham, Sweden) were used to 'fish out' thrombin. Pure α-syn was collected by centrifugation at 500 x g for 8 mins at 4°C. The α-syn concentration was estimated using a BCA assay (Pierce Biotechnology, Rockford, IL).

3.2.2 α-Syn purification and characterization.

Crude α-syn protein after GST tag cleavage was purified using reversed phase HPLC. The columns used were an analytical Phenomenex Jupiter C4 (250 3 4.6 mm) and a preparative Phenomenex Jupiter C4 (250 3 10 mm). α-Syn was loaded into the column and eluted with a linear gradient (30-80%) of acetonitrile (70% acetonitrile, 0.1% trifluoroacetic acid,) versus water (0.1% trifluoracetic acid) for 38 mins at a flow rate of 0.5 ml/minute. (Scheme 3.1). The homogeneity of α-syn protein was ascertained by using analytical HPLC and SDS-page (Scheme 3.1 A-C).



Scheme 3.1. HPLC analysis and characterization of crude α-syn. A. HPLC analysis was done using phenomenex Jupiter C4 (250 3 4.6 mm) column, with a gradient of 30%-80% solvent B in solvent A at 0.5 ml/minute over 38 mins. B. Coomassie blue staining of 15% SDS-PAGE for HPLC purified recombinant α-syn. C. Immunoblotting for HPLC purified α-syn detected by mAb 211.

3.2.3 Aggregation of α-syn *in vitro*.

The purity of the α-syn employed in this assay was >95% as estimated by SDS gels The α-syn samples placed in 1.5 ml sterile polypropylene tubes, topped with drops of mineral oil to prevent sample evaporation, and sealed with parafilm were incubated at 37°C for several days with continuous shaking at 800 rpm in a Thermomixer (Eppendorf). The samples were collected at certain points, while the aggregation of α-syn was monitored by the Thioflavin assay. The samples were stored at -80°C until required for further analyses.

3.2.4 Seeded polymerization assay.

The aggregation of monomeric wt or p-S129 α-syn with or without seeding was performed as described elsewhere (Di Giovanni, et al., 2010). The wt or p-S129 seeds were prepared by fragmenting the mature α-syn fibrils by sonication resulting in short fibrils. Monomeric α-syn at a concentration of 100 μM was seeded with different concentrations of seeds and incubated at 37°C with continuous shaking. The fibrillization was monitored by the Th-S binding assay as described above.

3.2.5 Thioflavin-S (Th-S) assay.

α-Syn fibril formation was monitored by Th-S binding assay. Th-S is a fluorescent dye that interacts with fibrils containing a β-sheet structure. For this assay, a total of 10 μl of the sample was diluted in 40 μl of Th-S in PBS (20 μM final concentration) and the mixture was dispensed in a 384-well, untreated black plate (Nunc, Denmark). The fluorescence was measured by a microplate reader (Victor X3 2030, Perkin Elmer) with the excitation and emission wavelengths set

at 450 and 510 nm, respectively. To allow for background fluorescence, the fluorescence intensity of a blank PBS sample was subtracted from all readings.

3.2.6 Preparation of α-syn crude seeds, oligomers and fibrils.

100 μM of monomeric α-syn was aggregated as described above (see Aggregation of α-syn *in vitro*) either for 2 or 7 days, leading to the α-syn samples referred to herein as 'crude oligomers' and 'crude fibrils', respectively. For the preparation of crude seeds, the crude fibrils were fragmented by ultrasonication while kept on ice using a sonic ruptor 250, equipped with a fine tip (5second pulses, output of 40 watts for 5 mins).

3.2.7 Preparation of α-syn pure fibrils and pure seeds.

100 μM of monomeric α-syn was aggregated as described above for 7 days. To prepare pure fibrils, the crude α-syn fibril sample was spun at 10,000 xg for 10 mins at 4°C in a refrigerated microfuge (Eppendorf). The supernatant was then discarded, and the pellet was washed twice with ultra-pure water, before resuspending in 1x PBS. For the preparation of pure seeds, the pure fibrils were fragmented by ultrasonication while kept on ice using a sonic ruptor 250, equipped with a fine tip (5 second pulses, output of 40 watts for 5 mins). For the estimation of α-syn concentration of the fibrils and seeds, the samples were denatured by 6M Guanidine-HCl and BCA assay which were used according to manufacturer's instructions.

3.2.8 Transmission electron microscopy (TEM).

Electron images were produced from aged α-syn either alone or in the presence of various percentages of p-S129 α-syn. The samples (5 μL) were deposited on Formvar-coated 400-mesh copper grids (Agar Scientific, UK), fixed

briefly with 0.5% glutaraldehyde (5 µl), negatively stained with 2% uranyl acetate (Sigma–Aldrich, USA) and examined in a Philips CM-10 TEM electron microscope.

3.2.9 Immunoblotting.

Samples of α-syn (20 ng) incubated alone or with various percentages of p-S129 were mixed with 1X sample buffer (250 mM Tris-HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue) and separated in 1 mm 15% SDS-PAGE gels. The separated proteins were transferred to 0.45 µm nitrocellulose membranes (Whatman Gmbh-Germany) at 90 V for 80 mins. The membranes were boiled for 5 min in PBS and then blocked for 1 hour with 5% non-fat milk prepared in PBS-Tween-20 (0.05%; PBST). The membranes were incubated overnight at 4°C with the primary mouse monoclonal anti-α-syn (211) antibody, which recognizes human α-syn (121-125) (Santa Cruz Biotechnology, USA), at a dilution of 1:1000. The membranes were then washed several times with PBST, followed by incubation with an HRP-conjugated goat anti-mouse antibody (Dako Ltd., Ely, UK) at a dilution of 1:70,000 for 60 mins at room temperature with gentle agitation. The membranes were then extensively washed for 25 mins, and immunoreactive bands were visualized with the Super Signal West Femto Chemiluminescent Substrate Kit (Pierce, Rockford, USA) according to the manufacturer's instructions. The amount of monomeric α-syn in the samples of α-syn aged alone or with various percentages of p-S129 α-syn was evaluated by densitometric analysis using ImageJ software and by comparing the monomeric bands of all samples with the band generated by a fresh α-syn sample containing only monomeric species.

3.2.10 Tissue culture of wt BE(2)-M-17 human neuroblastoma cells.

wt BE(2)-M-17 human neuroblastoma cells were routinely cultured in Dulbecco's MEM/Nutrient Mix F-12 (1:1) (Hyclone) containing 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (P/S; 100 U/ml penicillin, 100 mg/ml streptomycin-Sigma). The cells overexpressing wt α-syn were cultured in Dulbecco's MEM (Hyclone) containing 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin. To maintain the cells overexpressing α-syn, 250 µg/ml of G418 antibiotic was added to the culturing media for plasmid selection. The cells were maintained at 37°C in a humidified incubator with 5% CO_2/95% air.

3.2.11 Tissue culture of BE(2)-M-17 human neuroblastoma cells overexpressing wt-α-syn.

BE(2)-M-17 human neuroblastoma cells overexpressing wt-α-syn were routinely cultured in Dulbecco's MEM/High Glucose (Hyclone) containing 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin (P/S; 100 U/ml penicillin, 100 mg/ml streptomycin-Sigma) and 250 µg/ml of G418 antibiotic was added to the culturing media. The cells were maintained at 37°C in a humidified incubator with 5% CO_2/95% air.

3.2.12 Tissue culture of SHSY-5Y human dopaminergic neuroblastoma cells.

SHSY-5Y human dopaminergic neuroblastoma wt or overexpressing wt-α-syn cells were routinely cultured in Dulbecco's MEM/Nutrient Mix F-12 (1:1) (Hyclone) containing 15% fetal bovine serum (Hyclone), 1% penicillin-streptomycin (P/S; 100 U/ml penicillin, 100 mg/ml streptomycin-Sigma), and supplemented with 1% non-essential MEM amino acid supplement (Gibco) and 2

mM freshly prepared glutamine. The cells were maintained at 37°C in a humidified incubator with 5% CO2/95% air.

3.2.13 Measurement of cell viability.

The MTT assay was employed for the assessment of the cytotoxic effect of the different α-syn species, cells suspended in a culturing medium were plated at a density of 15,000 cells (100 µl /well) in a 96-well plate. After 24 hours, the medium was replaced with 100 µl of MEM-RS (Hyclone) serum-free medium containing the different solutions of α-syn species diluted in serum-free media to obtain the desired concentrations. The cells were then allowed to grow for 24 or 48 hours. A total of 20 µl of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) (6 mg/ml) in PBS was dispensed into each well, and the plate was incubated at 37°C for 4.5 hours. The MTT-containing medium was carefully removed, and replaced with 100 µl/well of lysis buffer (15% SDS, 50% N,N-dimethylformamide, pH 4.7). The lysis buffer was incubated at 37°C overnight. The absorbance values at 590 nm were measured in a microplate reader (Perkin Elmer). For the experiments assessing the toxic effect of seeded polymerization, the cells were prepared as mentioned above. The cells were then incubated at 37°C in 5% CO_2 for 1 hour and monomeric α-syn was then added to the cells treated with a different species, then the cells were incubated again for another 48 hours.

3.2.14 *In vitro* phosphorylation of α-syn.

Monomeric α-syn (wt) was phosphorylated by PLK2 (Invitrogen) at a concentration of 1.44 mg/ml (100 µM). The phosphorylation reaction was carried out in the presence of 1.09 mM ATP (Sigma), 1X reaction solution (20 mM

HEPES, 10 mM $MgCl_2$, 2 mM DDT, pH 7.4), and 1 µg of PLK2 at 30 °C for 24 hours without shaking. The reaction volume was 100 µl.

3.1.1. Wt α-syn gene SNCA small interfering RNA (siRNA) silencing.

The pre-designed siRNA sequence 5'-AGAGGGUGUUCUCUAUGUAtt-3' targeting human wt α-syn, as well as non-targeting scrambled siRNA were purchased from Ambion (Life technologies). BE(2)-M-17 human neuroblastoma cells grown in MEM serum-free media were plated either in 6-well or 96-well plates at a density of $3x10^5$ or 4,000cells /well respectively. The next day, the cells in the 6-well plates were transfected with a 200 pmol siRNA/well for immunoblotting, while the cells in the 96-well plates were transfected with 8 pmol siRNA/well for use in MTT assay. The siRNA was transfected using a transfection reagent (Santa Cruz Biotechnology, USA). After 6 hours of incubation, 1 ml of a normal growth medium containing 2 times the amount of bovine serum was added without removing the transfection mixture. The cells then incubated for 72 hours before applying the appropriate protocols (WB, MTT). For the MTT assay the cells were treated with pure fibrils, pure seeds or monomers.

3.1.2. Aggregation of p-S129 α-syn *in vitro*.

Recombinant α-syn was mixed with different percentages of *in vitro* prepared p-S129 α-syn (100, 50, 20 and 5%) in 1.5 ml sterile polypropylene tubes, topped with drops of mineral oil to prevent sample evaporation, and incubated at 37°C for several days with continuous shaking at 800 rpm in a Thermomixer (Eppendorf). Samples were then collected at certain points and a thioflavin-S assay was performed.

3.3 Results

3.3.1 Morphological and structural requirements for α-syn-mediated neuronal cell death

Several lines of evidence support the idea that toxic Aβ contains a mixture of high molecular weight aggregates with different sizes and structures (Jan, Hartley, & Lashuel, 2010; Lashuel et al., 2003; Walsh et al., 1999). To investigate whether this also applies to α-syn-mediated toxicity, we sought to determine the structural features of the most toxic species of α-syn. For this purpose we assessed the toxic effect of different species of α-syn on neuroblastoma BE(2)-M-17 and SHSY-5Y wt cells. The α-syn species employed for these studies ranged from monomeric to oligomeric/intermediate and fibrillar forms. The oligomeric and fibrillar species, which are referred to herein as 'crude oligomers' and 'crude fibrils', respectively, were prepared by aggregating 100 µM of monomeric α-syn at 37º C with continuous mixing for 2 and 7 days, respectively. The samples referred to as 'crude seeds' were in turn prepared by subjecting the crude fibrils to brief sonication as described above. The structure and the β-sheet content of these samples were evaluated by TEM and Th-S assay, respectively (Fig. 3.1 A, B). To assess the effect of the prepared species on the viability of neuronal cells an MTT assay was carried out employing wt SHSY-5Y and BE(2)-M-17 - human neuroblastoma cells, The total amount of α-syn expressed in these cells was estimated by immunoblotting as shown in (Fig. 3.1 C). For the MTT assay, the cells were treated with solutions of α-syn monomers and the various α-syn species at a range of final concentrations of 0.001- 20 µM for 24 and 48 hours.

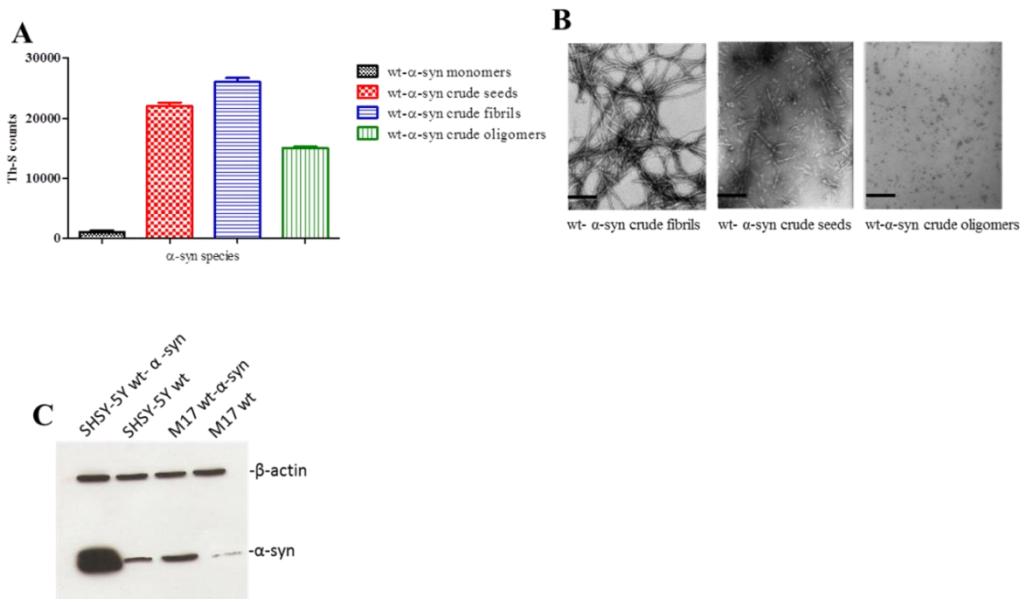


Figure 3.1. Characterization of the α-syn species and evaluation of the α-syn expression in the various neuroblastoma cells. A. The fibril content of the various α-syn species, namely crude oligomers, crude seeds, crude fibrils and monomers (5 μM) was evaluated by Th-S fluorescence. The assay was performed in triplicate, and the means ± standard deviations are shown. B. Electron microscopy images of negatively stained samples of α-syn species (100 μM). *Scale bar* 500 nm. C. The lysate (20 ng) of both the wt and the α-syn stably transfected neuroblastoma cells BE(2)-M-17 and SHSY-5Y were separated by electrophoresis in a 15% SDS-PAGE gel and transferred to nitrocellulose membrane, which was probed with anti-α-syn (211) and anti β-actin antibodies.

As shown in Fig. 3.1, A and B, treatment of BE(2)-M-17 -wt α-syn cells (*i.e.* the cells expressing only endogenous α-syn) with monomers had no effect on cell viability, while the treatment with the rest of the α-syn species (crude oligomers, seeds, fibrils) had a mild impact on both cell lines. This mild effect on the cell viability was comparable for all these three α-syn species, but was more prominent in the cells treated with the highest concentration of α-syn species (20 μM) and for a longer period (48 hours) (Fig. 3.2 B). Comparable results were obtained when SHSY-5Y expressing only endogenous α-syn was used (Fig. 3.2 C, D).

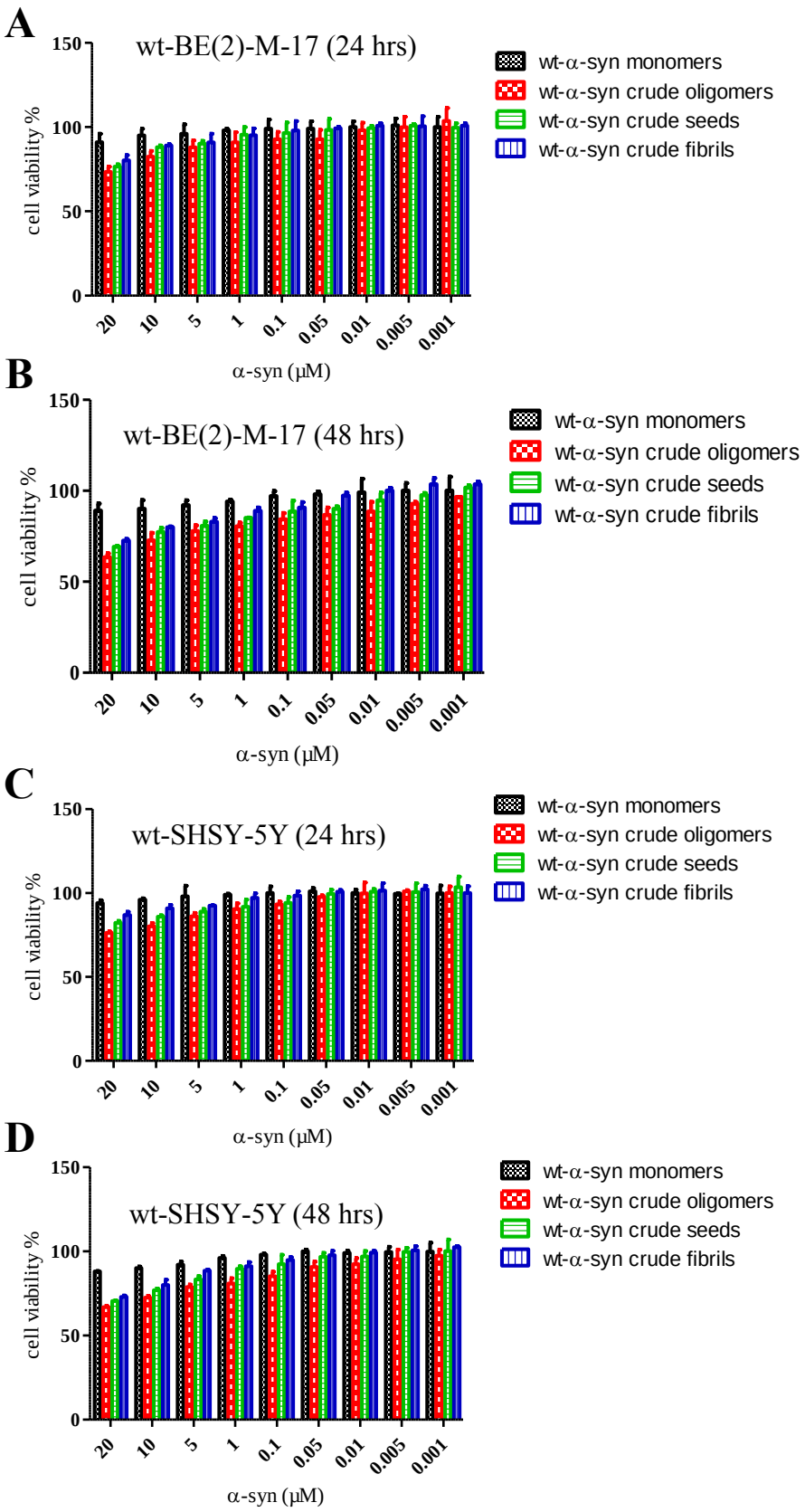


Figure 3.2. The effect of the various α-syn species on the viability of BE(2)-M-17 and SHSY-5Y wt neuroblastoma cells**.** The viability of BE(2)-M-17 human neuroblastoma cells was estimated by the MTT assay. The results are expressed as the percentage of the control average (*i.e.*, untreated cells). The cells were treated with different concentration of α-syn species either 24 or 48 hours prior to MTT

addition (average of 3 wells ± standard deviation). A. BE(2)-M-17 -wt cells incubated for 24 hours. B. BE(2)-M-17 -wt cells incubated for 48 hours. C. SHSY-5Y wt cells incubated for 24 hours. D. SHSY-5Y wt cells incubated for 48 hours. Statistical analysis was performed using one way anova, followed by Tukey's multiple comparison test using the GraphPad Prism 5 software. (***, $p< 0.001$; **, $p< 0.01$, *, $p< 0.05$).

The results obtained from the wt cells used in the above experiment, prompted us to use the same cells overexpressing wt-α-syn to distinguish any difference. However, in the case of the BE(2)-M-17 cells overexpressing wt α-syn (*i.e.* expressing α-syn in excess), α-syn monomers had no effect on cell viability. Treatment with α-syn oligomers, especially in higher concentrations (20 μM) reduced cell viability by approximately 50% and 65% after 24 hours and 48 hours of incubation, respectively (Fig. 3.3 A, B). At 20 μM, α-syn crude seeds and fibrils had a comparable effect on cell viability, reducing it by approximately 20% and 30% after 24 hours and 48 hours of incubation, respectively (Fig. 3.3 A, B). Overall, the impact of α-syn crude oligomers, seeds and fibrils on the viability of the BE(2)-M-17 cells overexpressing wt α-syn showed a dose-dependent relation (Fig. 3.3A, B).

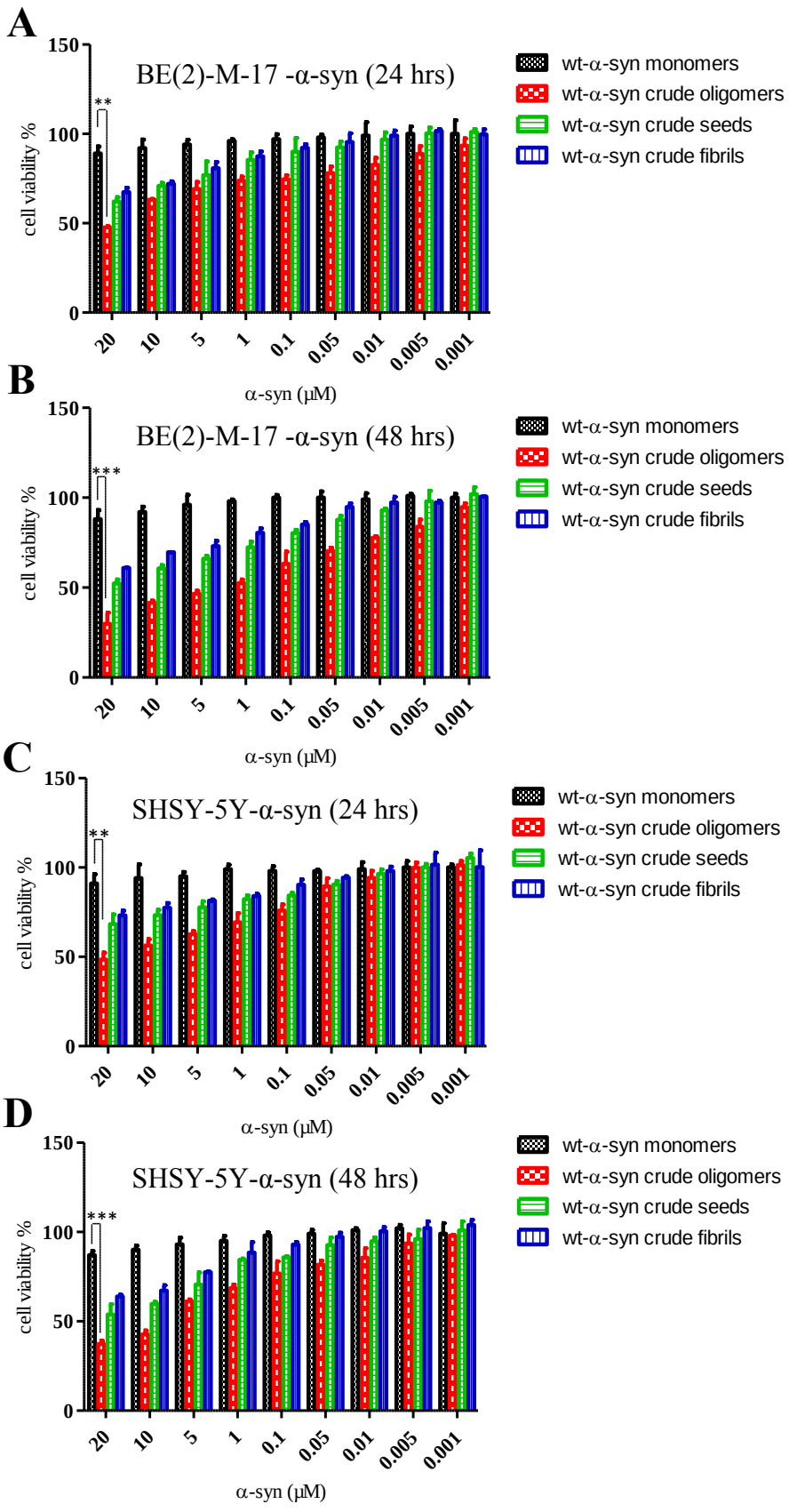

A
BE(2)-M-17 -α-syn (24 hrs)
cell viability %
150
100
50
0
**
20
10
5
1
0.1
0.05
0.01
0.005
0.001
α-syn (μM)
wt-α-syn monomers
wt-α-syn crude oligomers
wt-α-syn crude seeds
wt-α-syn crude fibrils
B
BE(2)-M-17 -α-syn (48 hrs)
***
C
SHSY-5Y-α-syn (24 hrs)
D
SHSY-5Y-α-syn (48 hrs)

Figure 3.3. The effect of the various α-syn species on the viability of BE(2)-M-17 and SHSY-5Y neuroblastoma cells overexpressing α-syn . The viability of SHSY-5Y human neuroblastoma cells was estimated by the MTT assay. The results are expressed as the percentage of the control average (*i.e.*, untreated cells). The cells were treated with different concentration of α-syn species either 24 or 48 hours prior to MTT addition (average of 3 wells ± standard deviation). A. BE(2)-M-17 overexpressing wt-α-syn cells incubated for 24 hours. B. BE(2)-M-17 overexpressing wt-α-syn cells incubated for 48 hours. C. SHSY-5Y cells over expressing wt α-syn incubated for 24 hours. D. SHSY-5Y cells over expressing wt α-syn incubated for 48 hours. Statistical analysis was performed using one way anova, followed by Tukey's multiple comparison test using the GraphPad Prism 5 software. (***, $p< 0.001$; **, $p< 0.01$, *, $p< 0.05$).

Taken together, the above results indicate that the different forms of aggregated α-syn species and especially the crude oligomers, can be more toxic in the presence of excess amounts of monomeric α-syn.

To further confirm these findings, this experiment was carried out using another SHSY-5Y cell over expressing wt α-syn. In (Figure 3.3 C and D) it is shown that treatment of SHSY-5Y cells (overexpressing wt α-syn) with the various α-syn species had an effect analogous to the one produced by the treatment of BE(2)-M-17 cells with the same α-syn species.

3.3.2 Addition of α-syn monomers to pre-treated with preformed aggregates cells enhances the α-syn-mediated toxicity

Given that α-syn crude oligomers had a stronger effect on cell viability compared to crude seeds and fibrils and taking into account that the crude oligomeric α-syn samples contain both monomers and protofibrils (Fig. 3.4 B), we assumed that the toxic effect of the oligomeric α-syn may be linked to its ability to seed the aggregation of monomeric α-syn. To investigate this hypothesis, we prepared α-syn species so that they contained the minimum number of monomers, namely pure seeds and fibrils (Fig. 3.4), and we evaluated their effect on the

viability of neuroblastoma cells. For this purpose, both BE(2)-M-17 and SHSY-5Y wt cells were treated with three different concentration (2, 5 and 10 μM) of pure seeds and fibrils for 48h..

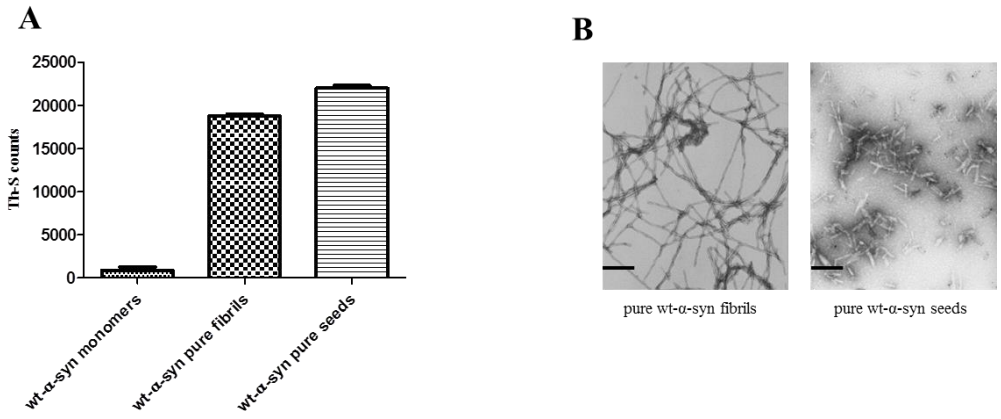


Figure 3.4. Characterization of α-syn pure fibrils and pure seeds. A. The fibril content of pure fibrils, pure seeds, and monomeric α-syn (5 μM) was evaluated by Th-S fluorescence. The assay was performed in triplicate, and the means ± standard deviations are shown. B. Electron microscopy images of negatively stained samples of α-syn species (100 μM). *Scale bar* 500 nm.

To assess the effect of these α-syn species in the presence of α-syn monomers, monomeric α-syn was added to the cells 1 hour later at a final concentration of 10μM. While both pure seeds and fibrils had an effect on the viability of the cells (both BE(2)-M-17 and SHSY-5Y) at all concentrations (Fig. 3.5), with seeds being more toxic than fibrils, the addition of monomeric α-syn rendered this effect even more pronounced

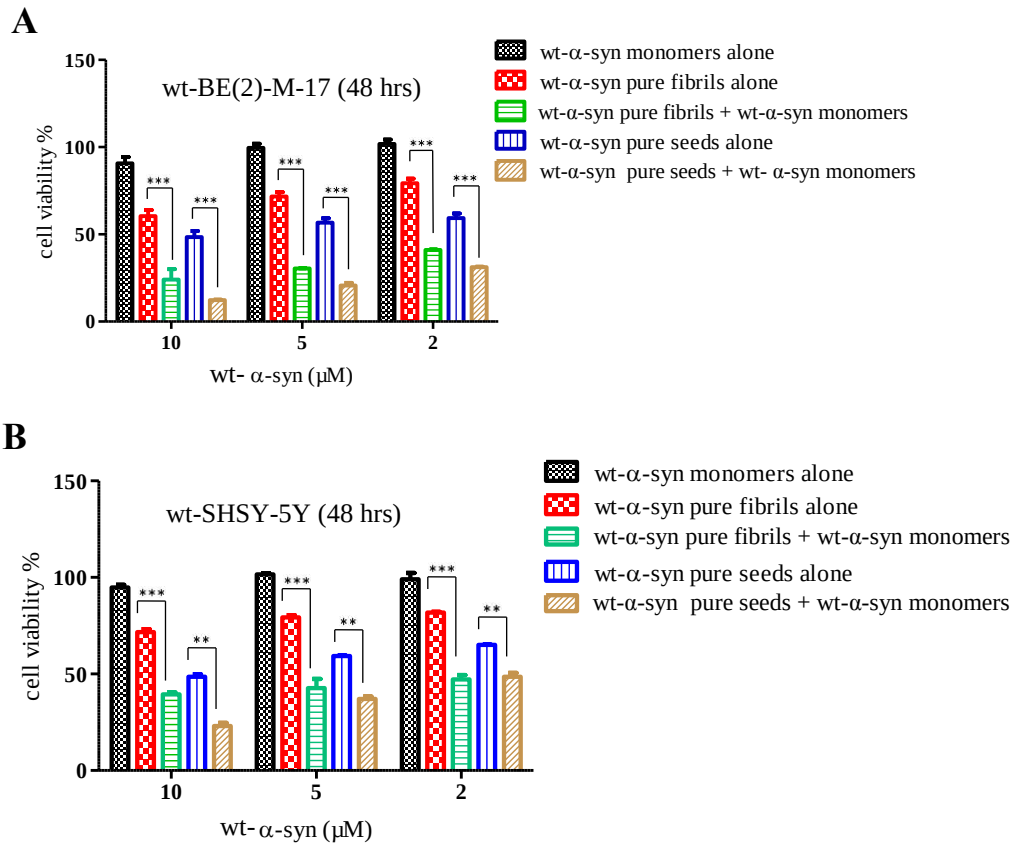
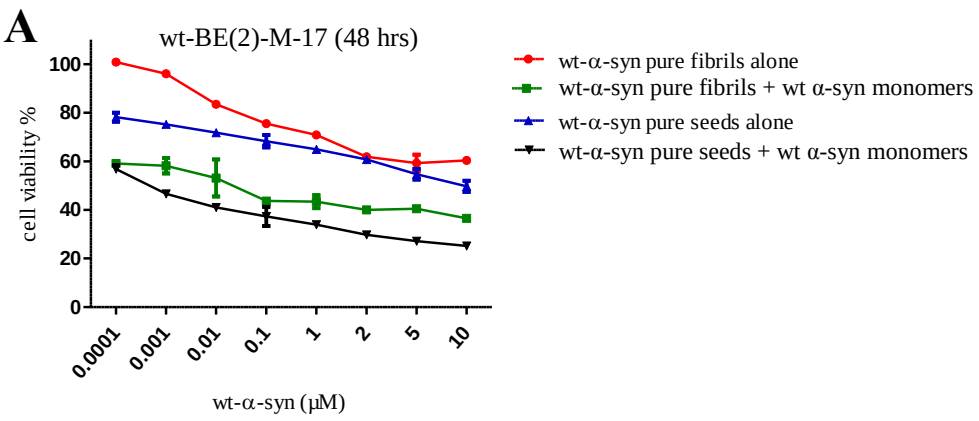


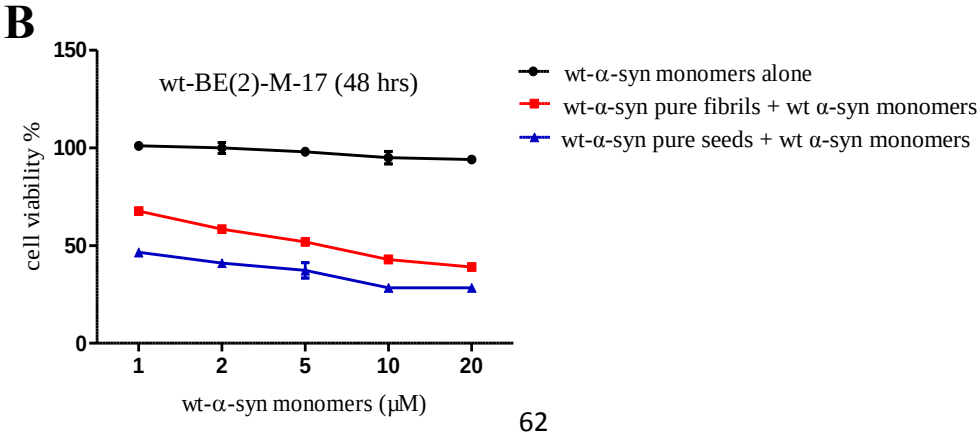
Figure 3.5. The effect of α-syn seeding on the viability of neuroblastoma cells. The viability of A. BE(2)-M-17 -wt cells, B. SHSY-5Y - human neuroblastoma wt cells was estimated by the MTT assay. The results are expressed as the percentage of the control average (*i.e.*, untreated cells). The cells were treated with different concentration of α-syn pure fibrils or pure seeds and one hour after treatment, monomeric α-syn to a final concentration of 10 μM was added. The cells were then incubated for 48 hours prior to MTT addition. (average of 3 wells ± standard deviation). Statistical analysis was performed using one way anova, followed by Tukey's multiple comparison test using the GraphPad Prism 5 software. (***, $p< 0.001$; **, $p< 0.01$, *, $p< 0.05$).

As the effect was independent of the concentration of the pure seeds and fibrils, we attempted to evaluate the toxicity of the cells varying both the concentration of seeds and fibrils (at a lower concentration scale, i.e. 0.0001-10 μM) in the presence of a constant concentration of α-syn monomers and vice versa. The results of this series of experiments are illustrated in Fig. 3.6. As

shown in Fig. 3.6 A and C, pure seeds and fibrils decrease cell viability in a concentration-dependent fashion, but they exert a more toxic effect in the presence of monomeric α-syn (final concentration of 10 μM), with seeds being once again more toxic than fibrils. Indeed, even at a concentration as low as 0.1 nM, pure seeds produced a strong toxic effect reducing cell viability by approximately 40%.

To investigate the effect of varying concentrations of monomeric α-syn (1-20 μM) on cell viability, the cells were first treated with a constant concentration (2 μM) of either pure fibrils or pure seeds. As shown in Fig. 3.6 B and D, the addition of monomers appears to be a prerequisite for cell toxicity when cells are pre-treated with pure fibrils and seeds (Fig. 3.6 B) as α-syn monomers alone had no effect on cell viability.





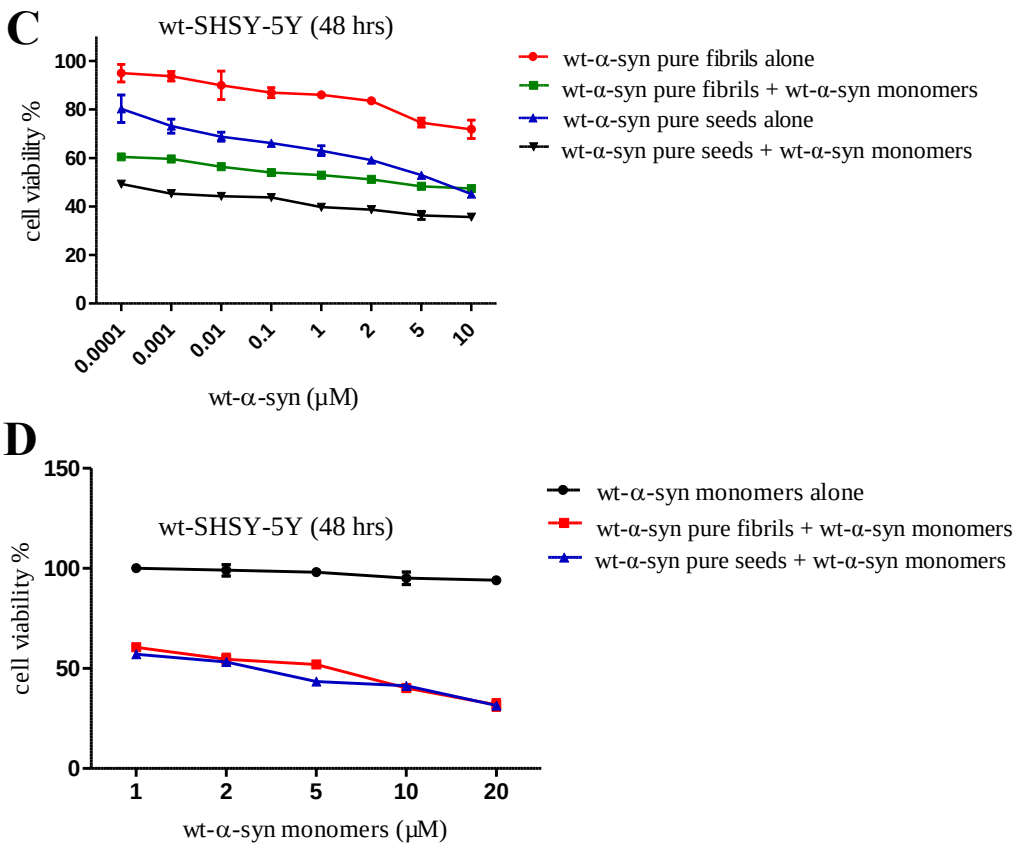


Figure 3.6. The effect of α-syn seeding on the viability of neuroblastoma cells**.** The viability of BE(2)-M-17 and SHSY-5Y human neuroblastoma wt cells was estimated by the MTT assay. The results are expressed as the percentage of the control average (*i.e.*, untreated cells). A. The BE(2)-M-17 -wt cells were treated with different concentration of α-syn pure fibrils or pure seeds (0.0001-10 μM) and one hour after treatment, monomeric α-syn to a final concentration of 10 μM of was added. B. The BE(2)-M-17 -wt cells were treated with 2 μM (final concentration) of α-syn pure fibrils or pure seeds and one hour after treatment, monomeric α-syn was added to a final concentration ranging between 1-20 μM. C. The SHSY-5Y-wt cells were treated with different concentration of α-syn pure fibrils or pure seeds (0.0001-10 μM) and one hour after treatment, monomeric α-syn was added to a final concentration of 10 μM. D. The BE(2)-M-17 wt cells were treated with 2 μM final concentration of α-syn pure fibrils or pure seeds and one hour after treatment, monomeric α-syn was added to a final concentration ranging between 1-20 μM. The cells were then incubated for 48 hours prior to MTT addition. (average of 3 wells ± standard deviation).

3.3.3 The effect of pure seeds and fibrils on the cell viability of BE(2)-M-17 cells, whose endogenous α-syn has been knocked down

To investigate further the role of monomeric α-syn in inducing the nucleation polymerization process of α-syn, we assessed the effect of pure seeds and fibrils on the viability of BE(2)-M-17 -wt cells whose endogenous α-syn was knocked down by siRNA silencing. The silencing was evaluated by immunoblotting and the expression level of α-syn was quantified by the image processing program ImageJ (Fig. 3.7 A, B). The siRNA transfected BE(2)-M-17 wt cells were treated with pure fibrils and pure seeds at four different concentrations (1, 5, 10 and 20 μM), while monomeric α-syn was employed as a control at the same concentrations. According to the MTT results (Fig. 3.7 C-E), the cells whose endogenous α-syn was knocked down were less susceptible to the toxic effects of pure fibrils (Fig. 3.7 C) and seeds (Fig. 3.7 D). However, the treatment of the cells with monomeric α-syn at any given concentration had a comparable impact on the viability of both the siRNA-transfected and the control cells (Fig. 3.7 E). These results confirm further the role of monomeric α-syn in the mechanism of polymerization/aggregation-induced toxicity

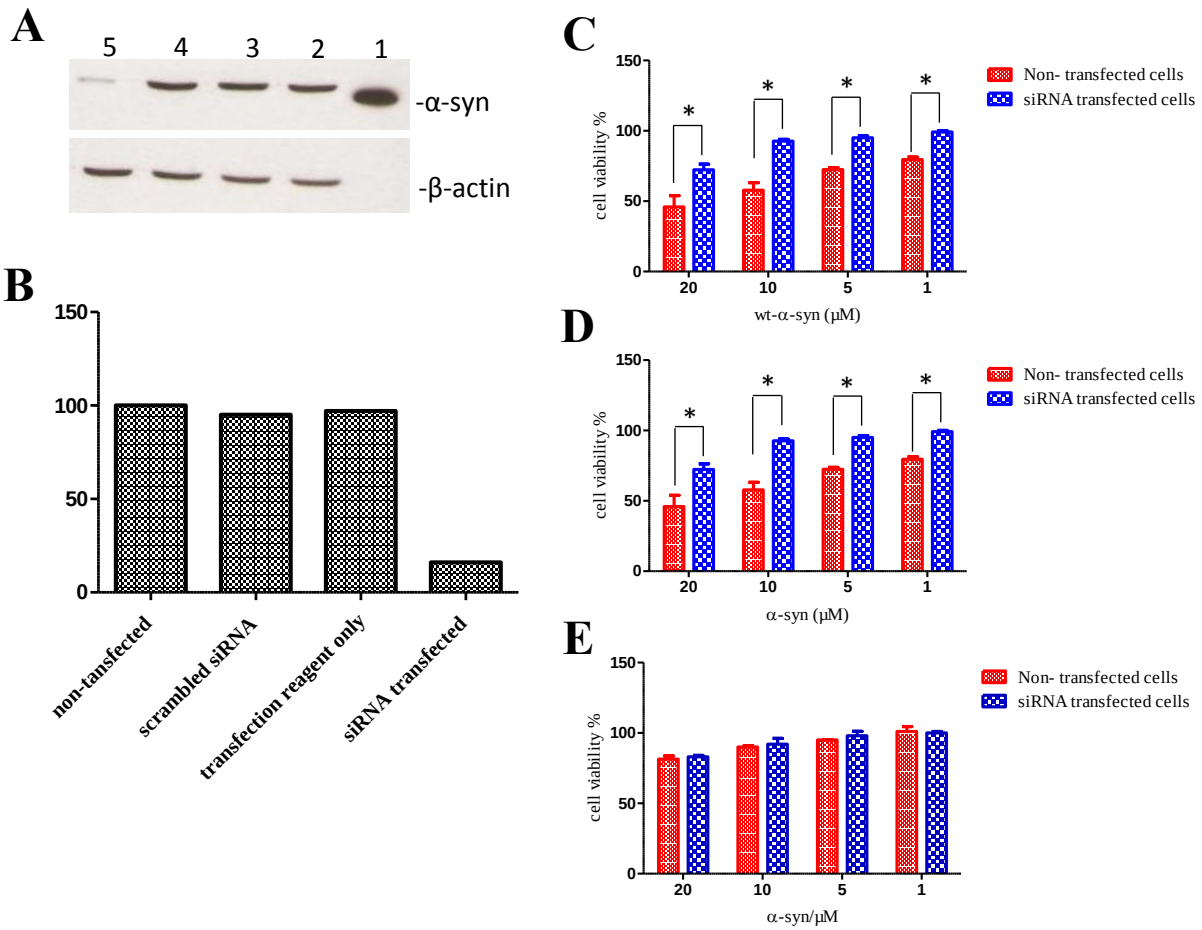


Figure 3.7. The effect of pure seeds and fibrils on the cell viability of BE(2)-M-17 cells, whose endogenous α-syn has been knocked down**.** Pre-designed siRNA sequence targeting human wt α-syn was used to silence the expression of α-syn, and non-targeting scrambled siRNA was used as negative control. A. Immunoblotting for cell lysate to detect total α-syn using the mouse monoclonal anti-α-syn (211) antibody and anti β-actin for loading control, lane 1: recombinant α-syn, lane 2: non- transfected cell lysate, lane 3: scambled siRNA transfected cell lyste, lane 4: R-12 peptide transfected cell lysate, lane 5: siRNA transfected cell lysate. B. Quantification of α-syn expression levels by densitometric analysis using the ImageJ software. C-E. The viability of BE(2)-M-17 human neuroblastoma wt cells was estimated by the MTT assay. The results are expressed as the percentage of the control average (*i.e.*, untreated cells). **C.** The BE(2)-M-17 wt cells were treated with different concentrations of wt-α-syn pure fibrils D. wt-α-syn puree seeds. E. Monomeric α-syn. The cells were then incubated for 48 hours prior to MTT addition, (average of 3 wells ± standard deviation). Statistical analysis was performed using one way anova, followed by Tukey's multiple comparison test using the GraphPad Prism 5 software. (*, $p< 0.05$).

3.3.4 Addition of p-S129 α-syn monomers to pre-treated with preformed aggregates cells does not promote the α-syn-mediated toxicity.

The finding that the majority of α-syn present in LBs is phosphorylated at S129, while the same modification appears only in 4% of the protein in the normal brain (Anderson, et al., 2006; Fujiwara, et al., 2002; Hirai, Fujita, Iwatsubo, & Hasegawa, 2004) indicates that the phosphorylation of α-syn at S129 plays a major role in PD. Despite the huge effort put into elucidating the role of p-S129 α-syn in the pathogenesis of PD, it remains unknown whether phosphorylation takes place before or after α-syn is deposited in the brain, while it is still debatable whether α-syn phosphorylation at S129 promotes or inhibits the aggregation and toxicity of the protein. To better understand the impact of phosphorylation on α-syn aggregation and toxicity, we sought to unravel the role of α-syn phosphorylation at S129 in the seeded polymerization and toxicity.

Our findings prompted us to investigate whether adding monomeric p-S129 α-syn to the cells pre-treated with either pure fibrils or pure seeds would have an effect on their toxicity. For this purpose, *in vitro* phosphorylated monomeric and aggregated recombinant α-syn (characterized by immunoblotting and Th-S binding Fig. 3.8 A and B) were used to assess the ability of various p-S129 α-syn species to seed the aggregation process. The phosphorylation of α-syn was assessed by immunoblotting employing a p-S129 α-syn-specific antibody (Fig. 3.8 C). Both wt and p-S129 α-syn pure seeds were characterized by EM and Th-S fluorescence (Fig. 3.8 D and E).

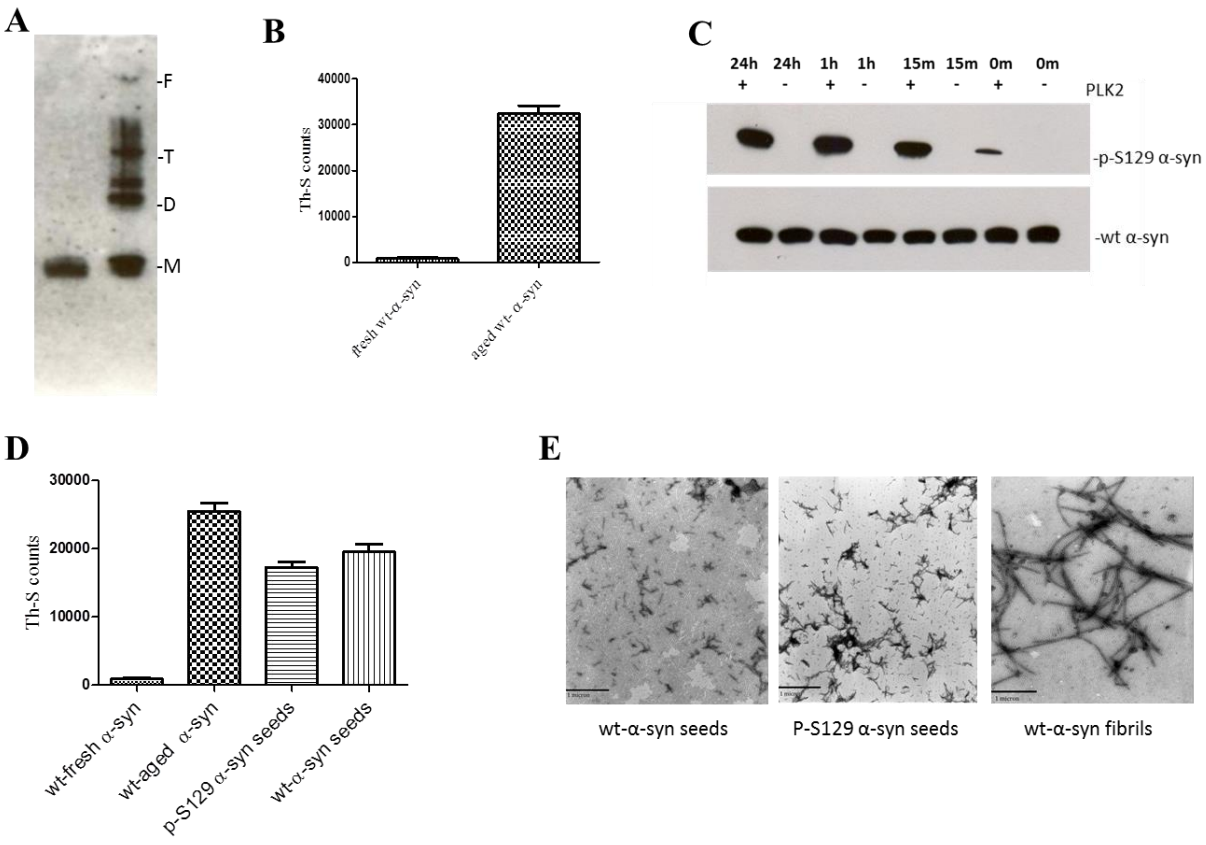
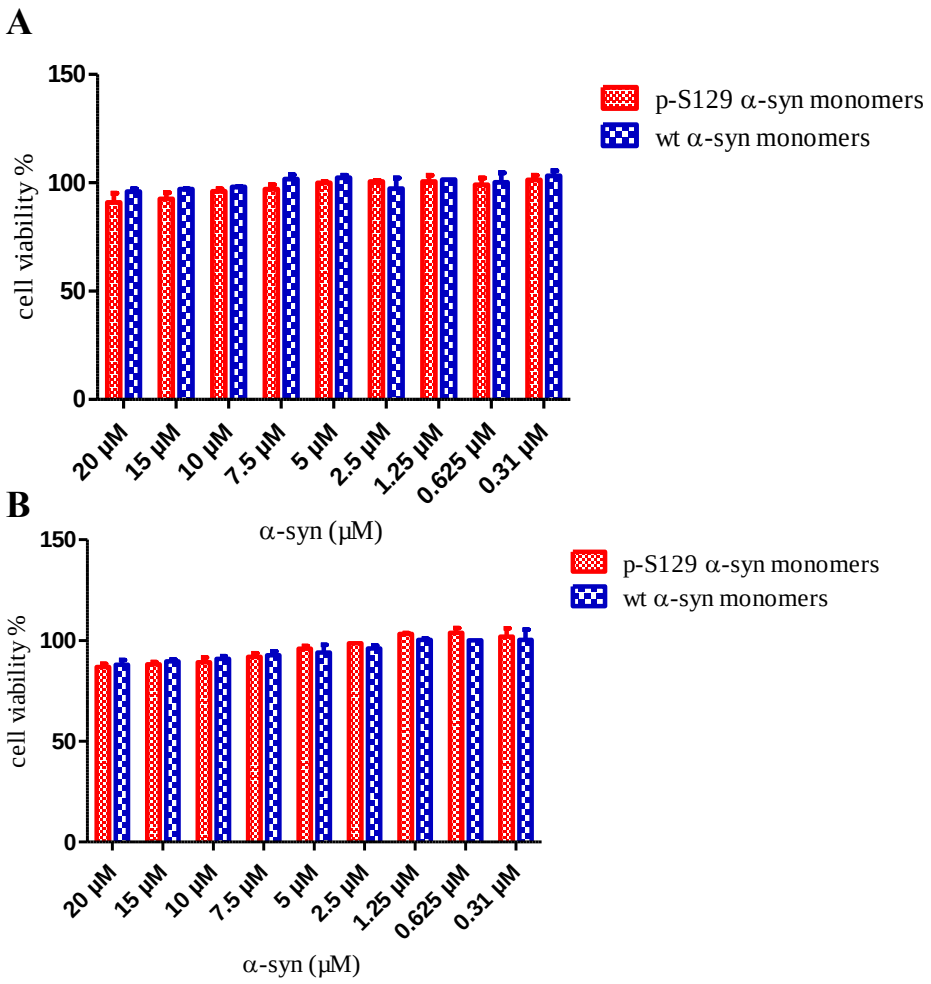


Figure 3.8. Characterisation of *in vitro* prepared p-S129 α-syn. A. Characterization of monomeric and aggregated α-syn by immunoblotting. B. Th-S fluorescence readings of the monomeric and aggregated α-syn samples. C. Immunobloting for the samples of α-syn incubated with PLK2 at different time points. Phosphorylation at S129 was detected by an p-S129 α-syn-specific antibody. Total a-syn was detected by anti-α-syn (211) antibody. D. Estimation of the fibril content in the p-S129 seeds, wt seeds and aged α-syn by Th-S fluorescnce. The assay was performed in triplicate and, the means ± standard deviations are shown. E. Electron microscopy images of negatively stained samples of p-S129 seeds, wt seeds and aged α-syn (100 μM). *Scale bar* 500 nm.

Prior to any toxicity experiment incorporating various species of α-syn, the effect of monomeric p-S129 α-syn alone compared with wt α-syn was assessed in both BE(2)-M-17 and SHSY-5Y wt cells. Monomeric p-S129 α-syn employed at a range of concentrations (0.31-20 μM) was not toxic to neither BE(2)-M-17 cells (Fig. 3.9 A) or to SHSY-5Y cells (Fig. 3.9 B).

For the experiments assessing the effect of monomeric p-S129 α-syn on the viability of the cells pre-treated with pure seeds or fibrils, BE(2)-M-17 and SHSY-5Y-wt cells were treated with different concentrations of either pure fibrils or pure seeds (10, 5 and 2 µM) and after one hour incubation, monomeric p-S129 α-syn was added to the cells at a final concentration of 10 µM. Surprisingly, as shown in Fig. 3.9 C and D, in contrast to the wt monomeric α-syn (Fig. 3.5), the p-S129 monomers had no effect on cell viability when pre-treated with any concentration of pure fibrils or pure seeds. Indeed, the toxicity levels observed after treatment with the monomeric p-S129 α-syn following treatment with pure seeds or fibrils were comparable (and statistically non-significant) to the ones seen in cells treated only with pure seeds or fibrils.



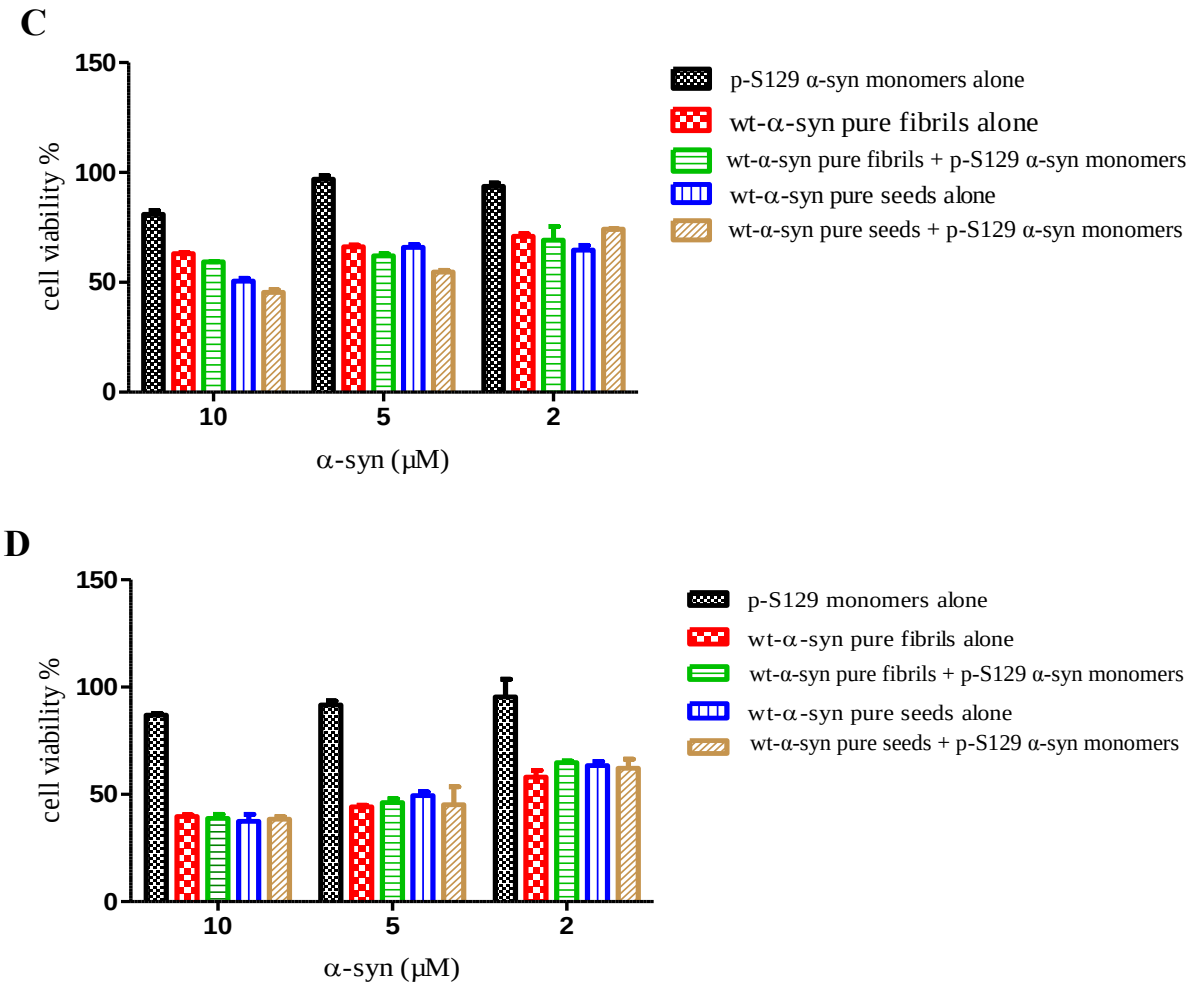


Figure 3.9. The effect of p-S129 α-syn seeding on the viability of neuroblastoma cells. The effect of monomeric wt and p-S129 α-syn on the viability of A. BE(2)-M-17 -wt cells, B. SHSY-5Y - human neuroblastoma wt cells was estimated by the MTT assay. The results are expressed as the percentage of the control average (*i.e.*, untreated cells). C. The BE(2)-M-17 and D. SHSY-5Y cells were treated with different concentration of α-syn pure fibrils or pure seeds and one hour after treatment, monomeric p-S129 α-syn was added to a final concentration of 10 μM. The cells were then incubated for 48 hours prior to MTT addition. (average of 3 wells ± standard deviation). Statistical analysis was performed using one way anova, followed by Tukey's multiple comparison test using the GraphPad Prism 5 software.

3.3.5 Assessing the ability of p-S129 to seed the aggregation of α-syn

To assess the ability of p-S129 α-syn to seed the aggregation of the protein, 100 μM of monomeric α-syn was incubated alone or in the presence of 2 μM (final concentration) wt or p-S129 α-syn seeds for 5 hours. Considering that α-syn aggregation is a nucleation-dependent process, the addition of exogenous preformed nuclei, namely the seeds, is expected to reduce the nucleation phase of the process, resulting in an immediate aggregation of the protein (Wood, et al., 1999; Conway, et al.,2000b). Indeed, as illustrated in Fig. 3.11, the addition of wt and p-S129 α-syn seeds accelerated the aggregation of monomeric α-syn as compared to the control, which showed comparable Th-S fluorescence readings throughout the 5-hour incubation process. It is noteworthy, that the wt α-syn seeds had a greater impact on the aggregation of monomeric α-syn compared to the p-S129 α-syn ones (Fig. 3.10), but since this effect was not statistically significant the experiment was repeated employing a lower concentration of seeds (0.1 μM) and/or a longer incubation period (48h).

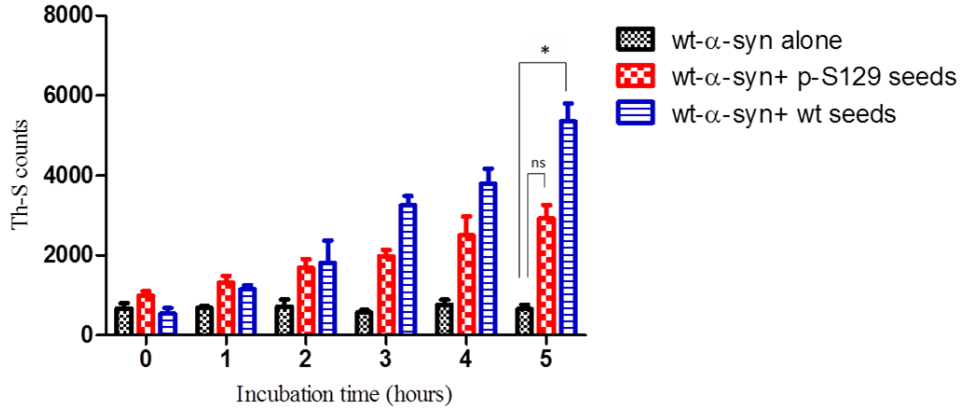
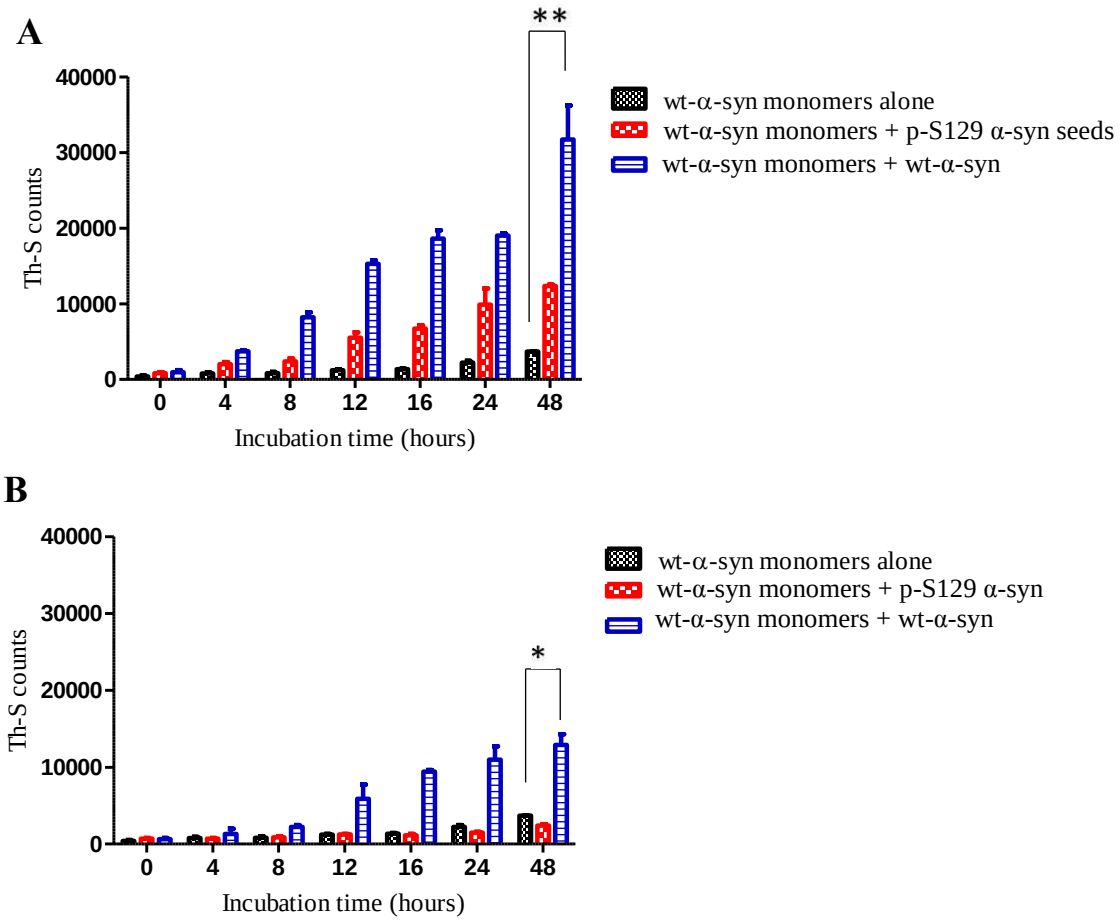


Figure 3.10. Assessing the ability of pS129 α-syn to seed the polymerization/aggregation of α-syn. Samples of monomeric α-syn (100 μM) were incubated for 5 hours at 37°C with continuous shaking in the presence or absence of 2 μM of wt or pS129 α-syn seeds. Fibril formation was measured by Th-S fluorescence. The assay was performed in triplicate, and the means ± standard deviations are shown.

The results of this series of experiments are shown in Fig. 3.11 A and B. As shown in Fig. 3.11 A, even after 48 hours of incubation, the seeding effect of 2 µM wt was statistically significant while 2 µM p-S129 α-syn seeds were not significant. However, at 0.1 µM, the impact of p-S129 α-syn seeding as compared to wt seeding was prominent. As shown in Fig. 3.11 B, at 0.1 µM, wt α-syn seeds accelerated the aggregation of α-syn, with the trend becoming apparent after 8-12 hours of incubation and increasing in a time-dependent manner thereafter. In contrast, at 0.1 µM, p-S129 α-syn seeds not only did not accelerate the aggregation of α-syn, but actually exerted an inhibitory effect on the process.



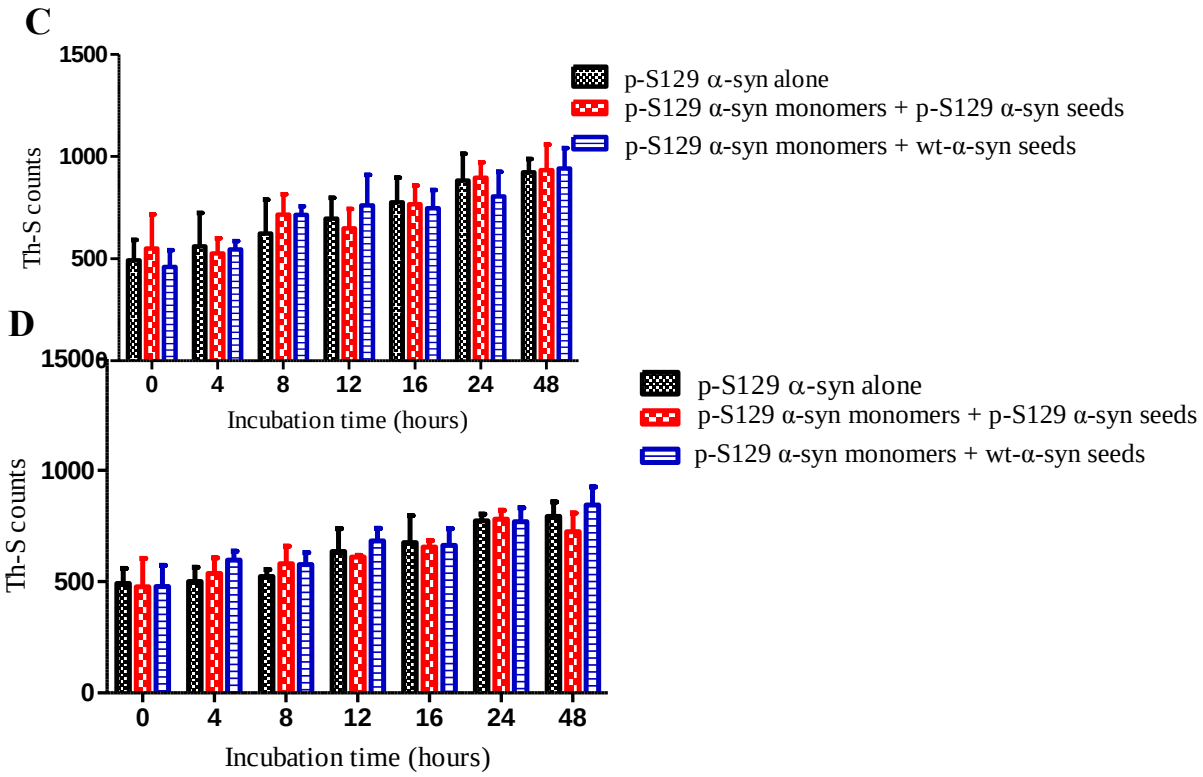


Figure 3.11. The ability of p-S129 α-syn to seed the on polymerization/aggregation of α-syn. A. Monomeric α-syn (100 µM) was incubated alone or with 2 µM or B. 0.1 µM (final concentration) of p-S129 or wt seeds for 48 hours. C. Samples of p-S129 monomeric α-syn (100 µM) was incubated alone or with 2 µM or D. 0.1 µM (final concentration) of p-S129 or wt seeds for 48 hours. The fibrillation was evaluated by Th-S fluorescence. The assay was performed in triplicate, and the means ± standard deviations are shown. Statistical analysis was performed using one way anova, followed by Tukey's multiple comparison test using the GraphPad Prism 5 software. (**, $p< 0.01$, *, $p< 0.05$).

To further investigate this rather unexpected finding, we assessed the ability of wt or p-S129 α-syn seeds to seed p-S129 α-syn monomers instead of wt. The monomeric p-S129 α-syn was incubated in the presence or absence of wt or p-S129 α-syn seeds at two different final concentrations (2 and 0.1 µM) for 48 hours. As shown in Fig. 3.11 C and D, regardless of the type or the concentration of the seeds, p-S129 α-syn failed to aggregate even after 48 hours of incubation,

indicating that phosphorylation at S129 has an inhibitory effect on the aggregation of the protein.

3.3.6 Assessing the effect of p-S129 α-syn on the aggregation of α-syn

To further explore the impact of phosphorylation at S129 on the aggregation of the protein, and considering that only 5% of α-syn is phosphorylated at S129 under physiological conditions (Hirai, et al., 2004), we investigated the effect of various amounts of p-S129 α-syn on the aggregation of α-syn. For this purpose, pure monomeric p-S129 α-syn was mixed with different concentrations non-phosphorylated α-syn to obtain the desired percentages of p-S129 α-syn monomers (0-100 % p-S129). These samples were incubated for up to 20 days, and the aggregation was assessed at different time points by Th-S assay. As shown in Fig. 3.12, the non-phosphorylated α-syn gradually aggregated reaching approximately 35,000 Th-S counts after 20 days of incubation. The sample containing 5% p-S129 α-syn exhibited a similar tendency to aggregate to the non-phosphorylated sample, with the only difference in the aggregation pattern of the two samples being the slightly slower rate of aggregation observed for the 5% p-S129 sample during the incubation period of between 3-12 days (Fig. 3.12). However, the sample containing 20% p-S129 α-syn showed a marked inability to aggregate, given that the reduction in Th-S fluorescence readings was more than 50%. In the case of the samples containing 50% and 100% p-S129 α-syn, the fibrillation was almost completely inhibited, with the Th-S fluorescence readings reaching approximately 2,000 counts after 20 days of incubation. Interestingly, the non-phosphorylated sample reached this aggregation level after only 2 days of

incubation (Fig. 3.12). These results further confirm that phosphorylation at S129 exerts an inhibitory effect on α-syn aggregation.

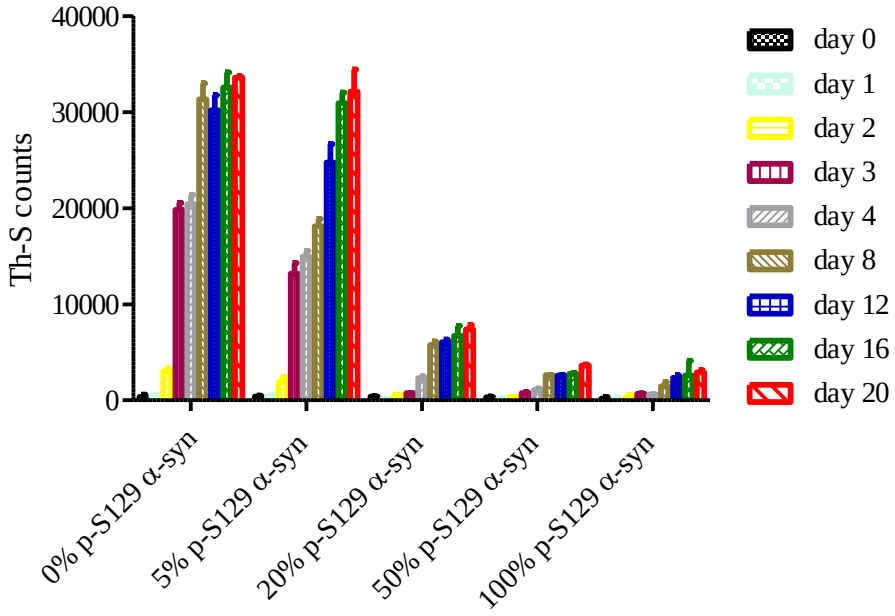


Figure 3.12. The effect of p-S129 on α-syn aggregation. Monomeric α-syn (100 μM) was incubated in the presence of various percentages (0, 5, 20 ,50 and 100%) of p-S129 monomeric α-syn for 20 days at 37°C with continuous shaking. The fibrillation was evaluated by Th-S fluorescence.The assay was performed in triplicate, and the means ± standard deviations are shown.

Collectively, the above results advocate a protective role for p-S129 α-syn, since it has been shown (i) not to be toxic to cells (Fig. 3.9 A and B) , (ii) not to promote the seeding-mediated toxicity (Fig. 3.9 C and D), (iii) not to aggregate (Fig. 3.11 C and D), (iv) to inhibit the seeded polymerisation of wt α-syn monomers (Fig. 3.11 A, B) and (v) to inhibit the aggregation of monomeric α-syn in a concentration-dependent manner (Fig. 3.12)

3.4 Discussion

Identifying the toxic species of α-syn together with the mechanism of their formation plays a central role in elucidating the α-syn-induced toxicity associated with PD and related disorders. Several lines of evidence indicate that the α-syn species driving neurodegeneration are the early aggregates, referred to as soluble oligomers, rather than mature amyloid fibrils (El-Agnaf, et al., 2003; Winner, et al., 2011). In order to identify specific toxic α-syn species, which contribute to the toxicity of the protein, we prepared various α-syn species (referred to as crude oligomers, crude seeds and crude fibrils) following a well-characterized *in vitro* aggregation protocol varying the incubation times from 2-7 days. The morphology and distribution of the aggregates present in the various preparations of α-syn were characterized by EM (Fig. 3.1). The toxic effect of these α-syn preparations was evaluated in two different neuroblastoma cell lines, BE(2)-M-17 and SHSH-5Y, either expressing only endogenous α-syn or overexpressing wt α-syn. The toxicity of the various α-syn species was first assessed in the cell lines expressing only endogenous α-syn. As shown in Fig. 3.2, the majority of the α-syn species employed in this experiment did not have a significant impact on the viability of the cells, with the crude oligomers being slightly toxic. However, when the same α-syn species were used for the treatment of the cell lines overexpressing wt α-syn, it became apparent that crude oligomeric preparation of α-syn, which is a heterogeneous mixture of monomeric and oligomeric α-syn (Fig. 3.1) was more toxic compared to crude seeds and fibrils (Fig. 3.3). Considering that the latter two preparations contain less or no monomeric or protofibrillar α-syn, and since they were more toxic in the cells overexpressing wt α-syn, we hypothesized that the lower levels of neurotoxicity observed for crude seeds and crude fibrils may

reflect their inability to grow. To support this finding, a previous study has shown that the toxicity of Aβ42 is not linked to specific prefibrillar aggregate(s), but to their ability to grow in the presence of Aβ42 monomers (Jan, et al., 2011; Wogulis, et al., 2005). To further investigate the impact of monomeric α-syn on the aggregation-induced toxicity of the protein, we assessed the effect of α-syn monomer-free preparations, namely pure fibrils and pure seeds (Fig. 3.4), on the viability of the wt BE(2) M-17 and SHSY-5Y cells expressing only endogenous α-syn in the presence or absence of exogenously added monomeric α-syn (10 μM final concentration). As illustrated in Fig. 3.5, the addition of monomeric α-syn to the cells pre-treated with pure seeds and fibrils increased the levels of toxicity when compared to the cells treated only with the pure seeds and fibrils. It is worth mentioning that, in the presence of α-syn monomers, pure seeds and fibrils induced a good level of toxicity, even at concentrations as low as 100 nM (Fig. 3.6 A and C).

The significance of α-syn monomers toxicity associated with the polymerisation/aggregation process was further confirmed with a series of experiments, during which the BE(2)-M-17 cells, whose endogenous α-syn was knocked down, were treated with pure fibrils and pure seeds. Compared to the wt cells, a significant level of protection against the seed and fibril growth-induced toxicity was achieved when the expression of endogenous α-syn was silenced (Fig. 3.7).

As the aim of the present study was to unravel the structural prerequisites of the seeding process and its relation to toxicity, we sought to unravel the role of p-S129 α-syn monomers in seeding the α-syn polymerisation/aggregation and to compare the mode of seeding action of wt and p-S129 α-syn. As shown in Fig.

3.9, addition of p-S129 α-syn monomers to the cells pre-treated with pure seeds or pure fibrils has no effect on cell viability. By comparing these findings with the corresponding data for the wt α-syn monomers (Fig. 3.5), which were shown to promote the toxicity of pure seeds and fibrils when added to the cells, it becomes apparent that p-S129 α-syn monomers behave differently from the wt α-syn monomers, suggesting a potential role of p-S129 α-syn in inhibiting the seeded polymerization process. It is still debatable whether α-syn phosphorylation at S129 promotes or inhibits the aggregation and toxicity of the protein, as far as the p-S129 α-syn-related toxicity is concerned, the results of the various studies are equally contradictory and inconclusive. Whereas studies on yeast, oligodendrial cells and a Drosophila model of PD have shown that α-syn phosphorylated at S129 (p-S129 α-syn) by various kinases [casein kinase 1 (CK1), CK1, Polo-like kinases (PLKs), G-protein-coupled kinases (GRKs)] correlates with α-syn toxicity (L. Chen & Feany, 2005; L. Chen, et al., 2009; Kragh, et al., 2009; Zabrocki, et al., 2008), studies employing the phosphomutants S129D and S129A are particularly contradictory. In some studies, S129D α-syn appears to be toxic (Chau, et al., 2009; L. Chen & Feany, 2005), while there are also reports supporting that the phosphomimic S129D α-syn plays a rather protective role (Gorbatyuk, et al., 2008; Kuwahara, et al., 2012; B. Wu, et al., 2011) or has no effect on the toxicity of the protein (Azeredo da Silveira, et al., 2009; Fischer, et al., 2009).

The findings above prompted us to further investigate the implications of p-S129 α-syn in the seeding of the aggregation process and according to the data obtained (Fig. 3.10 and Fig 3.12), monomeric p-S129 α-syn failed to seed the aggregation of wt α-syn at the concentrations used and for the given incubation

time. Moreover, prolonged incubation of wt α-syn with various percentages of p-S129 α-syn also resulted in a strong inhibitory effect, which was dependent on the percentage of p-S129 α-syn present in the samples (Fig. 3.12). These findings are in accordance with previous studies indicating that phosphorylation at S129 has a rather inhibitory effect on the aggregation of α-syn (Paleologou, et al., 2008; Waxman & Giasson, 2008).

Our data suggest that phosphorylation of α-syn at S129 may play a protective role against the toxic effect of α-syn by inhibiting the seeding polymerization process which was shown to underlie the α-syn-induced toxicity.

A deeper understanding of the role of p-S129 α-syn on cellular responses to nucleation dependent polymerization may provide a promising novel approach for treating PD and other associated disorders.

4 Chapter 4: Structure activity relationship of phenolic acid inhibitors of alpha-synuclein fibril formation and toxicity

4.1 Introduction

α-Syn aggregation proceeds through several key intermediate stages, with monomeric α-syn first assembling into oligomeric forms that gradually generate insoluble amyloid fibrils. Because α-syn aggregation plays a crucial role in PD pathogenesis and related synucleinopathies, intensive effort has been put into identifying compounds that could block or even reverse the aggregation process. Over the years, polyphenols, a set of more than 8000 compounds that contain one or more phenolic rings, have emerged as potent amyloid inhibitors, interfering with the *in vitro* fibril assembly of many amyloidogenic proteins including α-syn, β-amyloid (Aβ), tau-protein and prions (reviewed by Porat, et al., 2006).

Gallic Acid (GA) is a phenolic acid. Phenolic acids constitute a group of compounds, which are derived from benzoic acid and cinnamic acid, giving rise to hydroxybenzoic acids and hydrocinnamicacids, respectively. GA (3,4,5-trihydroxybenzoic acid) is a benzoic acid derivative that can be found in almost all plants, with the highest GA contents detected in gallnuts, witch hazel, pomegranate, berries such as blackberries and raspberries, sumac, tea leaves and oak bark. GA can also be isolated from the roots of Radix Paeoniae (white-flowered peony), which is commonly used to treat vascular and liver diseases in traditional Chinese medicine (J. H. Ho & Hong, 2011). It has been reported that GA possesses anti-oxidant (Y. J. Kim, 2007), anti-inflammatory (Kroes, van den

Berg, Quarles van Ufford, van Dijk, & Labadie, 1992) and anti-viral (Kreis, Kaplan, Freeman, Sun, & Sarin, 1990) properties, and a well-documented anti-cancer effect (H. H. Ho et al., 2013; K. C. Liu et al., 2011; C. S. Yang et al., 2000). Recently, GA has been reported to act as a potent anti-oxidant and free radical scavenger in a rat PD model (Sameri, Sarkaki, Farbood, & Mansouri, 2011). Additionally, GA was shown to efficiently inhibit α-syn and β-amyloid (Aβ) aggregation and toxicity *in vitro* (Bastianetto, Yao, Papadopoulos, & Quirion, 2006; Di Giovanni, et al., 2010).

The aim of the present chapter was to systematically assess the ability of GA to (a) inhibit α-syn oligomerization and fibrillation, (b) block α-syn-induced toxicity and (c) disaggregate preformed α-syn fibrils. To gain insight of the mechanism of action of GA against α-syn aggregation and toxicity and to establish a structure-activity relationship, we assessed the anti-fibrillogenic effect of eleven different hydroxybenzoic acid derivatives with chemical structures similar to GA. The selection of phenolic acids was based on the number of the hydroxyl moieties attached to the phenyl ring. To further investigate the role of hydroxyl groups in the inhibitory activity of phenolic acids, we also included and assessed the effect of three different benzoic acid derivatives that have fluorides and methoxy groups instead of hydroxyl moieties.

4.2 Experimental procedure

4.2.1 Expression and purification of recombinant human α-syn

As described in the experimental procedure 3.2.1

4.2.2 α-syn purification and characterization

As described in the experimental procedure 3.2.2

4.2.3 Aggregation of α-syn *in vitro*

Stock solutions of the compounds tested (10 mM) were prepared in DMSO. Solutions of lower concentrations were prepared by diluting the stock solutions to final concentration of 25-100μM. The amount of DMSO in the final samples was 1%. Samples of 25 μM (unless otherwise stated) α-syn in PBS were aged either alone or with phenolic acid at various molar ratios (phenolic acids to protein molar ratios of 4:1, 2:1 and 1:1). The samples were placed in 1.5ml sterile polypropylene tubes, sealed with parafilm and incubated at 37°C for 5 days with continuous shaking at 800 rpm in a thermomixer (Eppendorf). Samples were collected at regular intervals. The thioflavin-S (Th-S) fluorescence was measured immediately, while the rest of the samples were stored at -80°C until required for further analysis.

4.2.4 Thioflavin-S (Th-S) fluorescence assay

As described in the experimental procedure 3.2.5

4.2.5 Transmission Electron Microscopy (TEM)

Electron microscopy images were produced from α-syn aged in the presence or absence of GA. The samples (5 μL) were deposited on formvar-coated 400 mesh copper grids, fixed briefly with 0.5% glutaraldehyde (5 μl), negatively

stained with 2% uranyl acetate (Sigma-Aldrich) and examined in a Philips CM-10 TEM electron microscope.

4.2.6 Immunoblotting

Samples (20 ng) of α-syn incubated alone or with GA were mixed with a loading sample buffer (250 mM Tris-HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue) without SDS or boiling and then separated on 1 mm 15% SDS-PAGE gels. The separated proteins were transferred to nitrocellulose membranes (0.45 μm, Whatman Gmbh-Germany) at 90 V for 80 mins. The membranes were boiled for 5 mins in PBS and then blocked for 1 hour with 5% non-fat milk prepared in PBS-Tween-20 (0.05% PBST). The membranes were incubated overnight at 4°C with the primary antibody, namely the mouse monoclonal anti-α-syn (211) that recognizes human α-syn (121-125) (Santa Cruz Biotechnology, USA), at a dilution of 1:1000. The membranes were then washed several times with PBST, followed by incubation with HRP-conjugated goat anti-mouse antibody (Dako Ltd., Ely, UK) at a dilution of 1:70,000 for 60 mins at room temperature and with gentle agitation. The membranes were then extensively washed for 25 mins. The immunoreactive bands were visualized with a SuperSignal West Femto Chemiluminescent Substrate Kit (Pierce, Rockford, USA) according to the manufacturer's instructions.

4.2.7 Immunoassay for measuring oligomeric α-syn

A 384-well ELISA plate (Nunc Maxisorb, Nunc, Denmark) was coated with 1 μg/ml of non-biotinylated mouse monoclonal anti-α-syn antibody [mAb, 211 - recognizes amino acid residues 121-125 of human α-syn (Santa Cruz Biotechnology, California, USA)]diluted in 200 mM NaHCO3, pH 9.6 (50

µl/well) and incubated overnight at 4°C. The plate was then washed 4 times with PBST and blocked with 100 µl/well of blocking buffer (5% gelatin from cold water fish skin, 0.05% Tween 20 in 1X PBS pH 7.4) for 2 hours at 37°C. After being washed 4 times with PBST, 50 µl of the samples were dispensed in each well, and each sample was tested in duplicate. The plates were then incubated at 37°C for another 3 hours. After washing 4 times with PBST, 50 µl of biotinylated 211 antibody diluted in blocking buffer to a concentration of 0.4 µg/ml was added and incubated at 37°C for 2 hours. The wells were washed 4 times with PBST and incubated with 50 µl/well of extravidin-peroxidase (Sigma-Aldrich, GmbH-Germany) diluted 1:7,500 in blocking buffer and incubated for 1 hour at 37°C. The wells were then washed 4 times with PBST before adding SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, USA) (50 µL/well).The chemiluminescence in relative light units was measured immediately using a microplate reader (Perkin Elmer).

4.2.8 Culture of BE(2)-M-17 human neuroblastoma cells

As described in the experimental procedure 3.2.10

4.2.9 Measurement of cell viability

As described in the experimental procedure 3.2.13

4.2.10 Congo red binding assay

Congo red (20µM) was dissolved in PBS (pH 7.4) and filtered through a 0.45 µm filter. Samples of α-syn (5 µM), aged alone or with GA at different molar ratios, were mixed with Congo red (final concentration 5 µM), and the reaction samples were thoroughly mixed. The UV absorbance spectrum was recorded from 400 nm to 600 nm in a spectrophotometer (DU-800, Beckman-Coulter) using 10

mm quartz cuvettes (Hellma Analytics-Germany). Monomeric α-syn and Congo red alone were used as negative controls.

4.2.11 α-Syn disaggregation assay

Recombinant α-syn dissolved in sterilized PBS (pH 7.4) was aggregated at a concentration of 25 μM as indicated above. The resulting aggregated α-syn was incubated either alone or with GA at various molar ratios (GA: α-syn molar ratios 6:1, 4:1 and 2:1). It should be noted that for the purpose of the experiment, the concentration of α-syn was assumed to be the same as that of the fresh α-syn. The samples were incubated at 37°C for 3 days on a thermomixer with continuous shaking at 800 rpm. Samples were collected at regular intervals, and the Th-S fluorescence was measured immediately.

4.2.12 Seeding polymerization assay

The aggregation of monomeric α-syn with or without seeding was carried out as described elsewhere (Di Giovanni, et al., 2010). Mature α-syn fibrils were fragmented by sonication to obtain short fibrils, which were employed as 'seeds'. 100 μM monomeric α-syn was seeded with 2 μM seeds and incubated in the presence or absence of GA (10 μM and 50 μM) at 37°C for 6 hours with continuous shaking. Fibrillation was monitored by Th-S binding as described above.

4.2.13 Size Exclusion Chromatography (SEC) for separating α-syn oligomers and monomers

SEC was carried out using an AKTA FPLC system (GE Healthcare-Sweden) and a superdex 200 column at 4 °C, in order to separate the oligomers generated from the aggregation of α-syn with GA (GA: α-syn molar ratio of 4:1).

Monomeric α-syn at a concentration of 100 µM was aggregated in the presence of GA for 5 days as described above. At the end of the aggregation process, the sample was centrifuged for 45 mins at 14,000 x g at 4°C generating a supernatant free from insoluble material. Prior to injecting 80% of the supernatant generated, the column was thoroughly equilibrated with an SEC running buffer (1x PBS, pH 7.4) and the flow rate was set to 0.1 ml/min (0.5 ml/fraction). α-Syn elution was monitored at absorbance wavelengths of 215 nm, 254 nm, and 280 nm. To determine the elution time for monomeric α-syn, molecular weight standards (ferritin 440 kDa, aldolase 171 kDa, albumin 68 kDa and chymotrypsinogenA 25 kDa) and monomeric α-syn were co-injected into the column and eluted to the same standards as mentioned above. The fractions eluting between 7-9 ml CV were combined and labeled as oligomers (sample P1), whereas the fractions eluting in the 13–15ml CV were combined and labeled as monomers (sample P2). The P1 and P2 fractions were further characterized by western blotting and TEM.

4.2.14 UV scanning

The P1 and P2 samples, representing the oligomeric and monomeric fractions of SEC, respectively, were concentrated using a speed vac (CentriVap, Labconco). The content concentration of protein was estimated by using BCA assay. The UV absorbance spectrum was recorded from 200-600 nm in a spectrophotometer (DU-800, Beckman-Coulter) using 10 mm quartz cuvettes (Hellma Analytics-Germany) and employing equal concentrations of both P1 and P2. Fresh monomeric α-syn was used as a negative control.

4.2.15 NMR

For NMR studies, recombinant 15N-labeled α-syn was expressed and purified as previously described (Bussell & Eliezer, 2003a; Eliezer, Kutluay, Bussell, & Browne, 2001a), resuspended in PBS at pH 6.5. Two-dimensional proton-nitrogen correlation spectra were acquired for α-syn at a 200 μM concentration in the absence of GA and in the presence of increasing GA: α-syn stoichiometries of 0.5:1, 1:1, 2:1, 5:1 and 10:1. Data were collected on a Varian 600 MHz Unity Inova Spectrometer equipped with a cold probe.

4.3 Results

4.3.1 The effect of GA on α-syn fibril formation

To investigate the effect of GA on α-syn fibrillation, 25μM of α-syn was incubated with GA at varying molar ratios of GA: α-syn of 4:1, 2:1 and 1:1 for a period of 6 days. GA inhibited the formation of α-syn fibrils in a concentration-dependent manner as indicated by the Th-S assay (Fig. 4.1 A). Taking into account the lack of Th-S signal at the 4:1 ratio, GA exhibited an excellent inhibitory effect on α-syn fibrillation, inhibiting it completely during the 6-day incubation (Fig. 4.1 A). At a 2:1 ratio, GA also suppressed the formation of fibrils to a great extent, and the Th-S signal was detected only after 4 days of incubation, while after 6 days, GA reduced the Th-S counts to approximately one fifth of the control counts (Fig. 4.1 A). Even at a 1:1 ratio, GA hindered the fibrillation of α-syn but to a smaller extent than the other ratios (Fig. 4.1 A). The anti-fibrillogenic activity of GA was further assessed by using a Congo red binding assay. Congo red (CR) is a dye with high affinity for amyloid fibrils. The maximum absorption

of CR alone (5 μM) was 490 nm, while when incubated with α-syn amyloid fibrils, the maximum absorption shifted to 508 nm (Fig. 4.1 B).This shift in the peak absorption wavelength represents the binding of CR to the β-sheet-rich fibrils. However, this prominent shift was not observed for the fresh α-syn sample or when α-syn was aged in the presence of GA (Fig. 4.1 B), indicating that GA inhibited the formation of structures with a β-sheet conformation. In fact, GA blocked the formation of β-sheets in a dose-dependent manner, similar to the Th-S results. It was observed that the lower the GA concentration, the more prominent the peak absorbance wavelength shift was (485 nm, 493 nm and 500 nm for 100 μM, 50 μM and 25 μM GA, respectively). Electron microscopy images also confirmed that GA not only affected the extent of fibrillation in a dose-dependent fashion but also the morphology of α-syn fibrils. Indeed, instead of forming dense meshes of long fibrils (longer than 1 μm) as observed in aged α-syn alone (Fig. 4.1 C), α-syn aged in the presence of 100 μM (Fig. 4.1 D) and 50 μM of GA (Fig. 4.1 E) generated thin, sheared fibrils that were approximately 0.1-0.2 μm in length. α-Syn aged in the presence of 25 μM GA (Fig. 4.1 F) formed longer fibrils (approximately 0.5 μm long), which were arranged in dense networks.

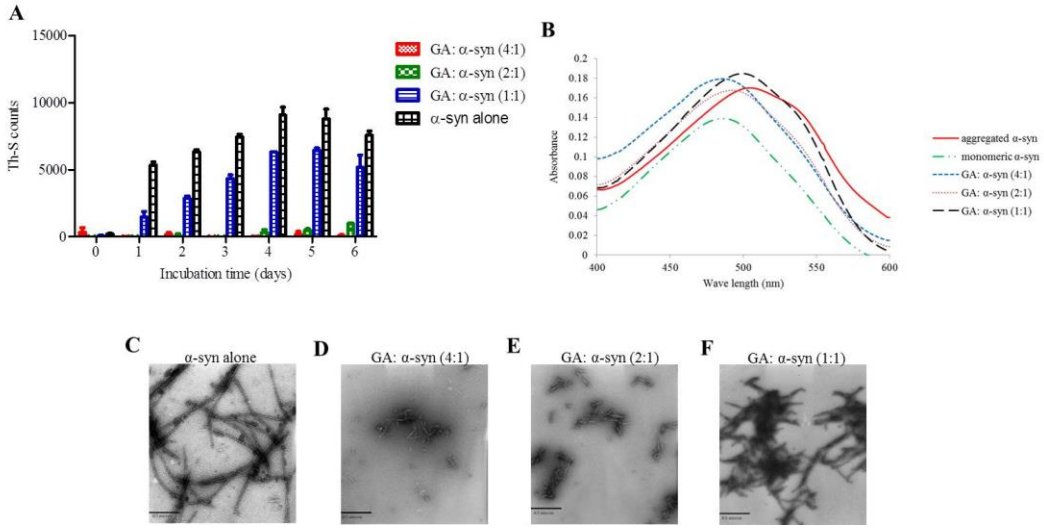


Figure 4.1. GA inhibits α-syn fibrillation in a concentration-dependent manner. A. Samples of α-syn (25 µM) were incubated alone or in the presence of GA (molar ration of GA: α-syn 4:1, 2:1, 1:1) for 6 days with continuous shaking at 37°C. Fibril formation was estimated by Th-S fluorescence. The assay was performed in triplicate (average of triplicate measurement ± standard deviation).B. Congo red binding for samples of α-syn (25 µM) incubated alone or in the presence of GA (molar ration of GA: α-syn 4:1, 2:1, 1:1) for 6 days with continuous shaking at 37°C. Samples of α-syn (5 µM) aged alone or with GA were mixed with Congo red at a final concentration of 5 µM. The reaction samples were thoroughly mixed and placed in a 10mm quartz cuvette. The UV absorbance spectra were recorded from 400 nm to 600 nm. C-F. Electron microscopy images of negatively stained samples of α-syn (25 µM) aged alone or in the presence of GA (molar ratio of GA: α-syn 4:1, 2:1, 1:1) for 6 days with continuous shaking at 37°C. C. Aged α-syn alone. D. α-Syn aged in the presence of GA at a molar ratio of GA: α-syn 4:1. E. α-Syn aged in the presence of GA at a molar ratio of GA: α-syn 2:1. F. α-Syn aged in the presence of GA at a molar ratio of GA: α-syn 1:1. Scale bar, 500 nm.

4.3.2 The effect of GA on α-syn oligomerization (early aggregates)

To evaluate the effect of GA on α-syn oligomer formation, samples of α-syn aged alone or in the presence of GA at different molar ratios were assessed for their oligomeric content by oligomer-specific ELISA and immunoblotting. For the detection of α-syn oligomeric species in the samples, we employed a novel ELISA developed in our laboratory at the UAEU (El-Agnaf, et al., 2006), which specifically recognizes the oligomeric species present in the samples. The ELISA results (Fig. 4.2 A) indicated that GA could only inhibit the formation of α-syn

oligomers when employed at the highest concentration namely 100 μM. Interestingly, at lower concentrations, GA appeared to enhance α-syn oligomerization as compared to the control (Fig. 4.2 A). The samples of α-syn aged in the presence of 25 μM and 50 μM GA exhibited gradually increasing oligomeric content, which was much higher than the control (Fig. 4.2 A).

Similarly, immunoblotting of the same samples (Fig. 4.2 B) indicated a decrease in the oligomeric species when α-syn was aged with 100 μM GA (Fig. 4.2 B), while there was a characteristic increase in the band corresponding to dimeric α-syn (Fig. 4.2 B). The same sample was also characterized by the presence of strong monomeric and trimeric (approximately 50 kDa) bands and the absence of the band corresponding to larger α-syn aggregates with a high molecular weight. However, in the presence of 50 μM and 25 μM GA, the bands corresponding to the oligomeric species were much stronger, with a concentration-dependent decrease in the bands corresponding to the monomeric, dimeric and trimeric species. Additionally, the intensity of the band corresponding to larger α-syn aggregates increased in a dose-dependent manner. These data indicate that GA at low concentrations may have the ability to stabilize α-syn oligomers.

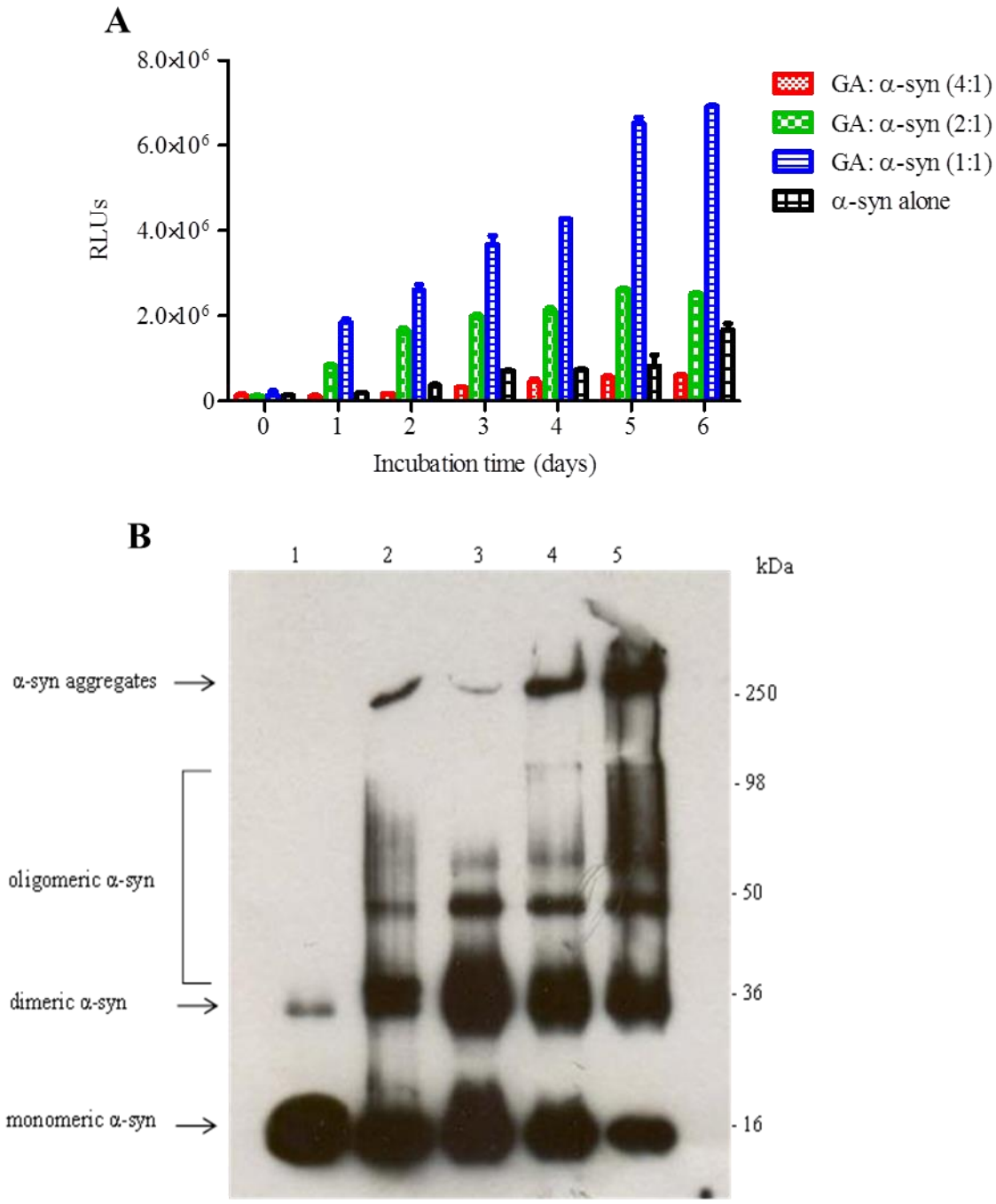


Figure 4.2. GA inhibits α-syn oligomerization at a high concentration, but at lower concentrations it promotes oligomerization. A. Samples of α-syn (25 μM) aged alone or in the presence of GA at different molar ratios for 6 days with continuous shaking at 37°C were assessed for their ability to inhibit the formation of oligomers by the oligomeric ELISA assay. The assay was performed in duplicate (average of duplicate measurements ± standard deviations). B. Immunoblot analysis of the effect of GA on α-syn oligomerization. Fresh or aged α-syn samples alone or in the presence of GA at molar ratios of GA: α-syn 1:1, 2:1 and 4:1 incubated for 6 days with continuous shaking at 37°C were separated by electrophoresis in a15% SDS-PAGE gel. Lane 1: fresh α-syn; lane 2: aged α-syn; lane 3: GA: α-syn molar ratio of 4:1, lane 4: GA:α-syn molar ratio 2:1 and lane 5: GA:α-syn molar ratio 1:1.

4.3.3 The effect of GA on preformed α-syn amyloid fibrils

Given that GA was shown to be such an effective inhibitor of α-syn fibrillation, we investigated whether it could also reverse fibrillation. Therefore, 25 μM of preformed α-syn fibrils (Fig. 4.3 A) were incubated at 37°C in the presence of GA at molar ratios of GA: α-syn of 6:1, 4:1 and 2:1 for a period of 48 hours. By measuring the Th-S fluorescence counts (Fig. 4.4 A) we estimated the fibril content at the indicated time points. At time 0, the Th-S counts were approximately 8000 for all samples (Fig. 4.4 A). The α-syn fibrils that were incubated alone continued to aggregate further, as indicated by the increase in Th-S counts (Fig. 4.4 A). However, the α-syn fibrils that were incubated in the presence of GA disaggregated over time as shown by the decrease in the Th-S counts (Fig. 4.4 A). It is noteworthy that after 24 hours of incubation, α-syn fibrils incubated without GA gave approximately 18,000 Th-S counts, while the fibrils incubated with all concentrations of GA tested produced less than 2,000 Th-S counts (Fig. 4.4 A). Thus, GA disaggregated preformed α-syn fibrils in a dose-dependent fashion. Furthermore, similar results were obtained when the experiment was performed using α-synuclein fibrils after reaching a plateau in Th-S assay (Fig. 4.3 B).

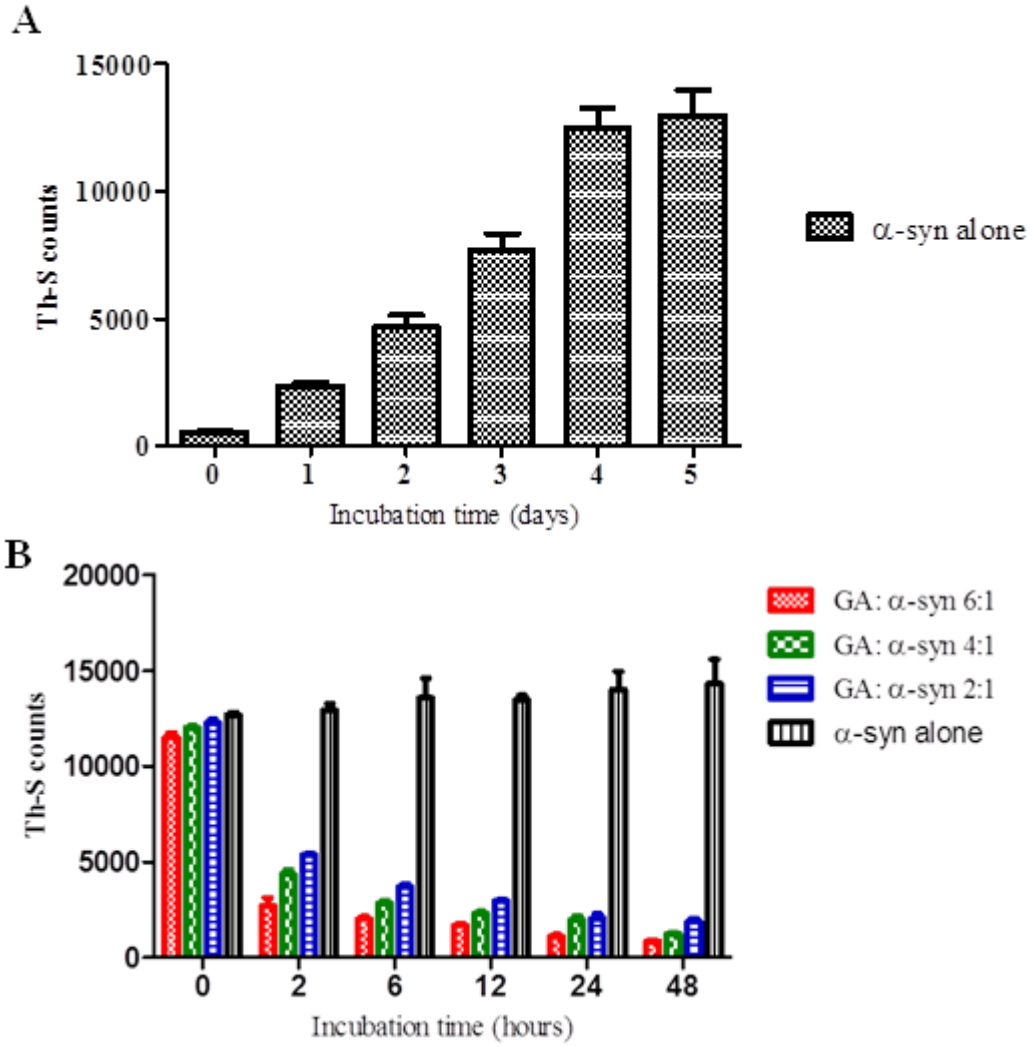


Figure 4.3. GA disaggregates preformed α-syn fibrils (after reaching plateau). A. Samples of α-syn (25 μM) were incubated alone for 5 days with continuous shaking at 37°C. Fibril formation was estimated by Th-S fluorescence. The assay was performed in triplicate (average of triplicate measurement ± standard deviation). B. Samples of aggregated α-syn were incubated for 48 hours at 37°C in the absence or presence of various concentrations of GA (GA: α-syn 6:1, 4:1, 2:1). The fibril content was then measured by the Th-S binding assay. The assays were performed in triplicate (average of triplicate measurements ± standard deviations).

After 48 hours of incubation, the samples were also tested for their CR binding (Fig. 4.4 B). As expected, α-syn fibrils incubated in the presence of GA produced spectra with a peak absorbance wavelength that was shifted slightly compared to the controls, indicating minimal β-sheet content. In contrast, the fibrils of α-syn that were incubated alone produced a peak absorbance at 550 nm, indicating a high β-sheet content (Fig. 4.4 B). These finding were confirmed by EM (Fig. 4.4 C). All samples at 0 were characterized by the presence of dense meshes of fibrils longer than 0.5 μm. However, the α-syn fibrils incubated in the presence of GA gradually became thinner and shorter and appeared fragmented, isolated and scarce, unlike the control, which continued to demonstrate networks of long fibrils (Fig. 4.4 C).

To assess whether GA disaggregation of α-syn fibrils was accompanied by a decrease in α-syn toxicity, we then evaluated the toxicity of the α-syn species that resulted from the disaggregation experiment on human neuroblastoma BE(2)-M-17 cells (Fig. 4.4 D). The samples were diluted to a final α-syn concentrations of 0.5 μM, 1 μM and 5 μM. Preformed α-syn fibrils incubated for 6 days in the absence of GA decreased cell viability in a dose-dependent manner. However, preformed α-syn fibrils incubated with GA for 6 days generated species that were less toxic compared to the control. This trend was most prominent for 5 μM α-syn, which, when incubated in the absence of GA, induced the death of 45% of the cells, but in the presence of GA, cell death was less than 25%.

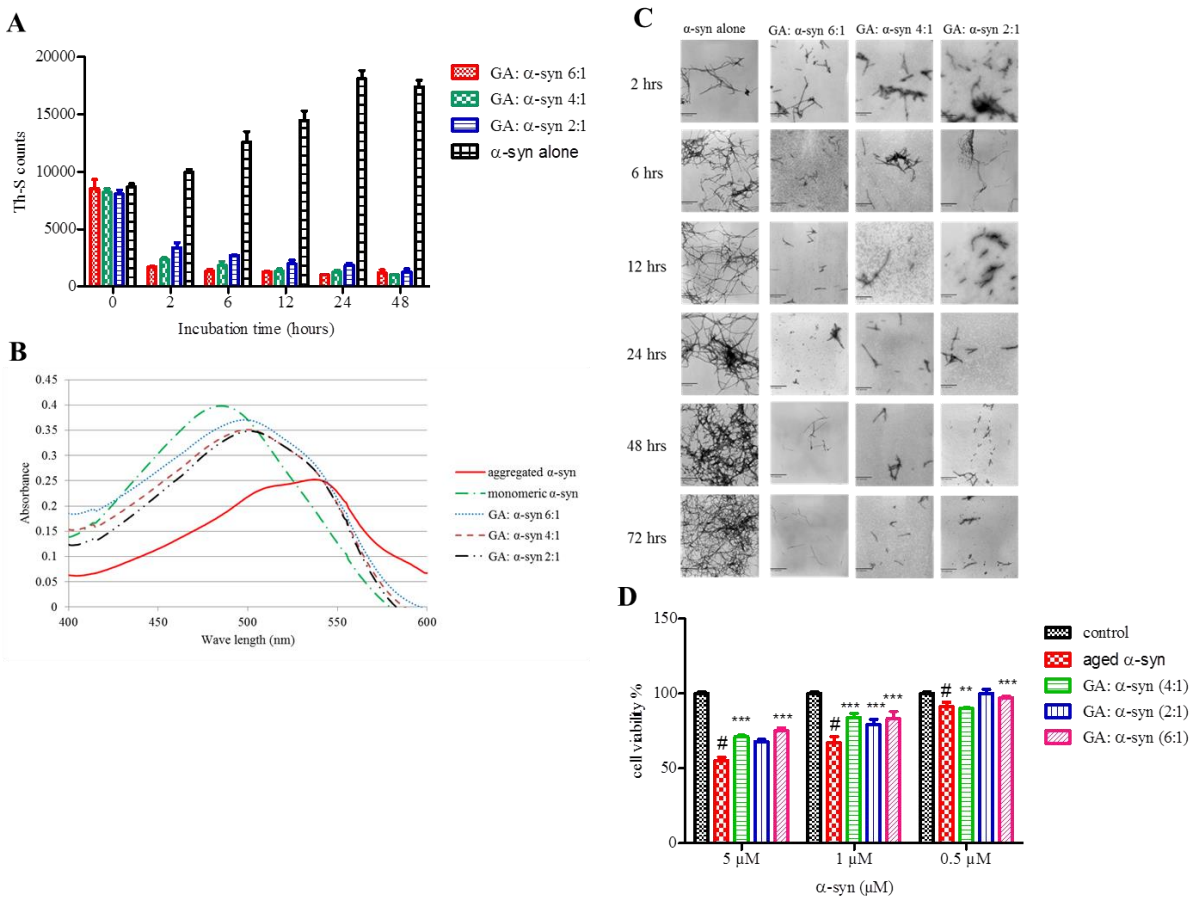


Figure 4.4. GA disaggregates preformed α-syn fibrils in a concentration-dependent manner**.** A. Samples of aggregated α-syn were incubated for 48 hours at 37°C in the absence or presence of various concentrations of GA (GA: α-syn 6:1, 4:1, 2:1). The fibril content was then measured by the Th-S binding assay. The assays were performed in triplicate (average of triplicate measurements ± standard deviations).B. Congo red binding to samples of pre-aged α-syn (25 μM) incubated alone or in the presence of GA (molar ratio of GA: α-syn 6:1, 4:1, 1:1) for 72 hours with continuous shaking at 37°C. Samples of α-syn (5 μM) incubated alone or with GA at different molar ratios were mixed with Congo red, at a final concentration of 5μM. The reaction samples were thoroughly mixed and placed in a 10mm quartz cuvette. The UV absorbance spectra were recorded from 400 nm to 600 nm. C. Electron microscopy images of negatively stained samples of pre-aged α-syn (25 μM) incubated alone or in the presence of GA for 72 hours with continuous shaking at 37°C. 1. α-Syn aged alone. 2. α-Syn aged in the presence of GA at a GA:α-syn molar ratio of 6:1. 3. α-Syn aged in the presence of GA at a GA:α-syn molar ratio of 4:1. 4. α-Syn aged in the presence of GA at a GA: α-syn molar ratio of 2:1. Scale bar, 500 nm. D. The disaggregation of preformed α-syn fibrils by GA generated species that were less toxic to the cells. The viability of BE(2)-M-17 human neuroblastoma cells was assessed by the MTT assay. The results are expressed as percentages of the control average (i.e., untreated cells). The α-syn species generated by 72h incubation of preformed α-syn fibrils in the presence or absence of GA were added to the cells 48 hours prior to MTT addition (average of 3 wells ± SD. Statistical analysis was performed using two tailed unpaired t-test, ***, $p < 0.001$; **, $p < 0.01$).

4.3.4 The effect of GA on the seeding of α-syn aggregation

It has been previously shown that the process of amyloid fibril formation follows a nucleation-dependent polymerization (Jarrett and Lansbury, 1992). According to this model, soluble species generated via the nucleation of oligomeric species (nucleation or lag time phase), which in turn polymerize (polymerization or growth phase) to generate fibrils, thus reaching a final plateau known as the equilibrium phase (Harper et al., 1999). Small aggregates or seeds have been shown to accelerate the nucleation phase of amyloid formation both *in vitro* and *in vivo* by a process known as seeding (Jarrett and Lansbury, 1993, Luk et al., 2012, Harper and Lansbury, 1997, Volpicelli-Daley et al., 2011). Given that GA inhibited both α-syn fibrillation and disaggregated preformed α-syn fibrils; we sought to identify the effect of this phenolic acid on the seeding of α-syn aggregation. More specifically, mature α-syn fibrils were fragmented by sonication to obtain short fibrils, which were employed as 'seeds' (Fig. 4.5 B). These short fibrillar 'seeds' were then added to monomeric α-syn, which was allowed to aggregate as described above. As expected, the addition of short fibrillar seeds accelerated the fibrillation of α-syn as indicated by the increased Th-S counts. Indeed, with seeding, the extent of α-syn fibrillation after 6 hours of incubation was comparable to the fibrillation of the protein incubated for 72 hours without seeding (Fig. 4.5 B and Fig. 4.1, respectively).

In order to assess the effect of GA on the seeding of α-syn aggregation, 10 and 50 μM GA was added to 100 μM monomeric α-syn containing seeds at a final concentration of 2 μM, and the mixture was incubated with continuous mixing at 37°C for 6 hours. GA at 50 μM inhibited the seeded fibrillation of α-syn by approximately 90% as indicated by the extremely low Th-S counts. At lower

concentrations (10 µM), GA also had an inhibitory effect on the seeded fibrillation of α-syn but to a smaller extent. At 10 µM, GA inhibited seeded fibrillation by 40-50% (Fig. 4.5 A). These findings were confirmed by TEM (Fig. 4.5 B).

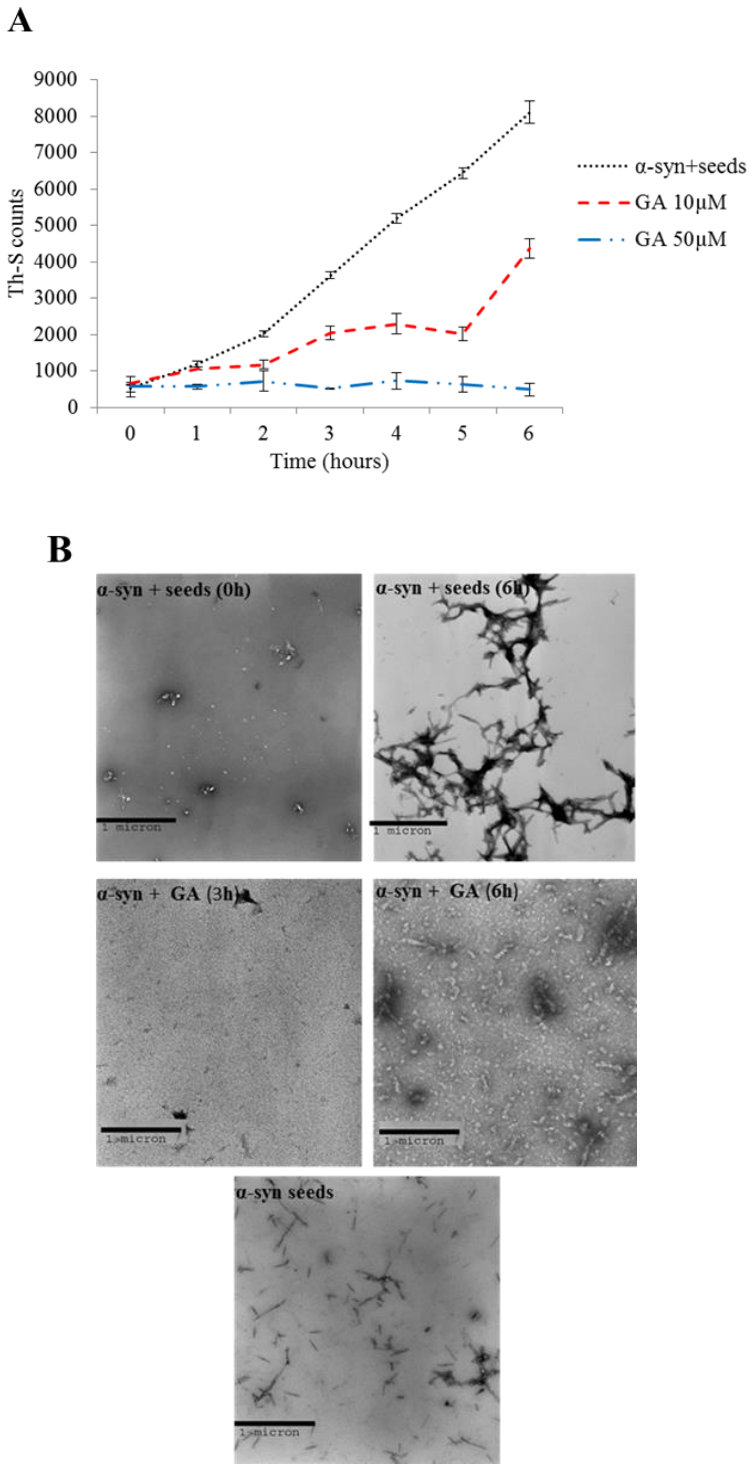


Figure 4.5. GA inhibits the seeded fibrillation of α-syn**.** A. Samples of α-syn (100μM) seeded with short fibrillar α-syn (2 μM) were incubated in the presence or absence of GA at different concentrations (10 μM and 50 μM) for 6 hours with continuous shaking at 37°C. The extent of fibrillation was estimated by the Th-S binding assay. The assays were performed in triplicate (average of triplicate measurements ± standard deviations). B. Electron microscopy images of negatively stained samples of seeds alone and α-syn+ seeds incubated alone or in the presence of GA (50 μM) for 6 hours with continuous shaking at 37°C. *Scale bar represents* 1000 nm.

4.3.5 The effect of GA on α-syn aggregates-induced toxicity

It has been reported that the oligomeric intermediates are the neurotoxic species in the amyloid fibrillation pathway (Allsop et al., 2001; Argyriou et al., 2012; Breydo, Wu, & Uversky, 2012; Colla et al., 2012; El-Agnaf, Nagala, Patel, & Austen, 2001; El-Agnaf, et al., 2006; Vendruscolo, Paci, Dobson, & Karplus, 2001; Walsh et al., 2002). As described above, at high concentration (4:1), GA inhibited both early and late aggregate formation, whereas at low concentrations (2:1 and 1:1), it stabilized α-syn oligomers (Fig. 4.2 A). To determine the effect of GA on the toxicity conferred by α-syn aggregates, a cell-based toxicity assay, the MTT assay, was conducted with human neuroblastoma BE(2)-M-17 cells. The samples were diluted to final α-syn concentrations of 5 μM and 0.5 μM. The MTT assay showed that α-syn aged in the absence of GA decreased cell viability in a dose-dependent manner (Fig. 4.6). However, when α-syn was aged in the presence of a high concentration of GA (4:1), there was visible neuroprotection of the cells observed (Fig. 4.6). At low concentrations (2:1 and 1:1), GA exhibited a minor protective effect against α-syn toxicity, possibly due to the increase in oligomers as demonstrated by the ELISA results and the immunoblotting analysis (Fig. 4.2, A and B, respectively). Our results clearly demonstrate that GA reduced the toxicity of α-syn at a high concentration (100

μM) by significantly inhibiting the formation of toxic α-syn oligomers. The toxicity of the oligomers resulted from α-syn: GA 1:4 was compared with α-syn oligomers prepared in the absence of GA as described in the materials and methods section by using MTT assay (Fig. 4.6 B). Both samples were characterized for the presence of oligomers by using SDS PAGE (Fig. 4.6 C) and native PAGE (Fig. 4.6 D). It was clear, that the oligomers resulting from the incubation of α-syn with GA were not toxic comparing with oligomers in absence of GA. Moreover, these oligomers were stable under denaturing conditions (Fig. 4.6 C) comparing with those formed in the absence of GA.

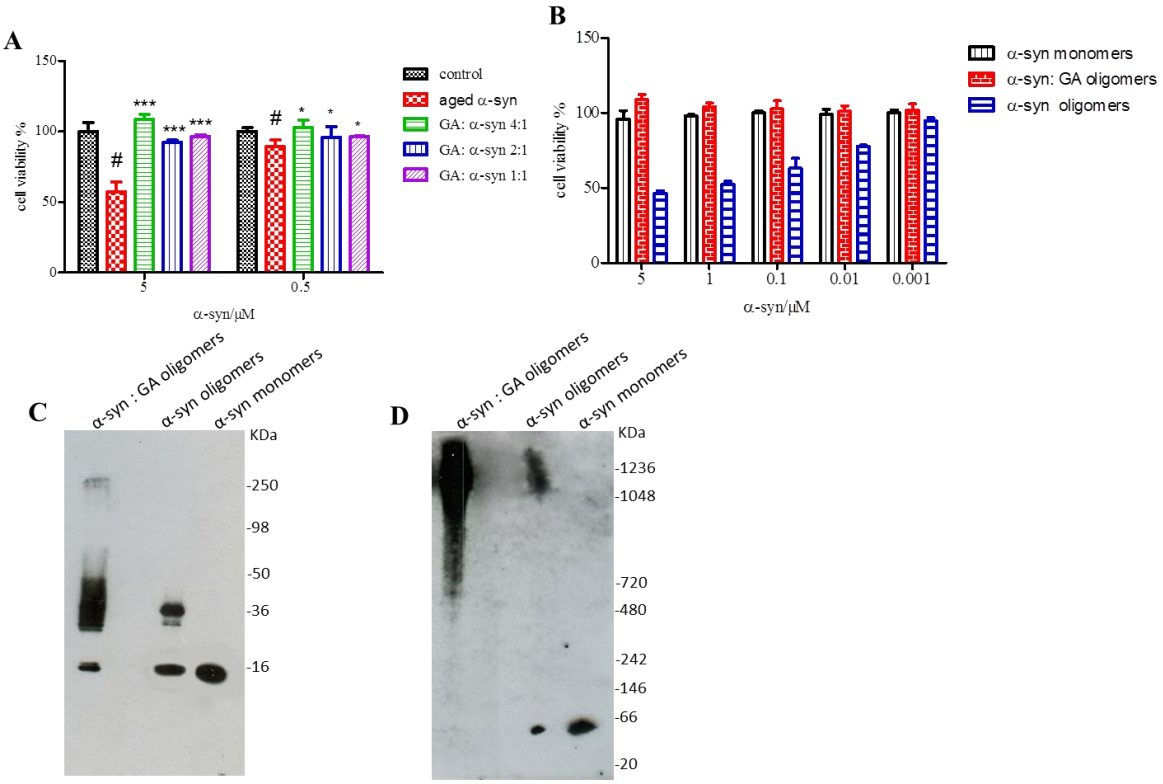


Figure 4.6. Effect of GA on the toxicity of aggregated α-syn**.** A.The viability of BE(2)-M-17 human neuroblastoma cells was estimated by the MTT assay. The results are expressed as the percentage of the control average (i.e., untreated cells). The cells were treated with aggregated α-syn with/without GA for 48 hours prior to MTT addition (average of 3 wells ± standard deviation. Statistical analysis was performed using two-tailed unpaired t-test, ***, $p< 0.001$; *, $p< 0.05$). B. The viability of BE(2)-M-17 human neuroblastoma cells was estimated by the MTT assay. The cells were treated with α-syn oligomers in absence or presence of GA for 48 hours prior to MTT addition (average of 3 wells ± standard deviation. C. Immunoblot analysis of α-syn oligomers generated in presence or absence of GA, separated by electrophoresis in a 15% SDS-PAGE gel. D.

Immunoblot analysis of α-syn oligomers generated in presence or absence of GA, separated by electrophoresis in a 3-12% Native-PAGE gel.

4.3.6 GA inhibition of α-syn fibrillation is mediated via binding to the intermediate species and forming stable oligomers

The strong inhibitory effect that GA exerted on fibrillation together with the stimulating effect it had on α-syn oligomerization at lower GA concentrations, prompted us to investigate further the interaction of GA with α-syn oligomers. For this purpose, monomeric α-syn (100 μM) was aggregated in the presence of GA (GA: α-syn 4:1). After 5 days of incubation the samples were centrifuged and the supernatant was injected in a superdex 200 SE column. The elution volume for monomeric α-syn was determined by a molecular weight standard (Fig. 4.7 A), and was eluted to a peak corresponding to a column volume of 13-15 mL (Fig. 4.7 B), while oligomeric α-syn eluted in a peak corresponding to column volume of approximately 7-9 ml (Fig. 4.8 A). The fractions corresponding to the oligomeric and monomeric α-syn peaks were pooled together giving rise to P1 and P2 samples, respectively (Fig. 4.8 A), which were concentrated using a speed vac. The α-syn species in samples P1 and P2 were characterized by western blotting (Fig. 4.8 B). According to the western blotting results, the oligomers generated during the incubation of α-syn with GA are stable under denaturing conditions (Fig. 4.6 C and 4.8 B). Electron microscopy of the same samples indicates the presence of different species of oligomers in P1 (Fig. 4.8 D), which agrees with the imunoblotting results (Fig. 4.8 B). In order to detect the incorporated GA in the P1 and P2 samples, we exploited the property of GA to produce UV absorbance spectra with two peaks, one at 225 nm and one at 260 nm. In the sample containing GA: α-syn at 4:1 molar ratio, we detected GA only in the

oligomeric P1 samples (Fig. 4.8 C). These findings support the hypothesis that GA binds to the oligomeric species and stabilizes them.

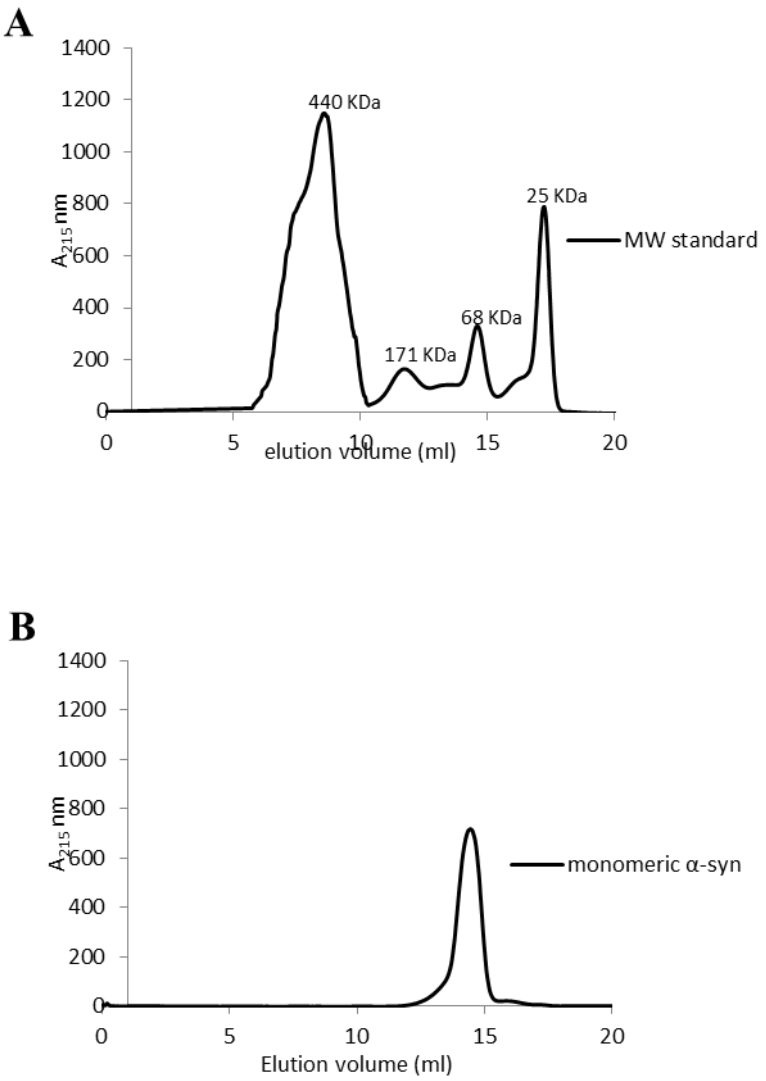


Figure 4.7. Size Exclusion Chromatography:A: Gel-filtration profile of MW standard containing Ferritin 440 KDa, Aldolase 171 KDa, Albumin 68 KDa and Chymotrypsinogen A 25 KDa using Superdex 200 column at 0.1 ml/min flow rate (0.5 ml/fraction). B: Gel-filtration profile for monomeric α-syn using Superdex 200 column at 0.1 ml/min flow rate (0.5 ml/fraction).

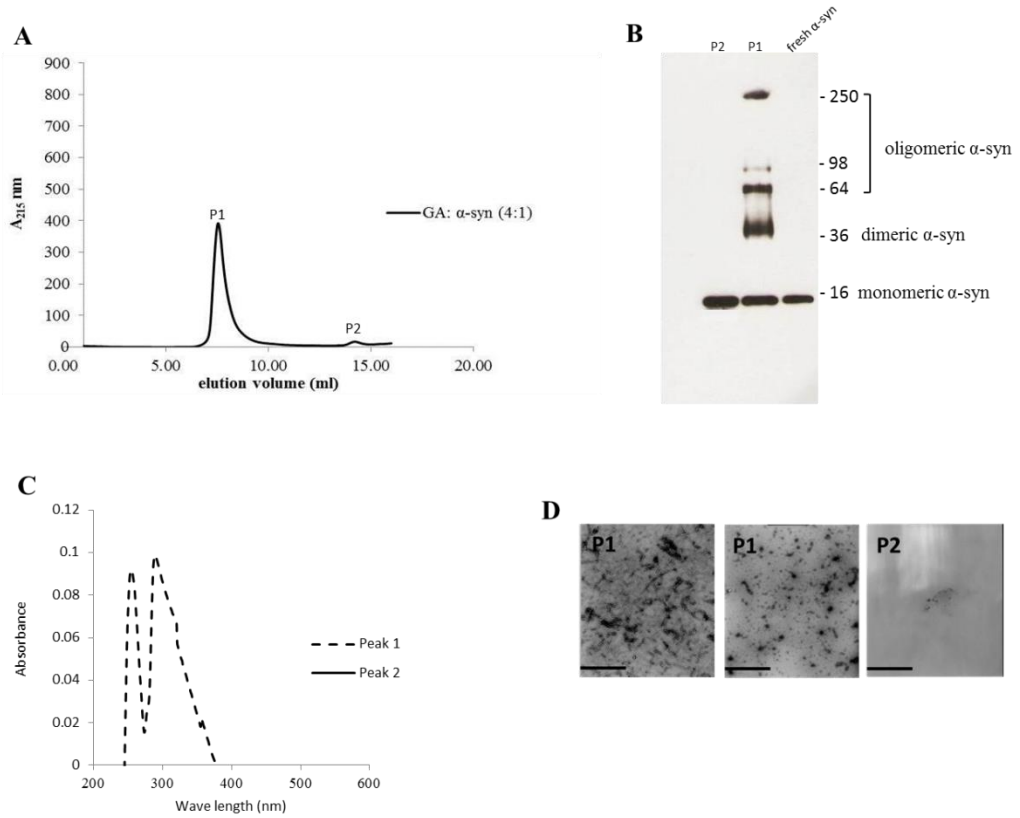


Figure 4.8. GA binds to α-syn oligomers (GA: α-syn molar ratio of 4:1). A. Gel filtration profile of the 5-day aggregated a-syn in the presence of GA at a GA: α-syn molar ratio 4:1 (α-syn concentration =100 µM) -using a superdex 200 SE column. P1 sample contains the isolated fractions corresponding to the oligomeric peak and P2 the isolated fractions corresponding to the monomeric peak. The elution was monitored at the absorbance wavelength of 215 nm. B. Immunoblot analysis of the samples P1 and P2 separated by electrophoresis in a 15% SDS-PAGE gel. C. UV absorbance spectra of samples P1 and P2. The UV absorbance was recorded between 200-600 nm employing a 10 mm quartz cuvette. D. Electron microscopy images of negatively stained samples P1 and P2 of α-syn in the presence of GA (molar ratio of GA: α-syn 4:1) purified by SEC. *Scale bar represents* 1000 nm.

To further evaluate whether GA interacts with α-syn monomers, we monitored a titration of GA into a solution of monomeric α-syn using two-dimensional NMR spectroscopy, which provides signals covering the entire amino acid sequence of α-syn (Fig. 4.9). At stoichiometries of up to 10:1 GA: α-syn we

observed no significant chemical shift or resonance intensity changes (Fig. 4.10 A-E), confirming that GA does not interact significantly with monomeric α-syn. This result is largely in agreement with NMR studies of related compounds Entacapone, Tolcapone and Quercetin (Di Giovanni, et al., 2010).

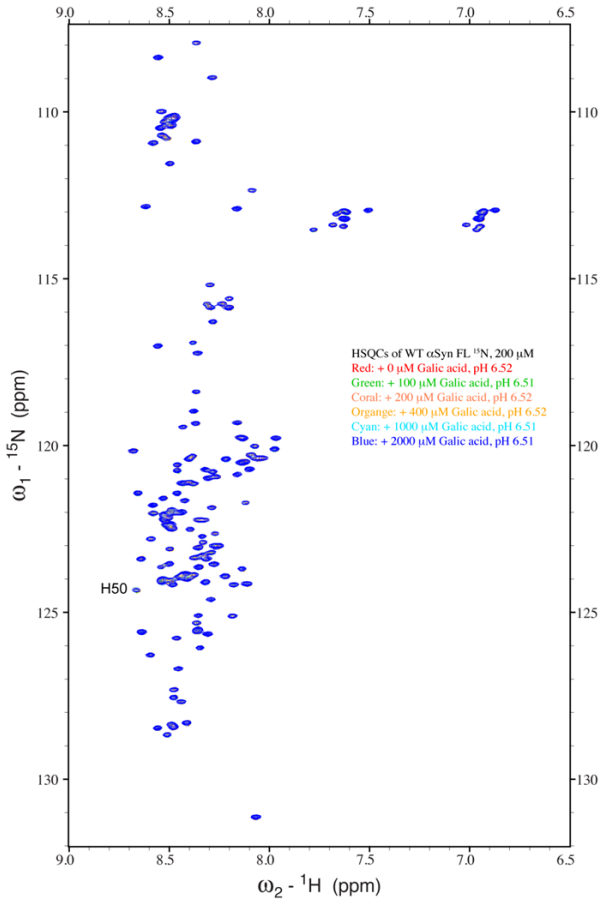


Figure 4.9. Analysis of GA binding to monomeric α-syn by NMR spectroscopy. Proton-Nitrogen correlation (HSQC) spectra of monomeric α-syn in the presence of increasing ratios of GA: α-syn demonstrating that there are no significant changes in the positions of the NMR resonances, indicating the lack of an interaction between GA and monomeric α-syn. Protein concentration was ca. 200 µM.

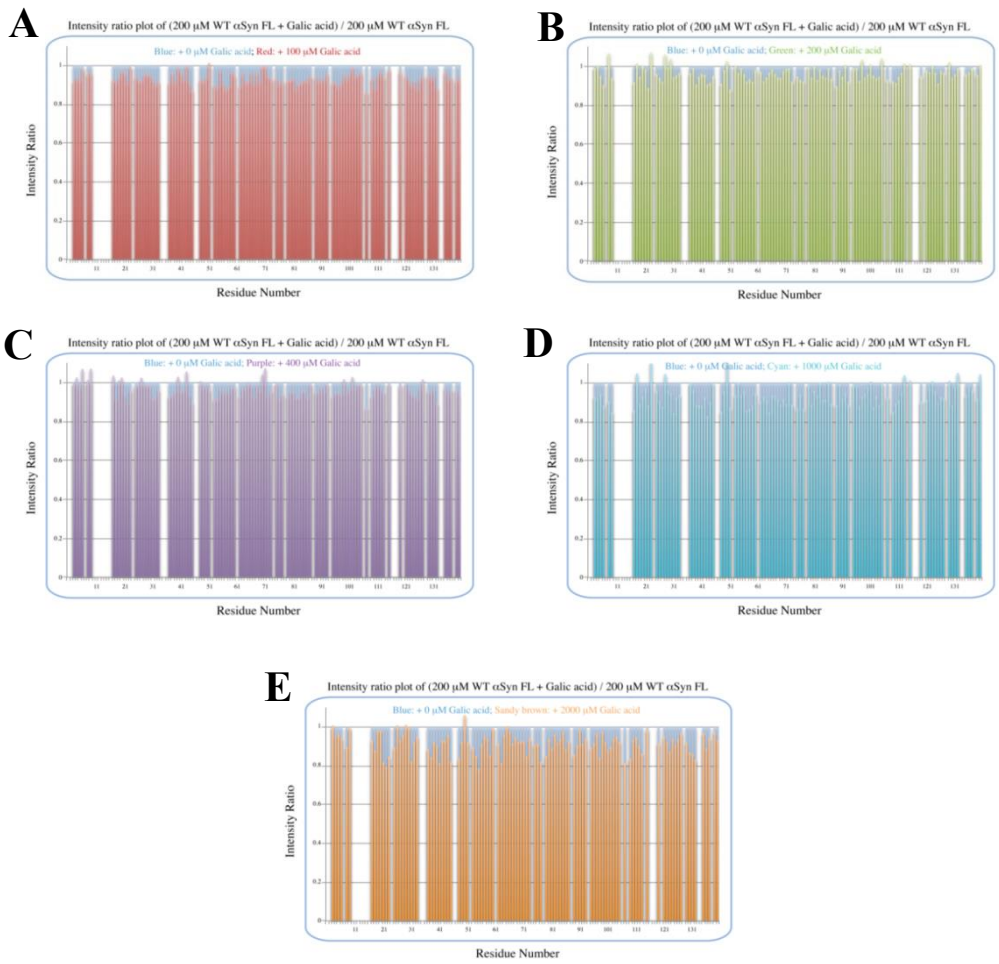


Figure 4.10. Analysis of GA binding to monomeric α-syn by NMR spectroscopy**.** Ratios of NMR resonance intensities from proton-nitrogen (HSQC) correlation spectra in the presence of increasing ratios of GA: α-syn (A - 0.5:1, B - 1:1, C - 2:1, D - 5:1, E - 10:1) to intensities observed in the absence of GA. The absence of any significant intensity changes in the presence of GA again confirms the lack of an interaction between GA and monomeric α-syn.

4.3.7 Investigating the structure-activity relationship of GA inhibition of α-syn fibrillation

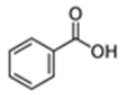
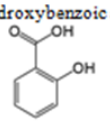
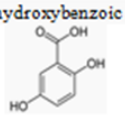
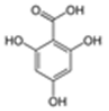
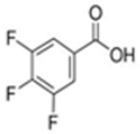
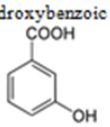
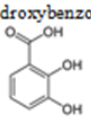
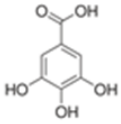
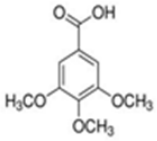
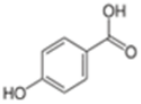
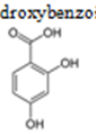
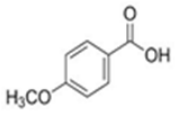
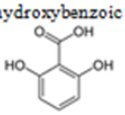
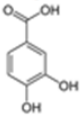
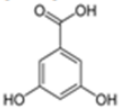
To define the most effective molecular scaffolds against α-syn fibril formation and establish a structure-activity relationship for GA, we exploited the structural diversity of benzoic acid derivatives (i.e., hydroxybenzoic acids). Twelve hydroxybenzoic acids, including GA, salicylic acid and gentisic acid (see Table 4.1) were assessed for their anti-fibrillogenic properties by the Th-S binding assay (Fig. 4.11).

Table 4.1. Description and names of the tested benzoic acid derivatives.

Name of compound	MW
Benzoic acid	122.12
2-Hydroxybenzoic acid (salicylic acid)	138.12
3-Hydroxybenzoic acid	138.12
4-Hydroxybenzoic acid	138.12
2,3-dihydroxybenzoic acid	154.12
2,4-dihydroxybenzoic acid	154.12
2,5-dihydroxybenzoic acid	154.12
2,6-dihydroxybenzoic acid	154.12
3,4-dihydroxybenzoic acid	154.12
3,5-dihydroxybenzoic acid	154.12
2,4,6-trihydroxybenzoic acid	188.13
3,4,5-trihydroxybenzoic acid (GA)	170.12
3,4,5-trihfluorobenzoic acid	176.09
3,4,5-trimethoxybenzoic acid	212.20
4-methoxybenzoic acid	152.15

The structure of the hydroxybenzoic acids tested was characterized by the presence of a phenyl ring that has a carboxyl group, and hydroxyl moieties (OH) attached to the ring at different positions. The selection of the compounds examined in the present study was based on the number (between 0-3) and position of the hydroxyl groups attached to the phenyl ring (see Table 4.2).

Table 4.2. The compounds were divided into four groups based on the number of hydroxyl groups attached to the phenyl ring.

No hydroxyl group	Single hydroxyl group	Two hydroxyl groups	Three hydroxyl groups
• Benzoic acid 	• 2-Hydroxybenzoic acid 	• 2,5-dihydroxybenzoic acid 	• 2,4,6-trihydroxybenzoic acid 
• 3,4,5-trihfluorobenzoic acid 	• 3-Hydroxybenzoic acid 	• 2,3-dihydroxybenzoic acid 	• 3,4,5-trihydroxybenzoic acid 
• 3,4,5-trimethoxybenzoic acid 	• 4-Hydroxybenzoic acid 	• 2,4-dihydroxybenzoic acid 	
• 4-methoxybenzoic acid 		• 2,6-dihydroxybenzoic acid 	
		• 3,4-dihydroxybenzoic acid 	
		• 3,5-dihydroxybenzoic acid 	

The kinetics of α-syn fibrillation was studied over a period of 5 days in the presence or absence of the phenolic acids. Based on the Th-S assay results (Fig. 4.11 and Table 4.3), the effect of the twelve phenolic acids on α-syn fibrillation varied greatly. The extent of the inhibition of α-syn fibrillation was ~ 99% for GA, ~ 72% for 2,4,6-trihydroxybenzoic acid, ~ 60% for 3,4-dihydroxybenzoic acid ~ 30% for five compounds including 2,6-dihydroxybenzoic acid and 4-hydroxybenzoic acid and only 5% for benzoic acid (see Fig. 4.11 and Table 4.3).

Table 4.3. Summary of Th-S results showing the percentage of inhibition of fibril formation comparing with number of OH groups around the phenyl ring.

Symbol	Compound	OH group	Inhibition% of α-syn aggregation
PA1	3,4,5-trihydroxybenzoic acid	3	99%
PA2	2,4,6-trihydroxybenzoic acid	3	72%
PA3	2,3-dihydroxybenzoic acid	2	44%
PA4	2,4-dihydroxybenzoic acid	2	35%
PA5	2,5-dihydroxybenzoic acid	2	30%
PA6	2,6-dihydroxybenzoic acid	2	16%
PA7	3,4-dihydroxybenzoic acid	2	60%
PA8	3,5-dihydroxybenzoic acid	2	0%
PA9	2-hydroxybenzoic acid	1	0%
PA10	3-Hydroxybenzoic acid	1	0%
PA11	4-Hydroxybenzoic acid	1	9%
PA12	Benzoic acid	0	5%
PA13	4-methoxybenzoic acid	0	0%
PA14	3,4,5-trimethoxybenzoic acid	0	0%
PA15	3,4,5-trifluorobenzoic acid	0	0%

Moreover, three compounds, 2-hydroxybenzoic acid (salicylic acid), 3-hydroxybenzoic acid and 3,5-dihydroxybenzoic acid, failed to inhibit α-syn fibrillation, with salicylic acid appearing to enhance the aggregation of α-syn compared to the control (see Fig. 4.11 and Table 4.3).

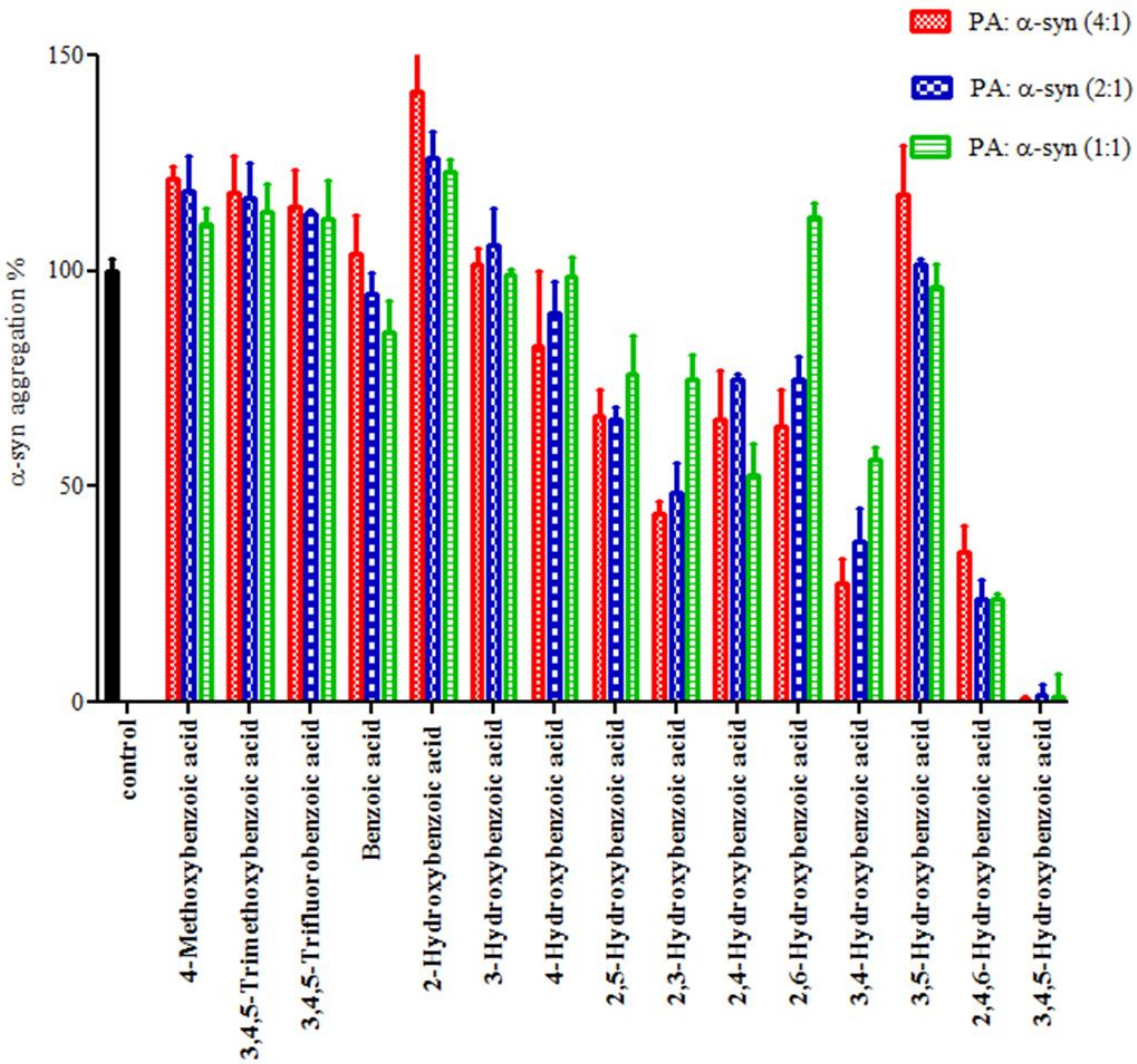


Figure 4.11. Effect of different benzoic acid derivatives (phenolic acids, PA) and the effect of methoxy and fluoro groups in benzoic acid derivatives on α-syn fibrillogenesis. Samples of α-syn (25 μM) were incubated alone or in the presence of different benzoic acid derivatives (PA: α-syn molar ratios of 4:1, 2:1, 1:1) for 6 days with continuous shaking at 37°C. The fibril formation was measured by the Th-S binding assay and expressed as a percentage of the fibril content of α-syn aged alone. The assay was performed in triplicate (average of triplicate measurements ± standard deviations).

4.4 Discussion

The formation of amyloid aggregates has long been considered responsible for the pathogenesis of several neurodegenerative disorders (Dorval & Fraser, 2007; Taylor, Hardy, & Fischbeck, 2002; Vekrellis, Xilouri, Emmanouilidou, Rideout, & Stefanis, 2011). Although amyloidogenic proteins, such as α-syn, ar e usually found unfolded in their native state (Fauvet et al., 2012; Uversky, 2008), in which they are soluble and non-toxic, they can undergo misfolding resulting in the formation of insoluble aggregates (Irvine, et al., 2008; Kahle, 2008; Seidler et al., 1996; Stefanis, 2012; Veldman, Wijn, Knoers, Praamstra, & Horstink, 1998). These aggregates deposit in various regions of the brain, constituting the main neuropathological feature of several neurodegenerative diseases such as PD, DLB, MSA and approximately 50% of Alzheimer's cases. In the case of α-syn, pathological, biochemical, genetic and animal modeling studies provide compelling evidence that α-syn aggregation plays a pivotal role in the pathogenesis of PD and related synucleinopathies. As a consequence, the identification of compounds that can block or reverse the aggregation process of α-syn is considered a vital therapeutic strategy against these diseases.

To this end, we assessed the effect of the phenolic compound GA (3,4,5-trihydroxybenzoic acid) on α-syn aggregation and toxicity, and we established a structure-activity relationship for GA. By employing an array of biophysical and biochemical techniques, we showed that at a high concentration (100 μM, represented by the molar ratio of 4:1), GA exerts significant inhibitory effect on both α-syn fibrillation (Fig. 4.1, A and B) and oligomerization (Fig. 4.2, A and B), as well as seeded fibrillation of α-syn (Fig. 4.5 A). In accordance with these findings, TEM images revealed that α-syn in the presence of GA at a high

concentration only formed a few short, thin fibrils that had a fragmented appearance (Fig. 4.1 D) accounting for the low Th-S counts (Fig. 4.1 A) and a minor wavelength shift in the CR binding assay (Fig. 4.1 B). At lower GA concentrations (especially at 1:1 molar ratio), however, the ability of the GA to inhibit α-syn fibrillation was less dramatic and accompanied by a striking increase in the oligomeric content (Fig. 4.2 A), indicating GA may stabilize the oligomeric structure. Size exclusion chromatography combined with UV spectroscopy for detection of the GA incorporated in α-syn confirmed that GA binds to α-syn oligomers (Fig. 4.8 C). Furthermore, GA was able to disaggregate preformed α-syn fibrils (Fig. 4.4, A-C), generating species that possessed a decreased β-sheet content (Fig. 4.4 B) and were less toxic to cells (Fig. 4.4 D). Previous studies have also shown that the phenolic flavonoid compound baicalein, as well as other antioxidant compounds inhibit α-syn fibrillation and disaggregate pre-aggregated fibrils (Ono & Yamada, 2006; Zhu et al., 2004). GA was also shown to alleviate α-syn aggregates associated-toxicity in neuroblastoma BE(2)-M-17 cells (Fig. 4.6 A and B).

These results are in agreement with previous studies indicating that GA possesses anti-fibrillation, oligomer-stabilizing and neuroprotective properties against α-syn and Aβ cytotoxicity (Di Giovanni, et al., 2010). Indeed, GA was shown to stabilize biotinylated $A\beta_{1-42}$ oligomers (LeVine, Lampe, Abdelmoti, & Augelli-Szafran, 2012) and to inhibit the *in vitro* conversion of low molecular weight $A\beta_{1-42}$ protofibrils into fibrils by 50% (Di Giovanni, et al., 2010). Additionally, GA was reported to block the fibrillation of α-syn *in vitro* and to suppress the ability of short α-syn fibrils to seed the aggregation of monomeric α-syn (Di Giovanni, et al., 2010). Furthermore, GA exerts a neuroprotective effect on cells against the

cytotoxicity of α-syn and $A\beta_{1-42}$ (Di Giovanni, et al., 2010), possibly due to the inhibition of the amyloid aggregation but potentially also due to the anti-inflammatory (D. H. Kim et al., 2011), anti-oxidant (Ban et al., 2008; S. Y. Hong, Jeong, & Jun, 2012) and anti-apoptotic properties it possesses (S. Y. Hong, et al., 2012).

Based on our findings using both SEC and NMR, as well as previous reports, GA inhibits α-syn fibrillation not by interacting with the monomeric α-syn - similar to many other phenolic compounds (reviewed by (Porat, et al., 2006), and preventing it from polymerizing but rather by stabilizing the structure of oligomeric α-syn (Fig. 4.2 B, 4.8 C) which appears to be non-toxic (Fig. 4.6). These findings are in accordance with previous studies indicating that polyphenolic compounds, such as baicalein, curcumin and epigallocatechin gallate (EGCG) induce the formation of soluble, non-toxic oligomers (Masuda et al., 2006; Zhu, et al., 2004). From a therapeutic point of view, this represents a major advantage, as GA may not interfere with the physiological function of the monomeric α-syn (Wagner et al., 2013). It has been reported that phenolic compounds such as curcumin inhibit Aβ aggregation due to conformation-dependent binding (F. Yang et al., 2005). Consistent with this model of inhibition, GA also inhibited the seeding ability of short fibrillar α-syn. Although the presence of oligomers can be transient as they assemble into fibrils (Fink, 2006; Kaylor et al., 2005a), there are studies revealing that in the presence of the polyphenolic compound EGCG, they can shift off-pathway from fibrillation (D. E. Ehrnhoefer, et al., 2008). Indeed, EGCG was shown to promote the formation of less toxic oligomers that were exceedingly stable and incapable of contributing to the aggregation process (D. E. Ehrnhoefer, et al., 2008). As it is also a phenolic compound, GA may interact with α-syn

oligomers by a mechanism similar to that of EGCG, accelerating the formation of off-pathway oligomers and leading to the accumulation of non-toxic oligomers and the interruption of the fibrillation process.

To gain insight into the mechanism of action of GA and to establish a structure-function relationship for this phenolic acid, we evaluated the effect of various structurally similar phenolic acids on α-syn fibrillation. The results generated from this approach point towards a structure-related effect on α-syn fibrillation in which the better inhibitors were observed to have a greater number of -OH groups attached to the phenyl ring that were directly conjugated to the carboxylic acid arm (see Fig. 4.11). In fact, the ranking of the anti-fibrillogenic potency of all the phenolic compounds tested could be represented as follows: trihydroxybenzoic acid>dihydroxybenzoic acid>monohydroxybenzoic acid = benzoic acid. This finding is in accordance with previous studies indicating that the potency of certain polyphenolic compounds to inhibit and disaggregate α-syn oligomers correlates with the number of vicinal - OH groups present on a single phenyl ring (Caruana et al., 2011; Masuda, et al., 2006). The significance of the presence of the hydroxyl group on the phenyl ring was further stressed by the fact that the compounds that possess appendages other than hydroxyl groups on the phenyl rings failed to inhibit α-syn aggregation. At this point, it is more than idle speculation to suggest that the inhibition capacity of the phenolic compounds is not only dependent on the number of hydroxyl moieties but also on their position and conjugation with respect to the benzoic acid appendage. Comparison of the two trihydroxybenzoic acids tested in this study, GA (3,4,5-trihydroxybenzoic acid) and 2,4,6-trihydroxybenzoic acid, indicates that although both compounds have a total of three - OH groups, the former is a much more potent inhibitor of α-

syn fibrillation compared to the latter, strongly suggesting that it is not only the total number of -OH groups present in the molecule but also their position that is important. Additionally, comparison of the dihydroxybenzoic acids employed in the present study reveals that the presence of -OH groups in two consecutive positions and conjugated with the carboxyl group (e.g., 3,4-dihydroxybenzoic acid (60% inhibition) and 2,3-dihydroxybenzoic acid (44% inhibition)) renders the compounds more active compared to those that have hydroxyl groups in non-consecutive positions (*e.g.*, 2,5-dihydroxybenzoic acid (30% inhibition) and 3,5-dihydroxybenzoic acid (0% inhibition) (see Fig. 4.11 and Table 4.3).The importance of the 4 - OH moiety is emphasized by the fact that of the three hydroxybenzoic acids tested, only 4-hydroxybenzoic acid could partially inhibit α-syn fibrillation. Among the dihydroxybenzoic acids, 3,4-dihydroxybenzoic acid was the most potent of all, inhibiting fibrillation by 60% (Table 4.3). Taken as a whole, these data show that the total number of - OH groups present in the molecule is important for the compound activity and that the position of the overall -OH groups also affects activity, with a hydroxyl group at position 4 appearing to play a significant role. Thus, the presence of three vicinal hydroxyl groups, as in 3,4,5-trihydroxy benzoic acid (GA), or the three homo-vicinal groups, as in 2,4,6-trihydroxy benzoic acid, with one - OH group at position 4, enhances the compounds able to inhibit α-syn fibrillation to a great extent.

Given that all the compounds tested in this study have a phenyl ring in their structures, the phenyl structure itself is clearly not sufficient to inhibit α-syn aggregation. Consequently, the inhibition ability of such compounds must be achieved by the presence of additional - OH groups attached to the phenyl ring.

The conjugated hydroxyl groups with the carboxylic acid appendage can easily form quinone structures (see Fig. 4.12). It is worth mentioning that quinone formation has been previously reported as the potential mechanism for the inhibition of α-syn fibrillation by the phenolic compound (Zhu, et al., 2004). The formation of such oxidized derivatives can increase the stability of the compound binding to the α-syn fibrils (Taniguchi et al., 2005), possibly accounting for the increased inhibitory potency of the compounds with more - OH groups present in their structures. Quinones are likely to interact with the aromatic residues of α-syn, interfering with their π−π stacking (Ebrahimi & Schluesener, 2012; Hamley, 2012; Porat, et al., 2006). Moreover, quinones have a planar orientation that allows them to align with the hydrophobic groove of α-syn fibrils, which possess an in-register organization of side chains in the regular cross-β-sheet structure (Ebrahimi & Schluesener, 2012; Hamley, 2012; Porat, et al., 2006). Therefore, the inhibition of α-syn fibrillation by GA and 2,4,6-trihydroxybenzoic acid could be attributed to the combined properties of their three -OH groups and quinone-forming structures. Based on these results, we propose a plausible model to interpret the inhibitory and disassembling effects of GA. Two dominant ligand-protein interactions contribute to inhibitor potency, (a) the dipole-dipole interactions of the quinone and (b) the dipole-dipole interactions of the diquinone group. The most potent inhibitors, with 3,4,5-trihydroxy and 2,4,6-trihydroxy substitution patterns (GA and 2,4,6-trihydroxy benzoic acid, respectively), (see Fig. 4.12 c), enable both hydrophobic and dipole-dipole interactions. The π−π stacking is known to play a role in the binding affinity of inhibitors to aggregates; increasing the potential for quinone formation of each compound generally leads to increased binding affinity and, therefore, increased inhibitor potency.

Consistent with this interpretation, ortho- and meta-hydroxybenzoic acid, as well as benzoic acid (see Fig. 4.12 f) showed no inhibition compared to their para- and ortho-paradihydroxy (Fig. 4.12 a) counterparts due to the inability of the former to form quinone-like ligands (Fig.4.12 f, d).

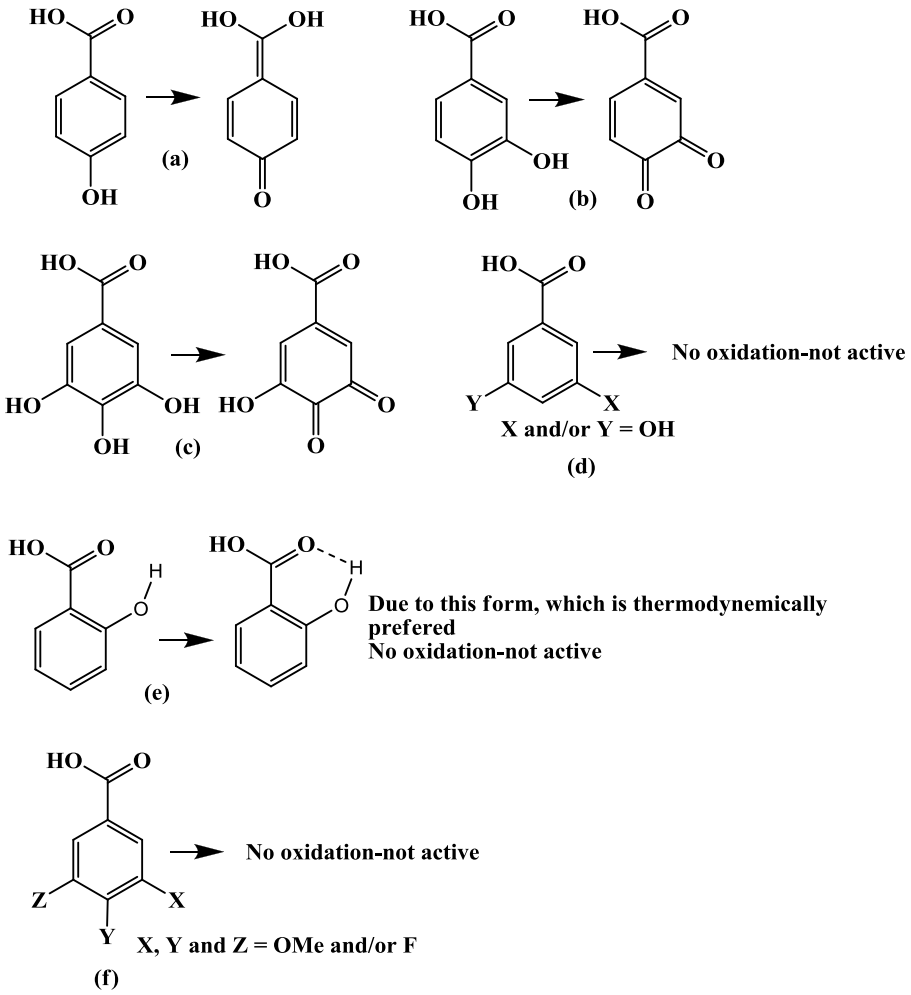


Figure 4.12. Mechanistic insight for the mechanism of quinone formation.

Although monocyclic compounds such as phenolic acids were previously shown to be very poor inhibitors of aggregation (Reinke & Gestwicki, 2007), later studies revealed that monocyclic compounds could suppress amyloid assembly by interacting with oligomers, stabilizing their structure and conformation, thus

inhibiting the transition to β-sheet structures (Levy-Sakin, Shreberk, Daniel, & Gazit, 2009). It is therefore likely that phenolic acids, especially GA, act in a similar way, interacting with oligomers and stabilizing their structure through the - OH groups, preventing the transition to β-sheet-rich structures. This may explain the increased oligomeric content detected at low GA concentrations and the low β-sheet content of α-syn species generated in the presence of GA.

In summary, we conducted a systematic study of the effect of GA on α-syn fibrillation, oligomerization and toxicity, and we conclude that GA inhibits α-syn fibrillation and toxicity by stabilizing the non-toxic oligomeric structure of α-syn through oligomer binding. Our study points toward a structure-related inhibition, with the number of - OH moieties and their position around the phenyl ring playing a fundamental role. Based on the above experimental results, we propose a model to interpret the inhibitory and disassembling effects of GA derivatives on α-syn aggregation (see Fig. 4.12). α-Syn aggregation is a sequential process in which unstructured α-syn monomers undergo conformational transition and re-ordering to form oligomers and then finally amyloid fibrils (routes a → b → c → d, Fig. 4.12). The analysis of the results obtained from this study shows that GA derivatives can prolong the nucleation process, suggesting that these scaffolds can bind to unstructured α-syn oligomers and prevent protein association (Fig. 4.12, route a → f) and/or slow down the conformational transition to structured oligomers, the precursors of amyloid fibrils. GA derivatives interact with α-syn monomers and/or oligomers through π-π stacking, leading to the formation of an energy barrier that prevents the association of α-syn molecules. Meanwhile, GA derivatives through their respective quinone oxidation products could induce structural disruption to the local β-sheet of α-syn fibrils via strong binding to the

β-sheet groove regions of α-syn fibrils, leading to fibril disaggregation (d → e). It should be noted that, due to the hydrophobic aromatic nature and planar structure of quinones, they might interact with α-syn fibrils by relatively nonspecific dipole-dipole interactions with β-sheet-rich side chains. This binding mode implies that quinones could have a general inhibitory effect on the aggregation of α-syn. A Schematic model for the anti-aggregation and disassembly effect of GA derivatives on α-syn is shown in (Fig. 4.13).

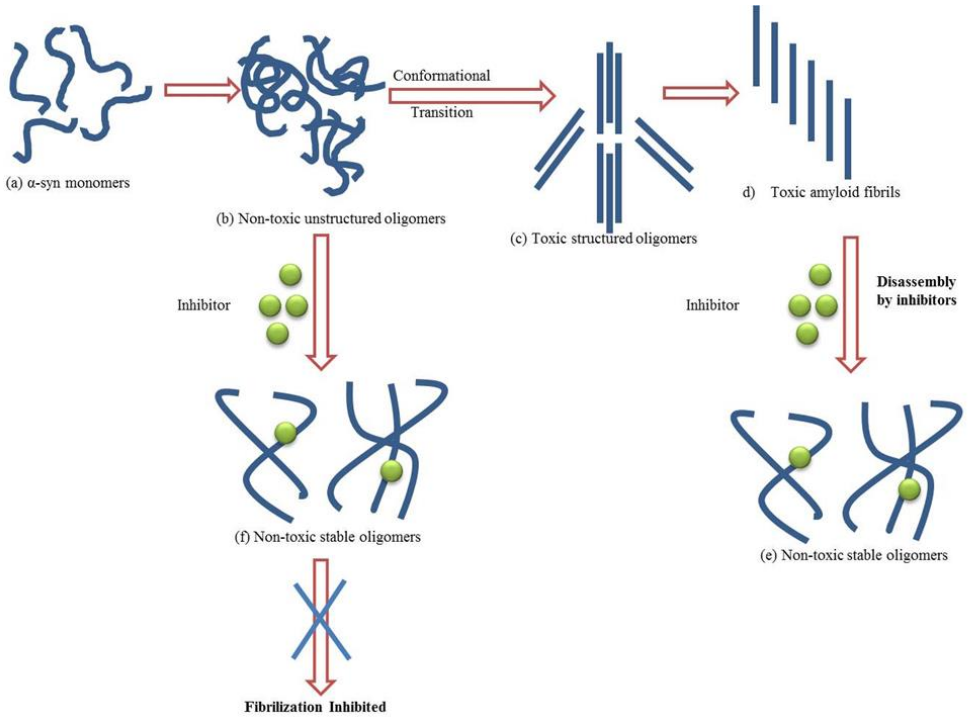


Figure 4.13. Schematic model for the antiaggregation and disassembly effect of GA derivatives on α-syn.

By comparing the structures of the most effective and least effective compounds, we concluded that better inhibition of fibrillation is observed when there are a large number of hydroxyl groups (- OH) attached to the phenyl ring of the compound. Moreover, the positions at which the hydroxyl groups are attached to the phenyl ring also appear to play a critical role. GA has hydroxyl groups

attached at positions 3, 4 and 5 (see Table 4.2) and is much more effective than 2,4,6-trihydroxybenzoic acid, which also bears three - OH groups but at different positions (Fig. 4.11). Interestingly, 3,4-dihydroxybenzoic acid, which only lacks an - OH group at position 5 compared to the very effective GA, is the third best inhibitor of α-syn fibrillation (Fig. 4.11). Among all the dihydroxybenzoic acids tested, the ones with - OH groups at two consecutive positions, i.e., 3,4-dihydroxybenzoic acid (60% inhibition) and 2,3-dihydroxybenzoic acid (44% inhibition), are more potent inhibitors compared to those that have -OH groups at non-consecutive positions, i.e., 2,5-dihydroxybenzoic acid (30% inhibition) and 3,5-dihydroxybenzoic acid (0% inhibition) (Fig. 4.11). Additionally, all compounds with one -OH group, as well as the benzoic acid without an OH group, failed to show any inhibitory effect on α-syn aggregation (Fig. 4.11).

To further investigate the significance of the (-OH) moieties on the activity of the GA and the other active phenolic acids, three additional compounds with either fluorides or methoxy groups rather than hydroxyl groups attached to their phenyl rings (4-methoxybenzoic acid, 3,4,5-trimethoxybenzoic acid and 3,4,5-trifluorobenzoic acid) (see table 4.2) were tested for their ability to inhibit α-syn fibril formation. As expected, none of the three tested compounds in any of the three molar ratios tested (compound: α-syn 4:1, 2:1 and 1:1) could inhibit α-syn fibrillation (Fig. 4.11). This finding provides additional support for the importance of the presence of the hydroxyl group in the phenyl ring.

5 Chapter 5: Ginsenoside Rb1 inhibits fibrillation and Toxicity of alpha-synuclein and disaggregates preformed fibrils

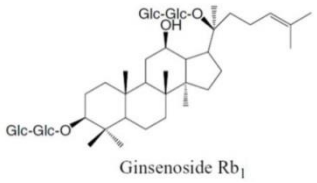
5.1 Introduction

α-Syn aggregation proceeds through the formation of oligomers (early aggregates), which ultimately convert into well-ordered fibrils (late aggregates) (Uversky, Lee, Li, Fink, & Lee, 2001). Recent studies indicate that early aggregates of α-syn, the so-called soluble oligomers, form the pathogenic species that drive neurodegeneration and neuronal cell death rather than mature amyloid fibrils (Conway et al., 2000b; El-Agnaf, et al., 2003; Winner, et al., 2011).

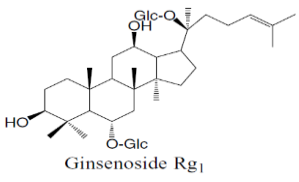
The drugs currently available for the treatment of PD provide symptomatic relief but do not alter the course of the disease (He et al., 2013). However, the effectiveness of these drugs diminishes after several years of treatment. Together with the increasing incidence of PD due to an aging population, these facts indicate the compelling need for more effective drugs and treatments for PD. Although modern therapeutic options that target disease modifications are on the rise, the multiple mechanisms involved in the pathogenesis of PD create considerable difficulties in producing effective treatments. Hence, the inhibition of α-syn aggregation may represent a viable strategy for therapeutic intervention in PD and related disorders. It is therefore essential to identify compounds that can serve as potent inhibitors and interrupt the early stages of aggregation.

Ginseng is a well-known medicinal herb that has been used for more than two thousand years in China, Korea and Japan to promote well-being and alleviate fatigue. Although there are eleven different species of ginseng that belong to the genus Panax of the Aralliaceae family, the most commonly used species are Panax ginseng (Asian or Korean Ginseng), P. quinquefolius (American Ginseng), P. japonicus (Japanese Ginseng) and P. notoginseng (Chinese Notoginseng or Sanchi) (F. Chen, Eckman, & Eckman, 2006). Named after its ability to treat several conditions - panax means panacea in Greek ginseng is now a well-documented anti-carcinogenic, anti-diabetic, anti-oxidant and vasorelaxing agent that exhibits immunomodulatory properties and improves the function of the central nervous system (Lü, Yao, & Chen, 2009). The numerous pharmacological properties of ginseng are attributed to its biologically active ingredients, the ginsenosides (reviewed by (Im & Nah, 2013), which can be extracted from many parts of the ginseng plant, including the root, the leaves and the berries (Attele, Wu, & Yuan, 1999). Ginsenosides, which are also referred to as ginseng saponins, are derivatives of triterpenoid dammarane with a four-ring, steroidal structure bearing sugar moieties and an aliphatic side chain (Wee JJ, 2011). The variations in the structure of ginsenosides, namely the type of aglycone (triterpene), the type of sugar moieties (glucose, maltose, fructose, saccharose etc), their number and their site of attachment (Wee JJ, 2011), give rise to three categories of ginseng saponins, the panaxadiol group, the panaxatriol group and the oleanolic acid group (Kim et al., 2013). More than 100 ginsenosides have been identified so far (Nag, et al., 2012), but the most frequently studied ones are Rb1, Rg1, Rg3, Rd, Re, Rh1 and Rh2.

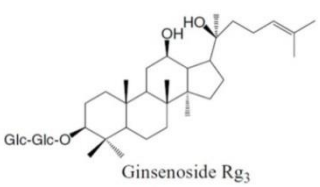
Ginsenosides have been shown to affect voltage-gated ion channels, such as the Ca2+, Na+, K+ channels, as well as the ligand-gated ion channels, such as the 5-HT3-, the α7 nicotinic acetylcholine and the N-methyl-d-aspartate (NMDA)-gated channels (reviewed by (Nag et al., 2012; Radad, Moldzio, & Rausch, 2011). This property of ginsenosides appears to underlie many of the pharmacological effects of ginseng, including neuroprotection (J. H. Kim et al., 2007), since it has a beneficial effect on many neurological conditions, including neurodegenerative diseases such as PD (reviewed by (Cho, 2012; H. J. Kim, Kim, & Shin, 2013). However, despite numerous studies exploring the effect of various ginsenosides on the nervous system, there are no reports on the effect of ginsenosides on the aggregation propensity of amyloidogenic proteins such as α-syn. As a consequence, we sought to determine the potential of the most frequently used and studied ginsenosides, namely Rb1, Rg1 and (20S)-Rg3, i.e. the stereoisomer of Rg3 with its C-20 OH being spatially close to the C-12 OH group (Scheme. 5.1).



Ginsenoside Rb_1



Ginsenoside Rg_1



Ginsenoside Rg_3

Scheme 5.1. Chemical structure of ginsenoside Rb1, ginsenoside Rg1, ginsenoside Rg3.

5.2 Experimental procedure

5.2.1 Expression and purification of recombinant human α-syn.

As described the experimental procedure 3.2.1

5.2.2 α-Syn purification and characterization.

As described the experimental procedure 3.2.2

5.2.3 Aggregation of α-syn *in vitro*.

Protein purity was estimated to be >95% using an SDS gel. The ginsenosides were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China (NICPBP). Ginsenosides stock solutions (10 mM) were prepared in 100% DMSO such that the final amount of DMSO in the sample solutions was 1%. Samples of 25 μM α-syn in PBS were aged alone or with ginsenosides at various molar ratios (ginsenoside: α-syn molar ratios of 4:1, 2:1 and 1:1). The samples were placed in 1.5 ml sterile polypropylene tubes, drops of mineral oil were added to prevent sample evaporation, and the tubes were then sealed with parafilm and incubated at 37°C for 5 days with continuous shaking at 800 rpm in a thermomixer (Eppendorf). Samples were collected at the indicated intervals, and a thioflavin-T assay was performed immediately at each time point, while the rest of the samples were stored at -80°C until required for further analyses.

5.2.4 Thioflavin-T (Th-T) assay

α-Syn fibril formation was monitored by Th-T binding assay. Th-T is a fluorescent dye that interacts with fibrils containing a β-sheet structure. A total of 10 μl of each sample was diluted in 40 μl of Th-T in PBS. Fluorescence was then measured in a 384-well, untreated black micro-well plate (Nunc, Denmark) using a microplate reader (Victor X3 2030, Perkin Elmer) with the excitation and

emission wavelengths set at 450 and 486 nm, respectively. To allow for background fluorescence, the fluorescence intensity of a blank PBS solution was subtracted from all readings.

5.2.5 Transmission electron microscopy (TEM).

As described in the experimental procedure 3.2.8

5.2.6 Immunoblotting.

As described in the experimental procedure 4.2.6

5.2.7 Congo red binding assay.

As described in the experimental procedure 4.2.10

5.2.8 Proteinase K (PK) digestion.

Samples of α-syn (25 μM), aged alone or with Rb1, Rg1 or Rg3 (α-syn: compound molar ratio of 1:1) were incubated for 15 mins at 37ºC with PK (Sigma-Aldrich, USA) to a 2.5 μg/ml. The reaction was stopped by the addition of 2x sample loading buffer (250 mM Tris-HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue, 8% SDS, 5% beta-mercaptoethanol), and then heated for 10 mins at 95ºC. The samples were loaded and run into 15% SDS-PAGE gels which were then silver stained.

5.2.9 Tissue culture of BE(2)-M-17 human neuroblastoma cells.

As described the experimental procedure 3.2.10

5.2.10 Measurement of cell viability.

As described in the experimental procedure 3.2.13

5.2.11 Immunocytochemistry staining.

BE(2)-M-17 cells expressing wt- α-syn were plated on 12-mm coverslips in 24-well plate for 24 hours. The cells were then washed twice with PBS and fixed with 4% paraformaldehyde for 15 mins at room temperature. Following two

washes (5 mins each with PBS) the cells were permeabilized by incubating for 10 mins at room temperature with PBS-1% Triton and then incubated in a blocking buffer [5% normal goat serum (NGS) in PBS + 1% Triton] for 1 hour at room temperature. Fixed cells were incubated with anti α-syn 211 Ab for 3 hours at room temperature and then washed three times with PBS. FITC conjugated goat anti-mouse secondary Ab (Sigma) was added to the cells for 1 hour at room temperature, and after three washes with PBS the cells were counterstained with DAPI to reveal nuclei, and mounted in Shandon immu-mount (Thermo Scientific). Fluorescent images were taken with an inverted Axiovert 40 CFL fluorescence microscope (Carl Zeiss), equipped with AxioCam HRc (Carl Zeiss) using the 63x oil objective.

5.2.12 α-Syn disaggregation assay.

α-Syn solution in PBS (pH 7.4) was aggregated at a concentration of 25 μM as indicated above. The resulting aggregated α-syn was incubated either alone or with Gn Rb1 at various molar ratios (Gn Rb1: α-syn molar ratios of 6:1, 4:1 and 2:1). It should be noted that for the purpose of the experiment, the concentration of α-syn taken into account was the concentration of fresh α-syn. The samples were incubated at 37°C for 48 hours on a thermomixer with continuous shaking at 800 rpm. Samples were collected at regular time points, and Th-T fluorescence was measured immediately.

5.2.13 Seeding polymerization assay.

The aggregation of monomeric α-syn with or without seeding was performed as described elsewhere (Di Giovanni, et al., 2010). The seeds were prepared by fragmenting the mature α-syn fibrils by sonication to obtain short fibrils, which were employed as ‘seeds’. Monomeric α-syn at a concentration of 100 μM was seeded with 2 μM of seeds and incubated in the presence or absence of Gn Rb1 (10 μM or 50 μM) at 37°C for 6 hours with continuous shaking. The fibrillization was monitored by Th-T binding assay as described above.

5.2.14 Size Exclusion Chromatography (SEC) for separating α-syn oligomers and monomers.

As described in the experimental procedure 4.2.13

5.2.15 UV scanning.

As described in the experimental procedure 4.2.14

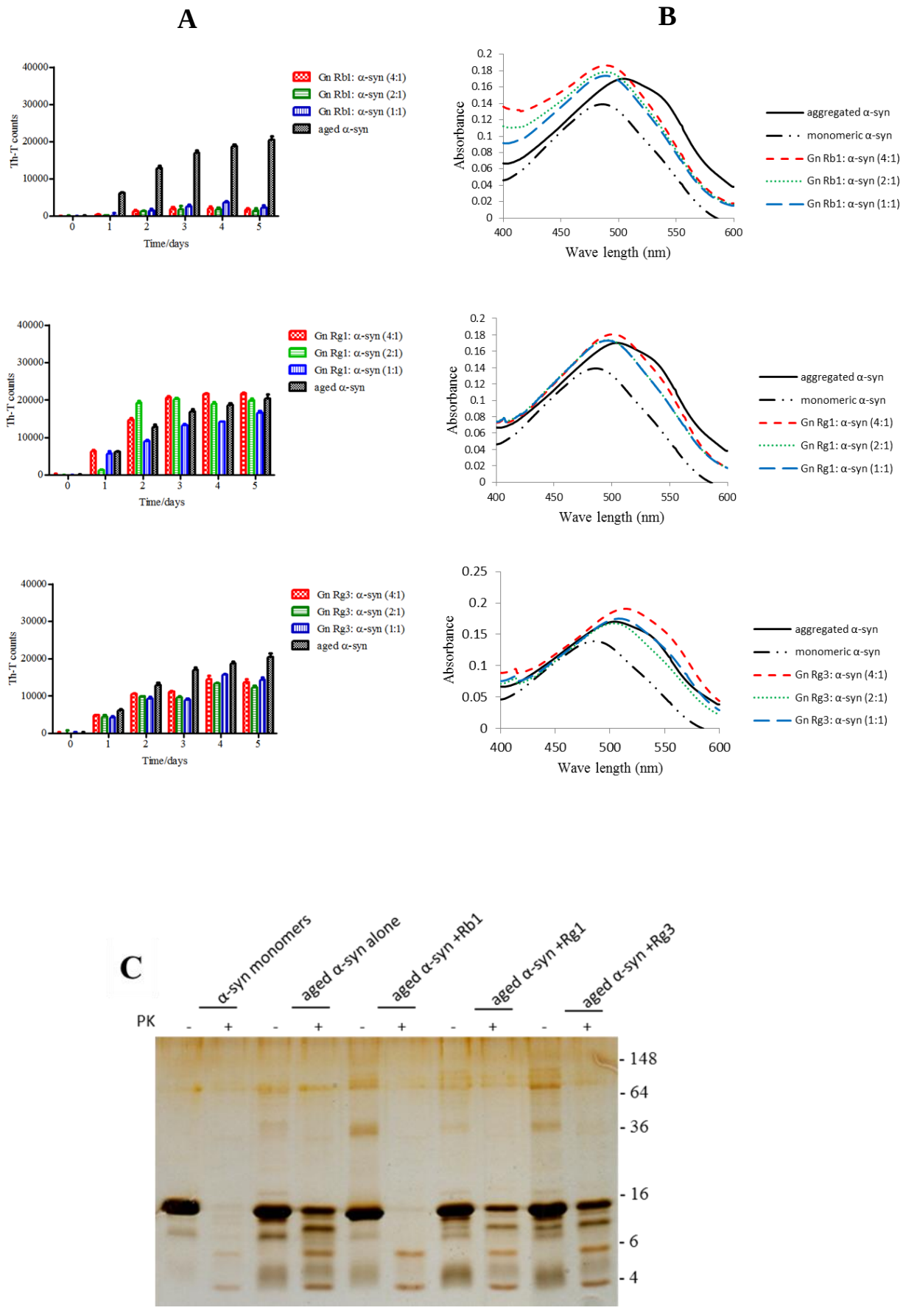
5.2.16 NMR studies.

As described in the experimental procedure 4.2.15

5.3 Results

5.3.1 The effect of ginsenosides on α-syn fibrillation

A 25 μM solution of α-syn , which was purified by reverse-phase HPLC chromatography and its purity was estimated by SDS-PAGE and immunoblotting (Scheme 3.1 B and C) was incubated at 37ºC with continuous shaking for 5 days, leading to fibril formation, which was monitored by Th-T fluorescence at regular time intervals. α-Syn was incubated with Gn Rg1, Gn (20S)-Rg3 and Gn Rb1 at molar ratios of 4:1, 2:1 and 1:1 (molar ratio ginsenosides: α-syn) with a constant α-syn concentration of 25 μM. From the three tested compounds, only Gn Rb1 exhibited a significant inhibitory effect on α-syn fibrillation as indicated by the reduced Th-T fluorescence at all tested concentrations (Fig. 5.1 A).


A
B
Th-T counts
Time/days
Gn Rb1: α-syn (4:1)
Gn Rb1: α-syn (2:1)
Gn Rb1: α-syn (1:1)
aged α-syn
Absorbance
Wave length (nm)
aggregated α-syn
monomeric α-syn
Gn Rb1: α-syn (4:1)
Gn Rb1: α-syn (2:1)
Gn Rb1: α-syn (1:1)
Gn Rg1: α-syn (4:1)
Gn Rg1: α-syn (2:1)
Gn Rg1: α-syn (1:1)
Gn Rg3: α-syn (4:1)
Gn Rg3: α-syn (2:1)
Gn Rg3: α-syn (1:1)
C
α-syn monomers
aged α-syn alone
aged α-syn +Rb1
aged α-syn +Rg1
aged α-syn +Rg3
PK
148
64
36
16
6
4

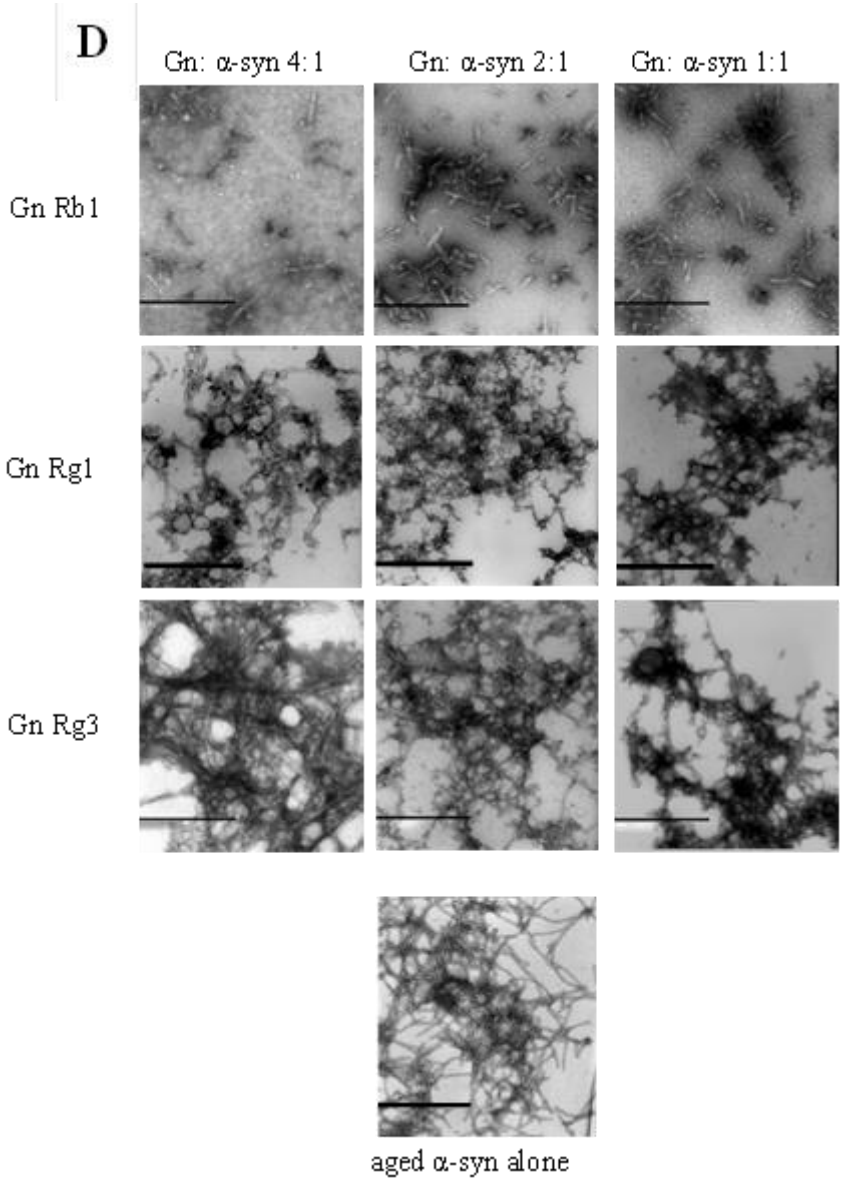


Figure 5.1. Gn Rb1 inhibits α-syn fibrillation. A. Samples of α-syn (25 μM) were incubated for 5 days at 37°C with continuous shaking in the presence of various concentrations of the ginsenosides Rb1, Rg1 and (20S)-Rg3 (100 μM, 50 μM and 25 μM). Fibril formation was measured by Th-T binding assay. The assay was performed in triplicate, and the means ± standard deviations are shown. B. Congo red binding for Gn Rb1, Gn Rg1 and Gn (20S)-Rg3. α-Syn (5 μM) aged alone or in the presence of different concentrations of ginsenosides was mixed with Congo red (final concentration of 5 μM). The UV absorbance spectrum was recorded from 400 to 600 nm in a spectrophotometer. C. Silver staining for 15% SDS gel of α-syn monomers, α-syn aged alone or in the presence of Rb1, Rg1 and Rg3 at molar ratio 1:1 after 2.5 μg/ml PK digestion. D. Electron microscopy images of negatively stained samples of α-syn (25 μM) aged alone or in the presence of the ginsenosides (gincenoside: α-syn molar ratios of 4:1, 2:1, and 1:1) for 5 days with continuous shaking at 37°C. *Scale bar*, 500 nm

This effect was observed as early as after 2 days of incubation, when the inhibition percentage was approximately 90%. After 5 days of incubation, the fibrillation of α-syn was reduced by approximately 80%. In contrast, Gn Rg3 showed a partial inhibitory effect, which was most prominent on the third day of incubation. After 5 days of incubation, Gn Rg3 inhibited the fibrillation of α-syn by approximately 25%, (Fig. 5.1 A). The extent of inhibition was comparable for all concentrations tested. The third compound tested, Gn Rg1, had no significant effect on α-syn fibrillation given the comparable Th-T measurements produced by the Gn Rg1 containing samples and the control, that is the α-syn solution aged alone (Fig. 5.1 A).

The ability of the three ginsenosides to block α-syn fibril formation was further assessed by the Congo red (CR) binding assay. CR is a dye with high affinity for amyloid fibrils (Groenning, 2010). Upon binding to α-syn fibrils, the absorption maximum of CR shifts from 490 to 508 nm. This shift was quite pronounced for the α-syn sample incubated in the absence of any of the three compounds (i.e. control) (Fig. 5.1 B). However, this characteristic shift was not observed for α-syn samples aged in the presence of Gn Rb1, indicating that this compound inhibited the formation of amyloid fibrils (Fig. 5.1 B). Indeed, the absorption maximum of CR bound to the α-syn samples containing Gn Rb1 (at all concentrations tested) only shifted a few nm and did not exceed the wavelength of 495 nm (Fig. 5.1 B). Interestingly, the CR shift was comparable to the shift observed for monomeric α-syn. In contrast, the samples incubated in the presence of Gn Rg1 and (20S)-Rg3 behaved similarly to the control sample, exhibiting the characteristic shift from 490 nm to approximately 508 nm. To test if the formed α-syn aggregates is resistant to PK digestion, samples of α-syn aggregated alone or

in the presence of Gn Rb1, Rg1 and Rg3 at molar ratio of 1:1 were treated with 2.5 µg/ml PK; the digested samples were then run on 15% SDS-PAGE and the gel was silver stained as described in the materials and methods. The α-syn aged in the presence of Rb1 was easily digested by PK and behaved exactly as the monomeric α-syn (Fig. 5.2 C), while the samples of aged α-syn alone or in the presence of Rg1 and Rg3 were resistant to digestion (Fig. 5.2 C) possibly due to presence of β-sheets in these samples (Fig. 5.1 A, B).

These findings were further confirmed by electron microscopy. TEM images of α-syn aged in the presence of Gn Rb1 showed that α-syn formed thin, short, rod-like fibrils, with a fragmented appearance (Fig. 5.1 D), unlike the dense meshes of long fibrils formed by α-syn aged alone (Fig.5.1). For both Gn Rg1 and Gn (20S)-Rg3, TEM confirmed that there was little or no change in fibril morphology compared with the control, consistent with the Th-T fluorescence and CR binding findings (Fig. 5.1 D).

5.3.2 The effect of ginsenosides on α-syn oligomerization

The effect of the Gn Rg1, Gn (20S)-Rg3 and Gn Rb1 over α-syn oligomerization was assessed by western blotting. The samples of α-syn aged alone or in the presence of the three ginsenosides were centrifuged (14,000 rpm, 15 mins, 4ºC) and the supernatants were separated on 15% SDS gels, transferred onto nitrocellulose membranes and probed with an antibody that recognizes the amino acid residues 121-125 of α-syn (anti-α-syn clone 211). Gn Rb1, which was shown to inhibit α-syn fibrillation, was also a potent inhibitor of α-syn oligomerization at all molar ratios (Fig. 5.2 A). More specifically, Gn Rb1 inhibited the formation of larger aggregates (MW >250 kDa) and high MW oligomers, generating a prominent monomeric band and a weak dimeric band

(Fig. 5.2 A). Densitometric analysis of the monomeric bands revealed that Gn Rb1 hindered α-syn oligomerization to the same extent in all tested concentrations, (Fig. 5.2 A). It should be noted that even at the lowest concentration, Gn Rb1 was able to inhibit the formation of larger aggregates.

The other two ginsenosides, Gn Rg1 and Gn (20S)-Rg3, failed to inhibit α-syn oligomerization, similarly to their effect on α-syn fibrillation. In fact, in the presence of Gn Rg1, α-syn oligomerization was enhanced compared with the control, as indicated by the detection of numerous strong bands corresponding to the oligomeric species (Fig. 5.2 B). Densitometric analysis of the monomeric bands showed that Gn Rg1 stimulated α-syn oligomerization to the same extent at all concentrations tested (Fig. 5.2 B). In the case of Gn (20S)- Rg3, at every concentrations tested of the compound, the separation of α-syn in the gel generated bands comparable with that of the control, indicating that Gn (20S)-Rg3 failed to inhibit α-syn oligomerization (Fig. 5.2 C).

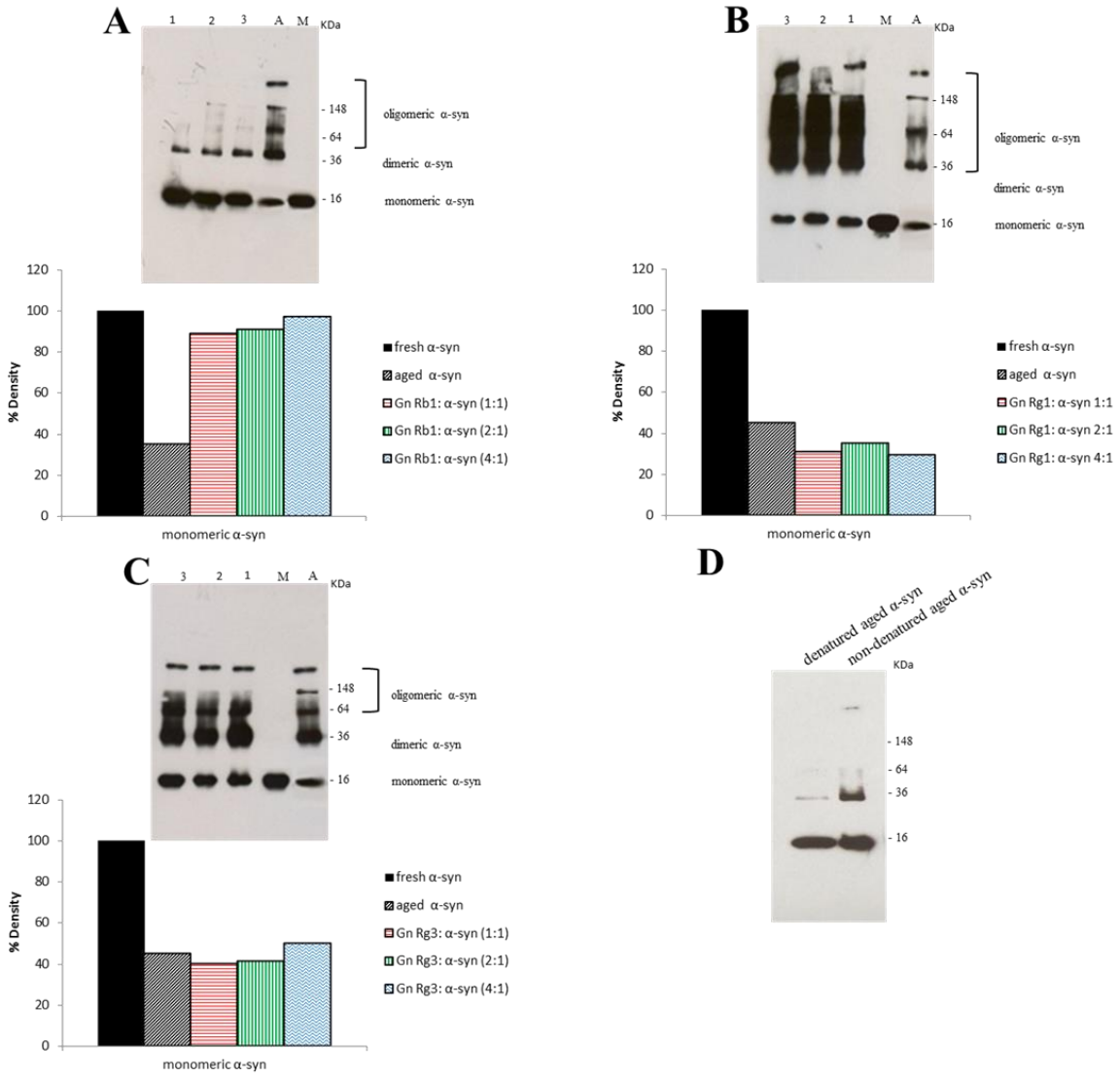


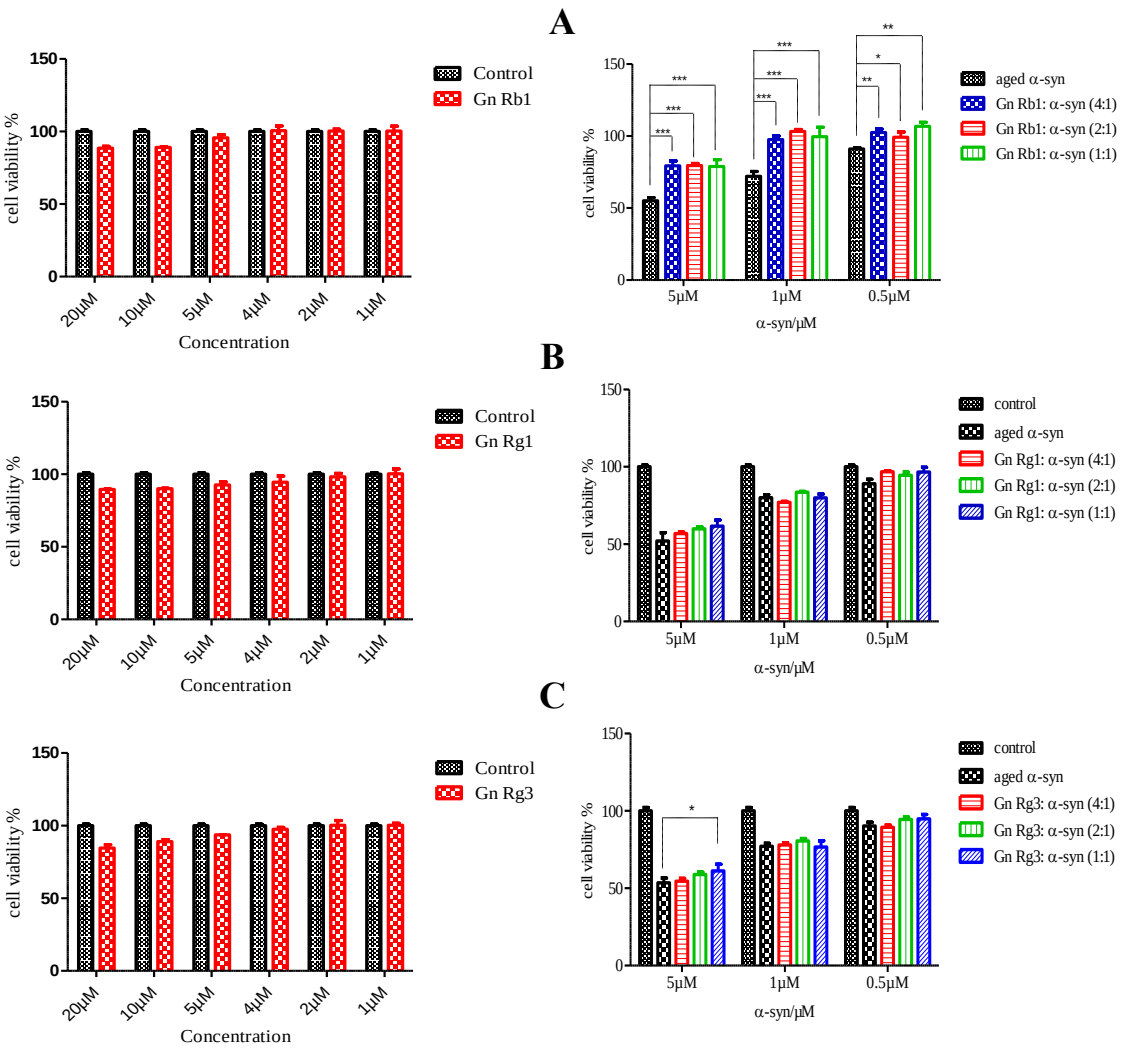
Figure 5.2. Immunoblot analysis showing the effect of ginsenosides on α-syn oligomerization. α-Syn alone or in the presence of ginsenosides at ginsenoside: α-syn molar ratios of 1:1, 2:1 and 4:1, was incubated for 5 days. Lane 1, ginsenoside: α-syn 1:1, lane 2, ginsenoside: α-syn 2:1, lane 3, ginsenoside: α-syn 4:1, A: aged α-syn and M: fresh α-syn. A**.** Gn Rb1, B. Gn Rg1, C. Gn Rg3. The amount of the monomeric α-syn in the samples was quantified using ImageJ software. D. Immunoblotting for aged α-syn samples under denaturing and non-denaturing conditions.

5.3.3 The effect of ginsenosides on α-syn-induced cytotoxicity

BE(2)-M-17 human neuroblastoma cells were treated with aged α-syn solutions at three different concentrations, 0.5 µM, 1 µM and 5 µM, either alone or in the presence of Gn Rb1, Gn Rg1 and Gn Rg3 and the viability of the cells

was determined by MTT assay. Prior to the experiments with α-syn and ginsenosides, we assessed the effect of the ginsenosides alone on cell viability (Fig. 5.3 left panel), employing the same non-toxic ginsenoside concentrations that were later employed for the experiments with aged α-syn.

As shown in Fig. 5.3 A-C, aged α-syn inhibited the reduction of MTT in a dose-dependent fashion. Given that MTT reduction is directly proportional to the number of surviving cells, it becomes apparent that fewer cells survived at higher concentrations of aged α-syn (Fig. 5.3 A-C).



D

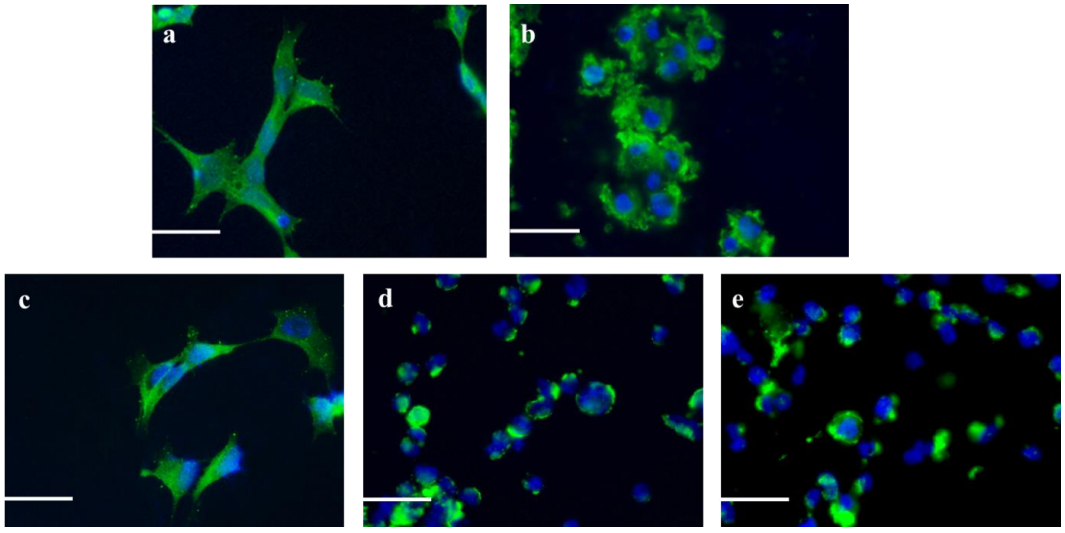


Figure 5.3. The effect of the ginsenosides on the toxicity induced by the aggregates of α-syn. The viability of BE(2)-M-17 human cells was evaluated by MTT assay. The results are expressed as percentages of the average of the control (*i.e.* untreated cells). The cells were treated with either α-syn aged with or without the A. Rb1, B. Rg1, and C. Rg3 for 48 hours prior to the addition of MTT. The graphs appearing on the left panel illustrate the toxicity of the compounds alone (average of 3 wells ± standard deviation). Statistical analysis was performed using a two-tailed unpaired t-test. ***, p< 0.001; **, p< 0.01; *, p< 0.05. D. Immunocytochemistry against α-syn of BE(2)-M-17 cells. *a.*The cells were either non-treated or treated for 48 hours with 5 µM of *b.* aged α-syn alone, or with ginsenoside *c.* Rb1, *d.* Rg1, *e.* Rg3. at a molar ratio of aged α-syn: compound 1:4. *Scale bar* 30 µm.

However, the α-syn aged in the presence of Gn Rb1 was less toxic to the cells, as indicated by the increase in MTT reduction (Figs. 5.3 A). Indeed, at 5 µM, the aged α-syn resulted in a decrease in the number of viable cells by almost 50%, whereas in the presence of all concentrations of Gn Rb1, the viability of the cells increased by approximately 30%, with approximately 80% of the cells surviving (Fig. 5.3 A). A similar trend was observed in the case of 1 µM aged α-syn; in all concentrations used, Gn Rb1 improved the viabilty of the cells treated with 1 µM aged α-syn by approximately 30 % (Fig. 5.3 A). Gn Rg1 and Gn

(20S)-Rg3, however, did not show any protective effect on neuroblastoma cells against the toxicity of aged α-syn (Fig. 5.3 B, C), possibly due to their inability to inhibit α-syn fibrillation (Fig. 5.1) and oligomerization (Fig. 5.2). To confirm the toxicity effect observed in the MTT assay, BE(2)-M-17 neuroblastoma cells were exposed to 5 μM α-syn aged in the presence or absence of the ginsenocide at a molar ratio of 1:4 (α-syn: Gn) and subjected to immunostaining for α-syn. Fluorescence microscopy revealed that, whereas untreated cells were healthy and displayed diffuse cytoplasmic α-syn staining (Fig. 5.3 D *a*), the cells treated with aged α-syn lost their neuronal shape, appearing rounded and unhealthy, with aggregated fibrils accumulating at the cell membranes (Fig. 5.3 D *b*). This effect was dramatically reversed when the cells were treated with α-syn aged in the presence of Gn Rb1, with no apparent aggregates detected on the cell membranes (Fig. 5.3 D *c*). In contrast, the cells treated with the α-syn aged in the presence of Rg1 and Rg3, which were shown above not to inhibit α-syn aggregation, appeared rounded, unhealthy and bearing extracellular, membrane-bound aggregates (Fig. 5.3 D *d*, *e*).

5.3.4 The effect of Gn Rb1 on preformed α-syn amyloid fibrils

Due to its high efficiency in inhibiting α-syn fibrillation, Gn Rb1 was also assessed for its effectiveness in reversing fibrillation. Hence, 25 μM of preformed α-syn fibrils were incubated at 37°C in the presence of Gn Rb1 at molar ratios representing Gn Rb1: α-syn of 6:1, 4:1 and 2:1 for a period of 48 hours. By measuring the Th-T fluorescence counts, the fibril content was estimated at different intervals. Even at time 0, the Th-T counts for α-syn incubated alone were much higher compared to the Th-T counts of the samples containing Gn

Rb1, especially the one with the highest concentration of that particular ginsenoside (Fig. 5.4 A). During the course of the experiment, the α-syn fibrils that were incubated in the absence of Gn Rb1 continued to aggregate further, as indicated by the increase in Th-T counts (Fig. 5.4 A), whereas the fibrils incubated in the presence of the ginsenoside disaggregated in a dose-dependent fashion, given the decrease in Th-T counts (Fig. 5.4 A). This trend was apparent after 4 hours of incubation, with the Th-T counts for the Gn Rb1 containing samples with approximately 1/5 of the Th-T counts of the control

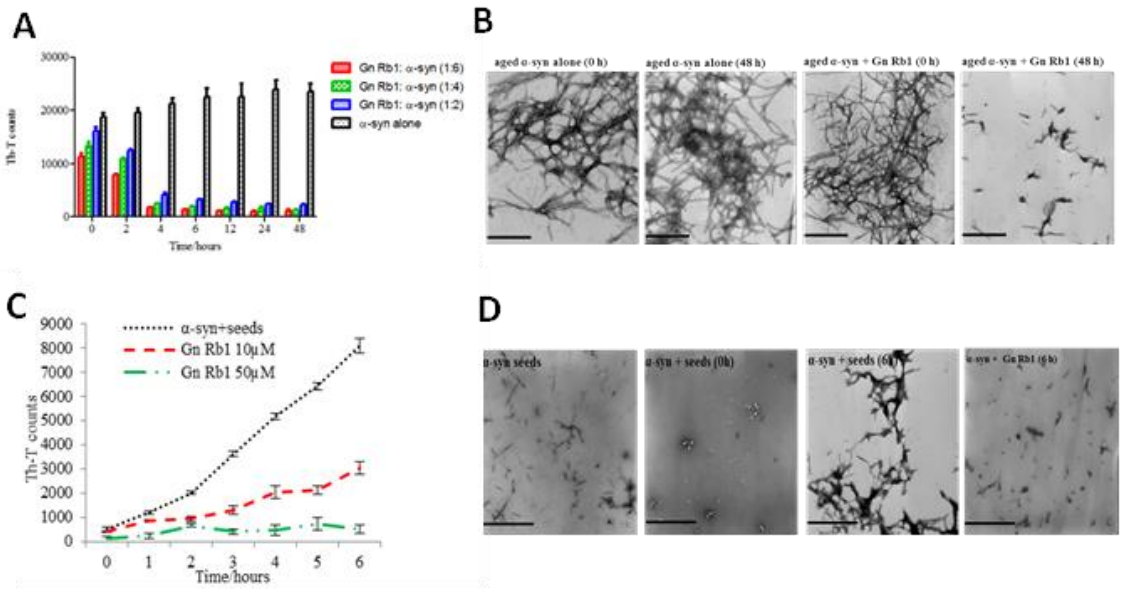


Figure 5.4. The effect of Gn Rb1 on preformed α-syn fibrils and on the seeded polymerization of α-syn. A. Samples of aggregated α-syn were incubated for 48 hours at 37°C in the absence or presence of various concentrations of Gn Rb1 (Gn Rb1: α-syn at 6:1, 4:1, and 2:1). The fibril content was then measured by the Th-T binding assay. The assay was performed in triplicate (average of triplicate measurements ± standard deviations). B. Electron microscopy images of negatively stained samples of the pre-aggregated α-syn incubated alone or in the presence of Gn Rb1 (1:4) for 0 and 48 hours with continuous shaking at 37°C. *Scale bar*, 500 nm. C. Samples of α-syn monomers (100 μM) were seeded with 2 μM sonicated α-syn fibrils, which were incubated in the presence or absence of Gn Rb1 at different concentrations (10 and 50 μM) for 6 hours with continuous shaking at 37°C. The extent of fibrillation was estimated by the Th-T binding assay. The assay was performed in triplicate (average of triplicate measurements ±

standard deviations). D. Electron microscopy images of negatively stained samples of the α-syn seeds alone and of the seeded α-syn incubated in the absence or presence of Gn Rb1 (50 μM) for 6 hours with continuous shaking at 37°C. *Scale bar*, 1,000 nm.

5.3.5 Gn Rb1 inhibition of α-syn fibrillation is mediated via binding to the intermediate species and formation of stable oligomers.

The strong inhibitory effect that Gn Rb1 showed on fibrillation, prompted us to investigate further the interaction of Gn Rb1 with α-syn oligomers. For this purpose, monomeric α-syn (100 μM) was aggregated in the presence of Gn Rb1 (Gn Rb1: α-syn molar ratio 4:1). After 5 days of incubation, the samples were centrifuged and the supernatant was injected in a superdex 200 SE column. The elution volume for monomeric α-syn was determined by molecular weight standards, and was eluted in a peak corresponding to column volume of 14-16 ml, while oligomeric α-syn eluted in a peak corresponding to a column volume of approximately 2-3 and 10-14 ml (Fig. 5.5 A).

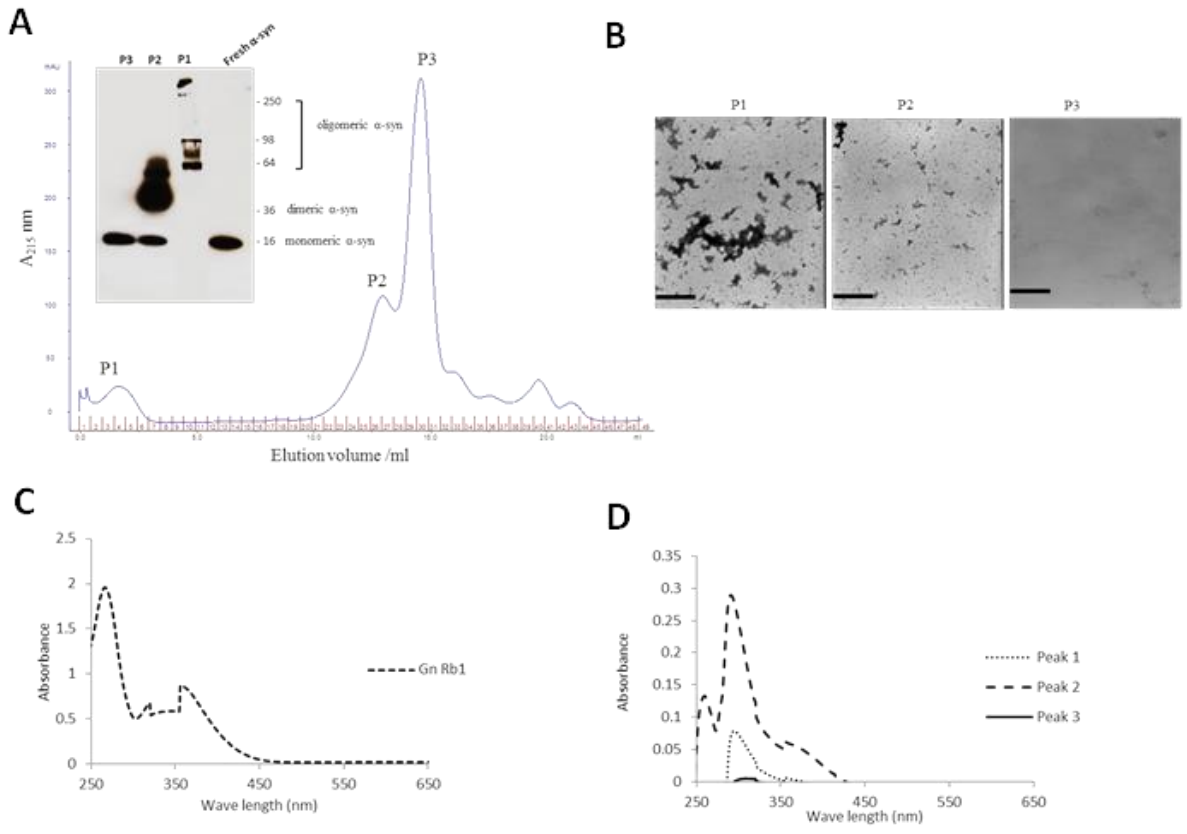


Figure 5.5. Gn Rb1 binds to α-syn oligomers (Gn Rb1: α-syn molar ratio of 4:1). A. Gel filtration profile of the 5-day aggregated α-syn in the presence of Gn Rb1 at a (Gn Rb1: α-syn) molar ratio 4:1 (α-syn concentration =100 μM) using a superdex 200 SE column. P1 and P2 samples contain the isolated fractions corresponding to the oligomeric peak and P3 the isolated fractions corresponding to the monomeric peak. The elution was monitored at the absorbance wavelength of 215 nm, immunoblot analysis of the samples P1 P2 and P3 separated by electrophoresis in a 15% SDS-PAGE gel. B. Electron microscopy images of negatively stained samples P1, P2 and P3 of α-syn in the presence of Gn Rb1 (molar ratio of Gn Rb1: α-syn 4:1) purified by SEC. *Scale bar,* 500 nm. C. UV absorbance spectra of Gn Rb1 alone. The UV absorbance was recorded between 200-600 nm employing a 10 mm quartz cuvette. D. UV absorbance spectra P1, P2 and P3. The UV absorbance was recorded between 200-600 nm employing a 10 mm quartz cuvette.

The fractions corresponding to the oligomeric and monomeric α-syn peaks were pooled together giving rise to P1, P2 and P3 samples (Fig. 5.5 A), which were concentrated using a speed vac. The α-syn species in the samples were

characterized by western blotting and EM (Fig. 5.5 A, B). In agreement with the imunoblotting results (Fig. 5.5 A), electron microscopy of the samples indicated the presence of different species of oligomers in P1 and P2 (Fig. 5.5 B). In order to detect the incorporated Gn Rb1 in the P1, P2 and P3 samples, we exploited the property of Gn Rb1 to produce UV absorbance spectra with three notable peaks (Fig. 5.5 C).

To further evaluate whether Gn Rb1 interacts with α-syn monomers, we monitored a titration of Gn Rb1 into a solution of monomeric α-syn using two-dimensional NMR spectroscopy, which provides signals covering the entire amino acid sequence of α-syn (Fig. 5.6). At stoichiometries of up to 6:1 Gn Rb1: α-syn, we observed no significant chemical shift or resonance intensity changes (Fig. 5.7 A-D), confirming that Gn Rb1, does not interact significantly with monomeric α-syn.

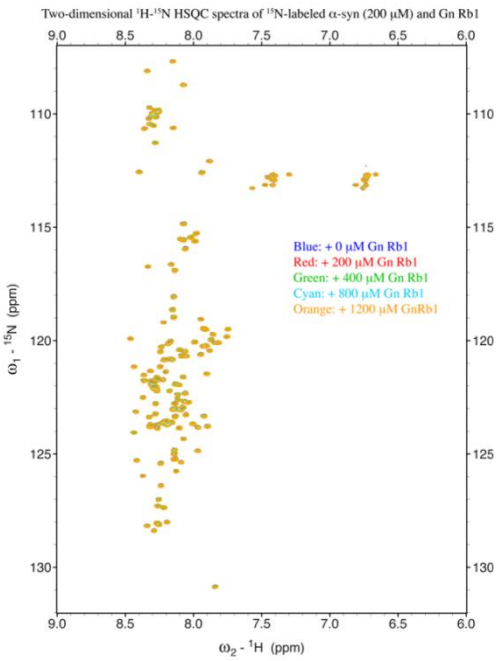


Figure 5.6. Analysis of Gn Rb1 binding to monomeric α-syn by NMR spectroscopy**.** Proton-Nitrogen correlation (HSQC) spectra of monomeric α-syn in the presence of increasing ratios of Gn Rb1: α-syn demonstrating that there are no significant changes in the positions of the NMR resonances, indicating the lack of an interaction between Gn Rb1 and monomeric α-syn. Protein concentration: 200 μM.

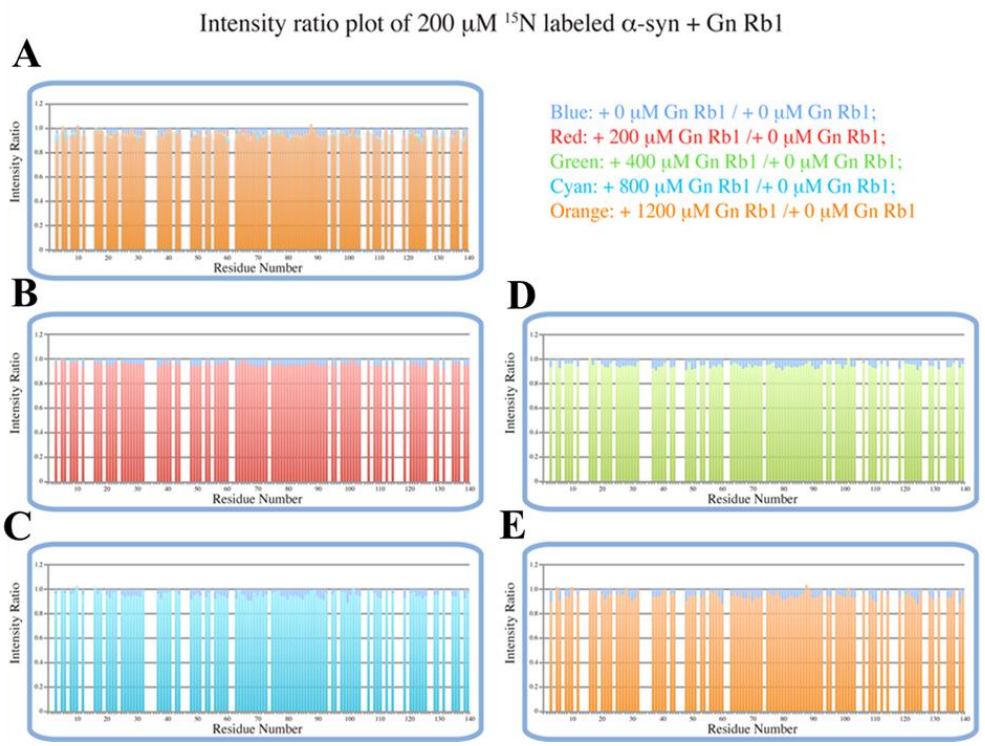


Figure 5.7. Peak intensity ratio plot of 200 μM 15N-labeled wt α-syn FL+ Gn Rb1 (Gn Rb1: α-syn 1:1, 2:1, 4:1 and 6:1). From the HSQC spectra and the intensity plot, there might be no interaction between α-syn monomer and Gn Rb1. A. all; B. 1:1/0:1 and 0:1/0:1; C. 2:1/0:1 and 0:1/0:1; D. 4:1/0:1 and 0:1/0:1; E. 6:1/0:1 and 0:1/0:1.

5.4 Discussion

PD is a progressive neurodegenerative disorder, the incidence of which is expected to rise sharply worldwide by 2030, with PD cases doubling by that time (Dorsey et al., 2007). Existing treatments for PD cannot cure the disease and can only offer moderate symptom relief, which is moreover accompanied by adverse side effects. Hence, there is a definite and imperative need for the development of new and more efficient treatments. Although PD pathology is considered to be induced by various etiological factors (reviewed by (Di Monte, 2003), there is ample evidence indicating that α-syn misfolding and aggregation play a central

role in the pathogenesis of PD and related disorders (Baba, et al., 1998; Spillantini, Crowther, Jakes, Hasegawa, & Goedert, 1998; Spillantini, et al., 1997). As a result, there is an ongoing endeavor to identify or design molecules that can suppress or even reverse α-syn aggregation (reviewed by (Findeis, 2000). Ginseng and its biologically active components, the ginsenosides, have been shown to have a beneficial effect on neurological conditions such as PD, due to their neuroprotective properties (Cho, 2012; H. J. Kim, et al., 2013), but their potential as anti-amyloidogenic agents have never been assessed. This gap in knowledge prompted us to investigate the effect of the most frequently used ginsenosides, namely Rb1, Rg1 and Rg3, on the formation of the early aggregates (soluble oligomers) and late aggregates (fibrils) of α-syn. For this purpose we employed an array of biophysical, biochemical and cell-cultured based techniques.

Among the ginsenosides screened, ginsenoside Rb1 was shown to be the only potent inhibitor of α-syn fibrillation that adequately blocked α-syn-induced toxicity; it disaggregated preformed α-syn fibrils and inhibited the seeded aggregation of α-syn. More specifically, our results show for the first time that Gn Rb1 can disrupt the fibrillation of α-syn *in vitro* (Fig. 5.1 A, B) allowing only the formation of small, sheared, rod-like species that are easily digested by PK (Fig. 5.1 C) and fail to elongate even after an extended aggregation period (Fig. 5.1 D). The accumulation of α-syn monomers was first detected by immunoblotting and densitometric analysis of the monomeric bands (Fig. 5.2 A) and was further confirmed by SEC (Fig. 5.5 A). Furthermore, Gn Rb1 could satisfactorily curb the neurotoxic effect that was provoked by the aged α-syn, demonstrating a marked

increase in cell survival and exhibiting a neuroprotective effect on neuroblastoma cells (Fig. 5.3 A and D). Gn Rb1 also disaggregated preformed α-syn fibrils (Fig. 5.4 A and B) and inhibited the seeded aggregation of α-syn in a concentration-dependent manner (Fig. 5.4 C and D),

These results are in accordance with previous reports that described Gn Rb1 as a neurotrophic and anti-inflammatory agent. Gn Rb1 has been previously shown to exert cell protective effects against the toxicity induced by $A\beta_{42}$ (Qian, Han, Hu, & Shi, 2009) and $A\beta_{25-35}$ (X. Xie et al., 2010). In animal models, Gn Rb1 was demonstrated to have anti-inflammatory activity (Wang et al., 2011) and to reduce soluble $A\beta_{40}$ in a dose-dependent fashion (Shi et al., 2010). Interestingly, Gn Rb1 was shown to attenuate the $A\beta_{25-35}$-induced hyperphosphorylation of tau protein *in vitro* (Y. H. Xie et al., 2007) and $A\beta_{25-35}$-induced memory impairment, axonal atrophy and synaptic loss *in vivo* (Tohda, Matsumoto, Zou, Meselhy, & Komatsu, 2004).

In terms of structure, Gn Rb1is a triterpene saponin, containing a four-ring steroidal structure that bears an aliphatic side chain and two disaccharide moieties attached to C-3 and C-20 of the triterpene structure (Scheme 5.1). Since one of the disaccharides of Rb1 is attached to the 3-position of the dammarane-type triterpine, Gn Rb1 is a protopanaxadiol-type of gindenoside, like Gn (20S)-Rg3. By comparing the structure of Gn Rb1 with that of Gn (20S)-Rg3 that partially inhibited fibrillation but failed to inhibit α-syn oligomerisation and toxicity (Scheme 5.1), it becomes apparent that the difference between the two ginsenosides lies in the presence of two additional sugar rings in Gn Rb1 at C-20 of the triterpene. As a consequence, Gn Rb1 possesses overall four sugar rings that are clustered in the form of disaccharides in each side of the dammarane-type

triterpene, thus conferring a more symmetrical structure to Gn Rb1 as compared to Gn (20S)-Rg3, which has only a disaccharide group attached to C-3 of the triterpene. Although structural symmetry is not a prerequisite for the inhibition of amyloid aggregation, there are many naturally occurring compounds whose structural symmetry renders them potent anti-aggregating agents *in vitro* (Caruana, et al., 2011; Porat, et al., 2006).

Furthermore, it is well understood that the specific action of each ginsenoside may depend on the type of sugar components, their number and their position on the aglycone (Z. Q. Liu et al., 2003; Nag, et al., 2012; Popovich & Kitts, 2002; Qi, Wang, & Yuan, 2010; Sun, Yang, & Ye, 2006). It is therefore likely that the number of sugar moieties may also play an important role in the anti-amyloidogenic properties of the ginsenosides. Gn Rb1 that was shown to be the most potent inhibitor possesses four sugar rings, while Gn (20S)-Rg3, which partially inhibited α-syn fibrillation but failed to exhibit any inhibitory effect in the rest of the assays, and possesses two sugar moieties. Although both Gn (20S)-Rg3 and Gn Rg1 (which failed to inhibit fibrillation and stimulated the oligomerisation of α-syn) contain two sugar rings, there is a major difference between the two ginsenosides. Gn (20)-Rg3 belongs to the protopanaxadiol group of gindenosides, whereas Gn Rg1 belongs to the protopanaxatriol group, which is characterized by the presence of an OH or a sugar group at C-6 of the triterpene. Gn Rg1 bears a sugar moiety at C-6 (Scheme 5.1). It has been reported before that the sugar linkage at C-6 is associated with a decreased ginsenoside anticancer activity compared to the linkages at C-3 or C-20 (R. J. Chen, Chung, Li, Lin, & Tzen, 2009). Also, the sugar linkage at C-6 has been shown to confer an antioxidant quality to the ginsenosides (Z. Q. Liu, et al., 2003). There is hence the

possibility that the C-6 linkage also renders the ginsenosides poor anti-amyloidogenic agents. Based on our findings using both NMR and SEC combined with UV spectroscopy, Gn Rb1 inhibits α-syn fibrillation not by interacting with the monomeric α-syn and preventing it from fibrillation, but rather by stabilizing the structure of soluble oligomeric α-syn without any β-sheet content (Fig. 5.2 A and 5.5 A), which appears to be non-toxic (Fig. 5.3 A and D). Since some interactions between α-syn and binding partners are not revealed in chemical shift changes, we also analyzed for changes to resonance intensities, which can sometimes reveal interactions that are otherwise missed. In this case, neither chemical shifts nor intensities changed. Nevertheless, it remains possible that a very weak interaction occurs that is missed by both measures, as indeed was the case in the analysis of Hsp70 binding by Dedmon et al. (Dedmon, Christodoulou, Wilson, & Dobson, 2005). The NMR data suggests that if any interaction is present with monomeric α-syn, it is a very weak and transient one. When combined with the size exclusion data, we conclude that the *predominant* interaction of Rb1 is with oligomeric α-syn rather than with the monomeric protein. These findings are in accordance with previous studies indicating that some polyphenolic compounds, such as baicalein, curcumin and epigallocatechin gallate induce the formation of soluble, non-toxic oligomers of α-syn or Aβ (Porat, et al., 2006).

Amyloid fibrils formed by aged α-syn are rich in β-sheet content and binding of these aggregates to the cell membrane results in membrane permeabilization and a resultant alteration in calcium homeostasis, which can cause cytotoxicity (El-Agnaf, Jakes, Curran, Middleton, et al., 1998; Pieri, Madiona, Bousset, & Melki, 2012; Reynolds et al., 2011). Indeed, the fluorescence pattern of α-syn aggregates

that we observed in cultured BE(2)-M-17 cells exposed to aggregated α-syn alone is suggestive of the localization of these aggregates to the plasma membrane, while the cells lost their characteristic neuronal shape as they appeared rounded and unhealthy. Treatment of the cells with α-syn aged in the presence of Gn Rb1, which did not result in α-syn fibrillation, appeared healthy without the accumulation of α-syn at the cell membranes, whereas exposure to α-syn aged in the presence of Rg1 or Rg3, which were poor inhibitors of α-syn fibrillation, led to the formation of membrane-bound aggregates, and the cells appeared unhealthy and rounded. This observation is in accordance with the results obtained from the MTT assay, wherein cells treated with aged α-syn alone or in the presence of Rg1 or Rg3 displayed cytotoxicity, which was not observed when the cells were exposed to α-syn aged in the presence of Rb1. Since Rb1 was found to inhibit α-syn fibrillation and stabilize soluble, non-toxic oligomers without a β-sheet content (Fig. 5.1), we hypothesize that the neuroprotective role of Rb1 stems from its ability to inhibit the formation of mature amyloid fibrils containing β-sheet structures and thereby prevent plasma membrane disruption in neuroblastoma cells. However, we cannot exclude the possibility that the anti-oxidant properties (Y. H. Xie, et al., 2007) and the anti-inflammatory activity (Wang, et al., 2011) of Gn Rb1 may also contribute to its neuroprotective effect. Interestingly, Gn Rb1 has previously been shown to be a much more potent anti-oxidant than Rg1, while Rg3 was shown to promote the free radical-induced heamolysis in human red blood cells (Z. Q. Liu, et al., 2003).

In conclusion, from the three tested ginsenosides, Rb1 was shown for the first time to inhibit α-syn fibrillation and toxicity *in vitro* and to be able to disaggregate

preformed fibrils and block the α-syn seeded polymerization possibly by binding and stabilizing non-toxic α-syn oligomers without β-sheet content. The next step is to assess its effect on PD animal models. Gn Rb1 could thus represent the starting point for designing new molecules that in turn could be used as new drugs for the treatment of PD and related disorders.

6 Chapter 6: Salvianolic acid b and Dihydromyricetin, inhibit the formation and toxicity of early and late alpha-synuclein aggregates.

6.1 Introduction

Drugs Currently available for the treatment of PD provide symptomatic treatment but do not alter the course of the disease (He, et al., 2013). However, the effectiveness of these drugs diminishes after several years of treatment. Together with the increasing incidence of PD due to an aging population, these facts indicate the compelling need for more effective drugs and treatment for PD. Modern therapeutic options that target disease modification are on the rise. However, the multiple mechanisms involved in the pathogenesis of PD create considerable difficulty in producing effective treatments. Hence, the inhibition of α-syn aggregation may represent a viable strategy for therapeutic intervention in PD and related disorders. It is therefore essential to identify compounds that can serve as potent inhibitors and interrupt the early stages of aggregation.

Chinese medicinal compounds (CMCs) extracted from medicinal herbs and plants have been used for the treatment of a wide range of diseases for over a millennium. Indeed, traditional Chinese medicine has employed various CMCs as remedies for disorders, including dementia (He, et al., 2013) and neurodegenerative

disorders (Kum et al., 2011), cardiovascular diseases (Ceylan-Isik, Fliethman, Wold, & Ren, 2008) and even cancer (reviewed by (X. Li et al., 2013). As a consequence, traditional Chinese medicine is considered a good resource for the treatment of several diseases, including PD. In fact, a lot of effort has been invested into unraveling the molecular basis of several CMCs employed in traditional Chinese medicine. Baicalein, the main component of the traditional Chinese herbal medicine *Scutellaria baicalensis*, has been reported to inhibit the fibrillation of wild-type (Zhu, et al., 2004) and E46K α-syn (Jiang et al., 2010) and to disaggregate preformed fibrils *in vitro* (Zhu, et al., 2004). Baicalein was also shown to alleviate the toxicity induced by E46K α-syn in a PD cell model (Jiang, et al., 2010). Additionally, curcumin, the main component of the traditional Chinese herbal medicine turmeric, isolated from the roots of Curcuma longa, has been shown to inhibit the aggregation and toxicity of β-amyloid peptide (F. Yang, et al., 2005).

The aim of this study was to assess the effects of a small library of CMCs on the oligomerization, fibrillation and toxicity of α-syn to identify potential inhibitors of α-syn aggregation. Eleven CMCs, which were extracted from Chinese herbal medicines (CHMs) including ginseng, gardenia, ginkgo biloba, Japanese raisin tree, Japanese arrowroot, peony, licorice, and danshen, were systematically tested using biophysical, biochemical and cell-culture techniques.

We found that among the 11 CMCs, salvianolic acid B (CMC1) significantly inhibited α-syn oligomerization and fibril formation and protected against α-syn-induced toxicity, while dihydromyricetin (CMC7), was shown to inhibit α-syn oligomerization and fibrillation, but exhibited only mild neuroprotective activity.

6.2 Experimental procedure

6.2.1 Expression and purification of recombinant human α-syn

As described in the experimental procedure 3.2.1

6.2.2 α-syn purification and characterization

As described in the experimental procedure 3.2.2

6.2.3 Aggregation of α-syn *in vitro*.

As described in the experimental procedure 3.2.3

6.2.4 Thioflavin-T (Th-T) assay

α-Syn fibril formation was monitored by Th-T binding assay. Th-T is a fluorescent dye that interacts with fibrils containing a β-sheet structure. A total of 10 µl of each sample was diluted in 40 µl of Th-T in PBS. Fluorescence was then measured in a 384-well, untreated black micro-well plate (Nunc, Denmark) using a microplate reader (Victor X3 2030, Perkin Elmer) with the excitation and emission wavelengths set at 450 and 486 nm, respectively. To allow for background fluorescence, the fluorescence intensity of a blank PBS solution was subtracted from all readings.

6.2.5 Transmission electron microscopy (TEM)

As described in the experimental procedure 3.2.8

6.2.6 Immunoblotting

As described in the experimental procedure 4.2.6

6.2.7 Congo red binding assay

As described in the experimental procedure 4.2.10

6.2.8 Tissue culture of BE(2)-M-17 human neuroblastoma cells

As described the experimental procedure 3.2.10

6.2.9 Measurement of cell viability

As described in the experimental procedure 3.2.13

6.2.10 Seeding polymerization assay

As described in the experimental procedure 5.2.13

6.2.11 α-Syn disaggregation assay

As described in the experimental procedure 5.2.12

6.2.12 Size Exclusion Chromatography (SEC) for separating α-syn oligomers and monomers

As described in the experimental procedure 5.2.14

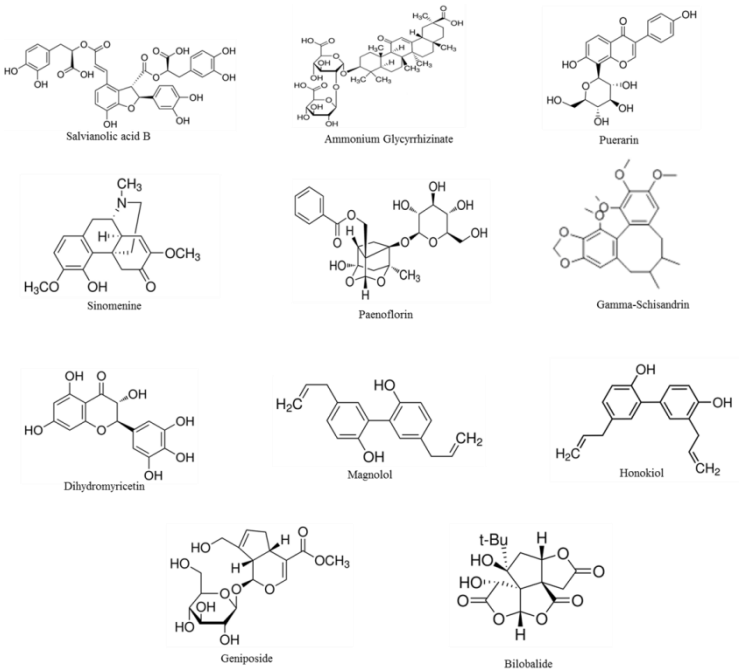
6.2.13 UV scanning.

As described in the experimental procedure 5.2.15

6.3 Results

6.3.1 The effect of salvianolic acid B and dihydromyricetin on α-syn fibrillation

α-Syn solution at a concentration of 25 μM was incubated at 37ºC with continuous shaking for 5 days, leading to fibril formation, which was monitored by Th-T fluorescence at regular time intervals. α-Syn was incubated with each of the eleven CMCs (see Table. 6.1 and Scheme 6.1) at molar ratios of 4:1, 2:1 and 1:1 (molar ratio CMC: α-syn) with a constant α-syn concentration of 25 μM.



Scheme 6.1: Chemical structure for the tested compounds. Salvianolic acid B, Ammonium Glycyrrhizinate, Puerarin, Sinomenine, Paenoflorin, Gamma-Schisandrin, Dihydromyricetin, Magnolol, Honokiol, Geniposide, Bilobalide.

Table 6.1. The small compounds from Chinese herbal medicines and their effect on fibril formation, oligomerization and toxicity

ID	Name	Inhibition of fibril formation[1]	Inhibition of oligomer formation[2]	Inhibition of fibril toxicity[3]
CMC 1	Salvianolic acid B	+ +	+	+ +
CMC2	Ammonium Glycyrrhizinate	-	-	-
CMC3	Puerarin	-	-	-
CMC4	Sinomenine	-	-	-
CMC5	Paenoflorin	-	-	-
CMC6	Gamma-Schisandrin	-	-	-
CMC7	Dihydromyricetin	+	+	+
CMC8	Magnolol	-	-	-
CMC9	Honokiol	-	-	-
CMC10	Geniposide	-	-	-
CMC11	Bilobalide	-	-	-

[1] Inhibition of α-syn fibril formation was tested by Th-T assay as described in the materials and methods. [2] Inhibition of α-syn oligomer formation was tested by immunoblotting as described in the materials and methods. [3] Inhibition of α-syn fibril toxicity toward M-17 - neuroblastoma cells was tested by MTT assay as described in the materials and methods. (+ + +, very potent; + +, potent; +, moderate; -, no effect).

Two compounds, salvianolic acid B (CMC1) and dihydromyricetin (CMC7), inhibited α-syn fibrillation as indicated by reduced Th-T fluorescence (Fig. 6.1). More specifically, at concentrations of 100 and 50 μM, CMC1 exhibited a complete inhibitory effect, which was prominent on the third day of incubation. After 5 days of incubation, CMC1 at 100 μM completely abolished the fibrillation of α-syn, while at 50 μM; it inhibited fibrillation by almost 80%. At a lower concentration (i.e., 25 μM), CMC1 also induced the inhibition of α-syn fibrillation, the percentage of which was reduced by approximately 35% after 5 days of incubation (Fig. 6.1). These results indicate that CMC1 inhibited the formation of fibrils in a concentration-dependent manner. CMC7 was observed as a good inhibitor of fibrillation, although its inhibitory effect was not comparable with of CMC1. Indeed, after 5 days of incubation, CMC10 inhibited α-syn fibrillation by 80 and 40% at 100 and 50 μM, respectively, while at 25 μM, the compound failed to inhibit fibrillation (Fig. 6.1).

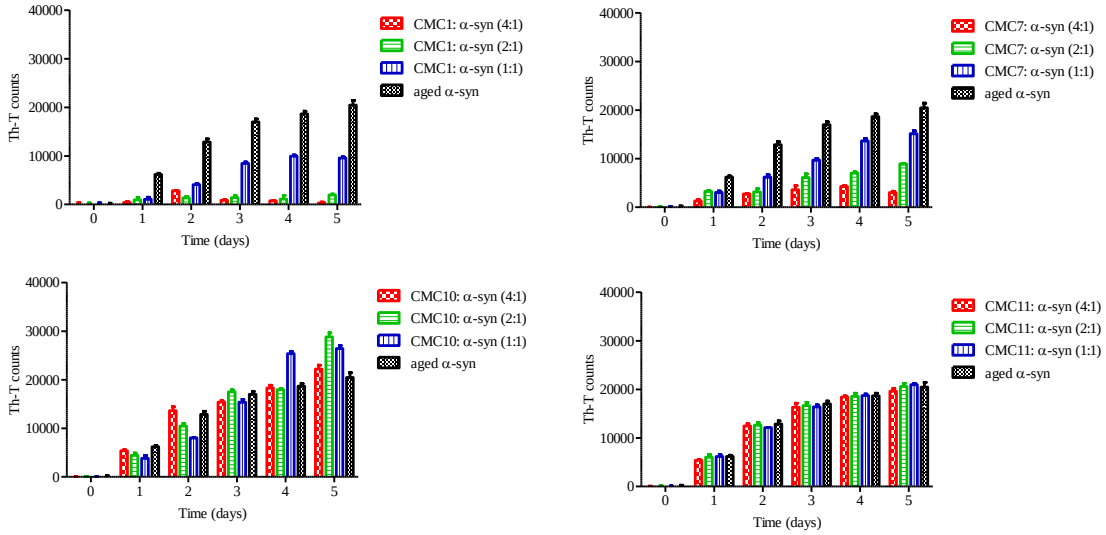


Figure 6.1. Th-T assay for CMCs. CMCs inhibit α-syn fibrillation in a concentration-dependent fashion. Samples of α-syn (25 μM) were incubated for 5 days at 37°C with continuous shaking in the presence of various concentrations of CMCs (100, 50 and 25 μM) Salvianolic acid B (CMC1), Dihydromyricetin (CMC7), Geniposide (CMC10) and Bilobalide (CMC11). Fibril formation was then measured by Th-T binding assay. The assays were performed in triplicate, and the means ± standard deviations are shown.

In contrast, the compounds ammonium glycyrrhizinate (CMC2), puerarin (CMC3), sinomenine (CMC4), paeoniflorin (CMC5), gamma-schisandrin (CMC6), magnolol (CMC8), honokiol (CMC9),and bilobalide (CMC11) had no significant effect on α-syn fibrillation because Th-T measurements indicated that the α-syn fibril formation was comparable to that for the control α-syn solution aged alone, (see Table 6.1). Geniposide (CMC10) had a slight stimulating effect on fibrillation with increased Th-T measurements (Fig. 6.1).

The ability of CMC1 and CMC7 to block α-syn fibril formation was further explored using the Congo red (CR) binding assay. CR is a dye with high affinity for amyloid fibrils (Groenning, 2010). Upon binding to α-syn fibrils, the absorption maximum of CR shifts from 490 to 508 nm, and this shift is quite pronounced for α-syn samples incubated in the absence of CMCs (Fig. 6.2). However, this characteristic shift was not observed for α-syn samples aged in the presence of CMC1 or CMC7, indicating that these compounds inhibited the formation of amyloid fibrils (Fig. 6.2), comparing with CMC10 and CMC11 which did not inhibit the fibril formation (Fig. 6.2). Indeed, the absorption maximum of CR bound to the α-syn samples containing CMC1 and CMC7 (at all tested concentrations) only shifted a few nm and did not exceed the wavelength of 495 nm (Fig. 6.2).

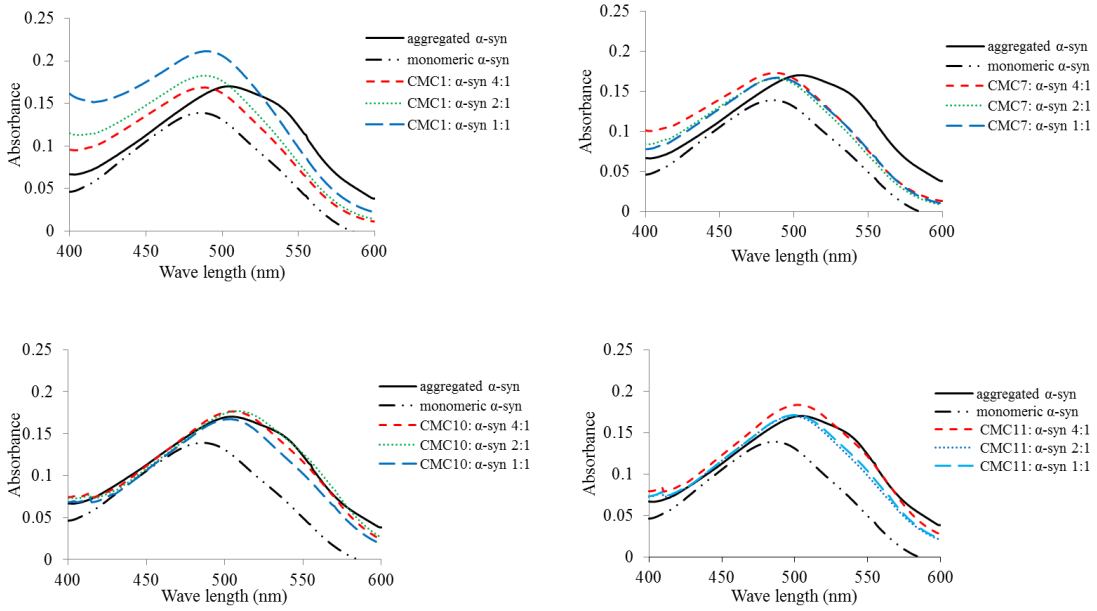


Figure 6.2. Congo red binding assay assay for CMCs. Congo red binding for CMCs. α-Syn solution samples (5 µM) either aged alone or with different molar ratios of CMCs were mixed with a final concentration of 5 µM Congo red. The UV absorbance spectrum was recorded from 400 to 600 nm in a spectrophotometer

Then the α-syn formed aggregates were tested to see if they were resistant to PK digestion, samples of α-syn aggregated alone or in the presence of CMC1, CMC7, CMC10 and CMC11 at molar ratio of 1:1 were treated with 2.5 µg/ml PK; the digested samples were then run on 15% SDS-PAGE and the gel was silver stained. The α-syn aged in the presence of CMC1 and CMC7 was easily digested by PK and behaved exactly as monomeric α-syn (Fig. 6.3), while the samples of aged α-syn alone or in the presence of CMC10 and CMC11 were resistant to digestion (Fig. 6.3) possibly due to the presence of β-sheets in these samples (Fig. 6.1 and 6.2).

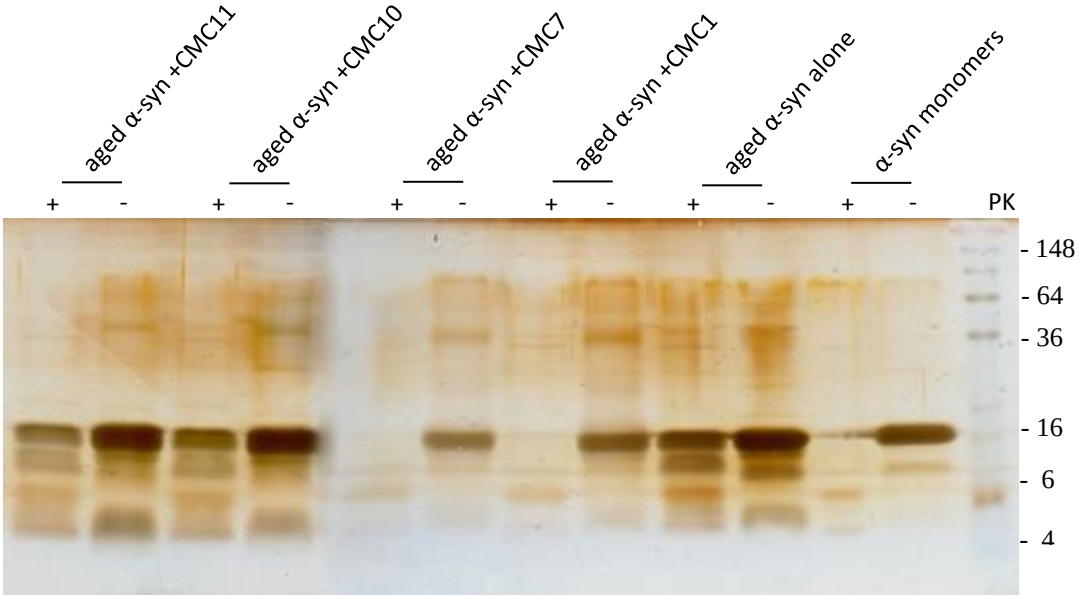


Figure 6.3. Proteinase K digestion assay. Silver staining for 15% SDS gel of α-syn monomers, α-syn aged alone or in the presence of CMC1, CMC7, CMC10 and CMC11at molar ratio 1:1 after 2.5 µg/ml PK digestion.

These findings were further confirmed by electron microscopy. TEM images of α-syn aged in the presence of CMC1 and CMC10 showed that α-syn formed thin, short rod-like fibrils, with a fragmented appearance (Fig. 6.4), which was unlike the dense meshes of long fibrils formed by α-syn aged alone (Fig. 6.4). For the rest of the compounds, TEM images confirmed that there were few or no changes in fibril morphology compared with the control, which is consistent with the Th-T fluorescence findings (results shown only for CMC10 and CMC11) (Fig. 6.4).

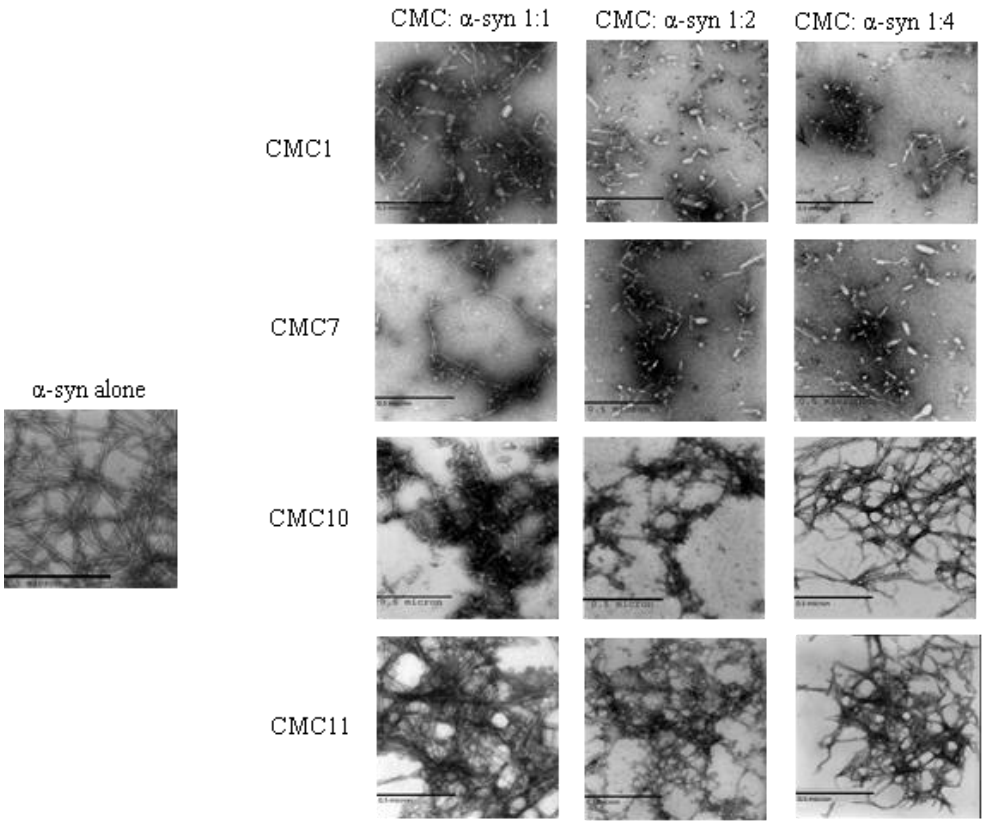


Figure 6.4. Electron microscopy images for CMCs. Electron microscopy images of negatively stained samples of α-syn (25 μM) aged alone or in the presence of CMCs (CMC: α-syn molar ratios of 4:1, 2:1, and 1:1) for 5 days with continuous shaking at 37°C. Scale bar, 500 nm.

6.3.2 The effect of salvianolic acid B and dihydromyricetin on α-syn oligomerization

The effect of the compounds on α-syn oligomerization was also assessed by immunoblot analysis. The same fresh and aged solutions of α-syn alone or with CMCs were analyzed, and the majority of freshly prepared α-syn migrated as a band at ~16 KDa, corresponding to α-syn monomers (Figs. 6.5 A-D). CMC1 and CMC10, which were shown to inhibit α-syn fibrillation, were also observed as potent inhibitors of α-syn oligomerization. In fact, similar to their effect on α-syn fibrillation, CMC1 and CMC7 inhibited α-syn oligomerization in a dose-dependent fashion (Figs. 6.5 A and B).

More specifically, CMC1 inhibited the formation of larger aggregates (MW >250 kDa) and high MW oligomers bands corresponding to monomeric and dimeric α-syn were the most prominent species, and a band corresponding to trimeric α-syn was faint (Fig. 6.5 A). With decreasing CMC1 concentration, the dimeric, trimeric and oligomeric bands were increasingly stronger, indicating that CMC1 inhibited α-syn oligomerization in a dose-dependent fashion (Fig. 6.5 A). It should be noted that even at the lowest concentration, CMC1 still induced the inhibition of larger aggregates. CMC7 also suppressed the formation of oligomeric forms in a concentration-dependent manner; the highest concentration was the most potent of the three concentrations tested (Fig. 6.5 B). At the highest concentration of CMC7, α-syn produced faint dimeric and trimeric bands (Fig. 6.5 B). However, these bands were progressively stronger with decreasing CMC7 concentration, consistent with dose-dependent inhibition (Fig. 6.5 B).

The rest of the compounds tested had an effect on α-syn oligomerization analogous to their effect on fibrillation. These compounds either had no effect on α-syn oligomerization or they stimulated it. For the sake of simplicity, we will only refer to two compounds: CMC10, which represents the group of CMCs that induced oligomerization (Fig. 6.5 C), and CMC11, which represents the group of CMCs had no effect on oligomerization (Fig. 6.5 D). Thus, In the presence of CMC10, α-syn oligomerization was enhanced compared to the control, as indicated by the detection of numerous strong bands corresponding to the oligomeric species (Fig. 6.5 C), in the presence of all CMC11 concentrations, separation of α-syn in the gel generated bands comparable with that of the control, indicating that CMC11 failed to inhibit α-syn oligomerization (Fig. 6.5 D), which is similar to its observed effect on fibrillation. The amount of monomeric α-syn in the α-syn aged alone samples and in

the presence of CMCs was quantified against a fresh α-syn sample that contained only the monomeric species using ImageJ software (Figs. 6.5 A-D).

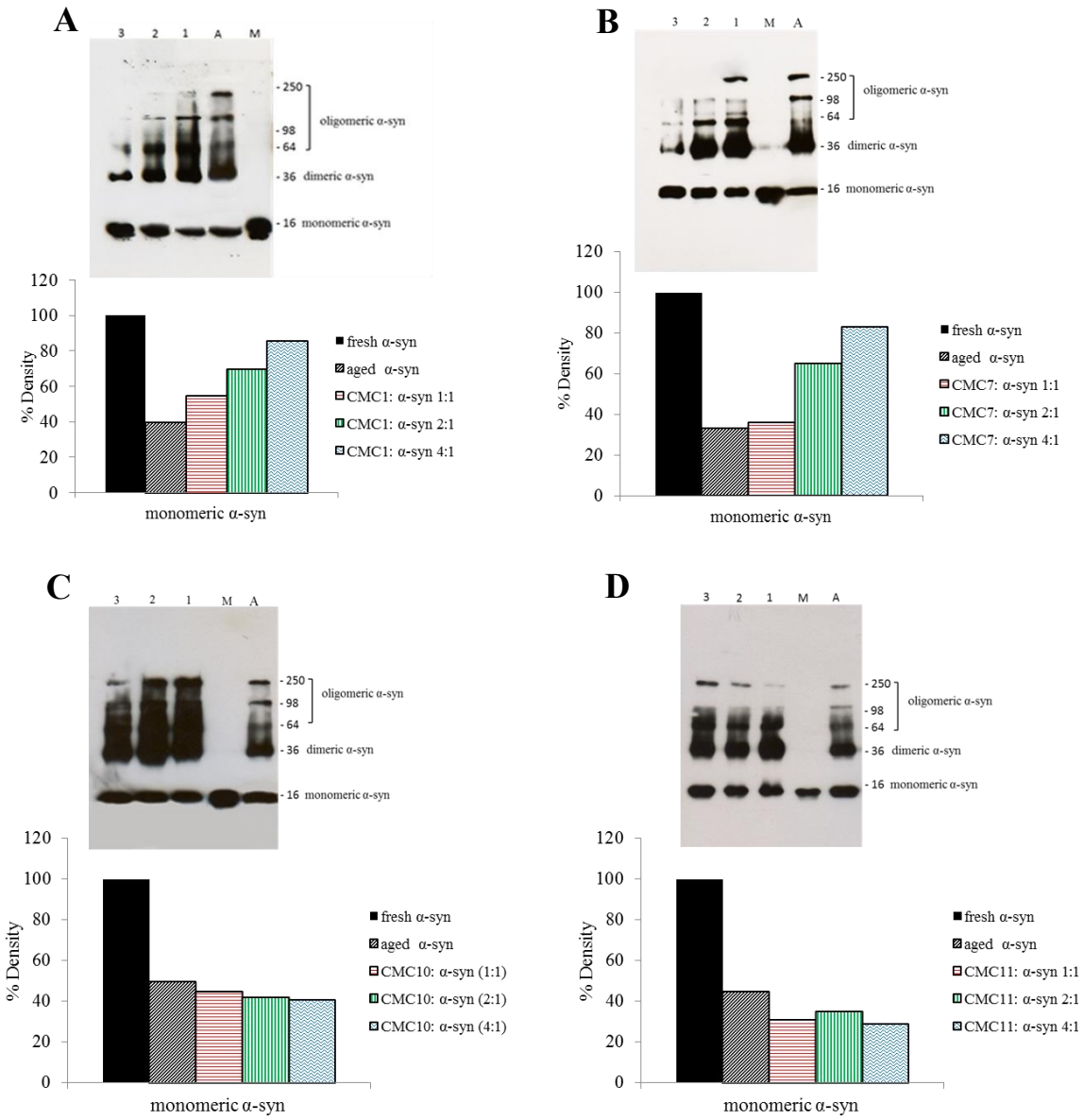


Figure. 6.5. Immunoblot analysis of the effect of CMCs on α-syn oligomerization. α-Syn alone or in the presence of CMCs at CMC: α-syn molar ratios of 1:1, 2:1 and 4:1, incubated for 5 days. Lane 1, CMC: α-syn 1:1, lane 2, CMC: α-syn 2:1, lane 3, CMC: α-syn 4:1, A: aged α-syn and M: monomeric α-syn. A. Salvianolic acid B (CMC1), B. Dihydromyricetin (CMC7), C. Geniposide (CMC10), D. Bilobalide (CMC11). The amount of the monomeric α-syn was quantified for α-syn samples incubated with or without the CMCs at different molar ratios using ImageJ software.

6.3.3 The effect of salvianolic acid B and dihydromyricetin on α-syn-induced cytotoxicity

BE(2)-M-17 human neuroblastoma cells were treated with aged α-syn solutions at three different concentrations, 0.5, 1 and 5 μM, either alone or in the presence of CMCs. The CMCs tested included CMC1, CMC7, CMC10 and CMC11. The last two compounds CMC10 and CMC11 were included as negative controls because they failed to inhibit or even stimulate the formation of α-syn aggregation.

The viability of cells treated with α-syn solution aged in the presence or absence of CMCs was determined with an MTT assay. Prior to experiments, the effect of CMCs alone on cell viability was assessed, employing the same non-toxic CMCs concentrations that were later employed for experiments with aged α-syn solutions (Figs. 6.6 left panel)

Aged α-syn inhibited the reduction in MTT in a dose-dependent fashion (Fig. 6.6). Because a reduction in MTT is directly proportional to the number of surviving cells, it is apparent that fewer cells survived at higher concentrations of aged α-syn (Fig. 6.6). However, aged α-syn solutions in the presence of CMC1 and CMC7 were less toxic to cells, as indicated by the increase in MTT reduction (Fig. 6.6), which was directly proportional to the number of living cells. Indeed, 5 μM of α-syn aged alone induced the reduction in viable cells by almost 50%, whereas in the presence of CMC1, cell survival dramatically improved, reaching 95% for the molar ratios of 4:1 and 2:1 (Fig. 6.6). Interestingly, at these molar ratios, CMC1 proved to be a good inhibitor for fibril formation, as indicated by the reduction in the Th-T counts (Fig. 6.1). CMC7 also reduced the α-syn toxicity, but it was not as potent as CMC1 (Fig. 6.6). CMC7 exhibited slight protection for cells at the highest concentration, which is represented by the molar ratio of 4:1, thus improving cell viability by approximately

10%. These findings are in accordance with the Th-T fluorescence measurements (Fig. 6.1) and immunoblotting analyses (Fig. 6.5).

As expected, the rest of the tested compounds, CMC10 and CMC11, did not show any protective effect for neuroblastoma cells against the toxicity of aged α-syn (Fig. 6.6).

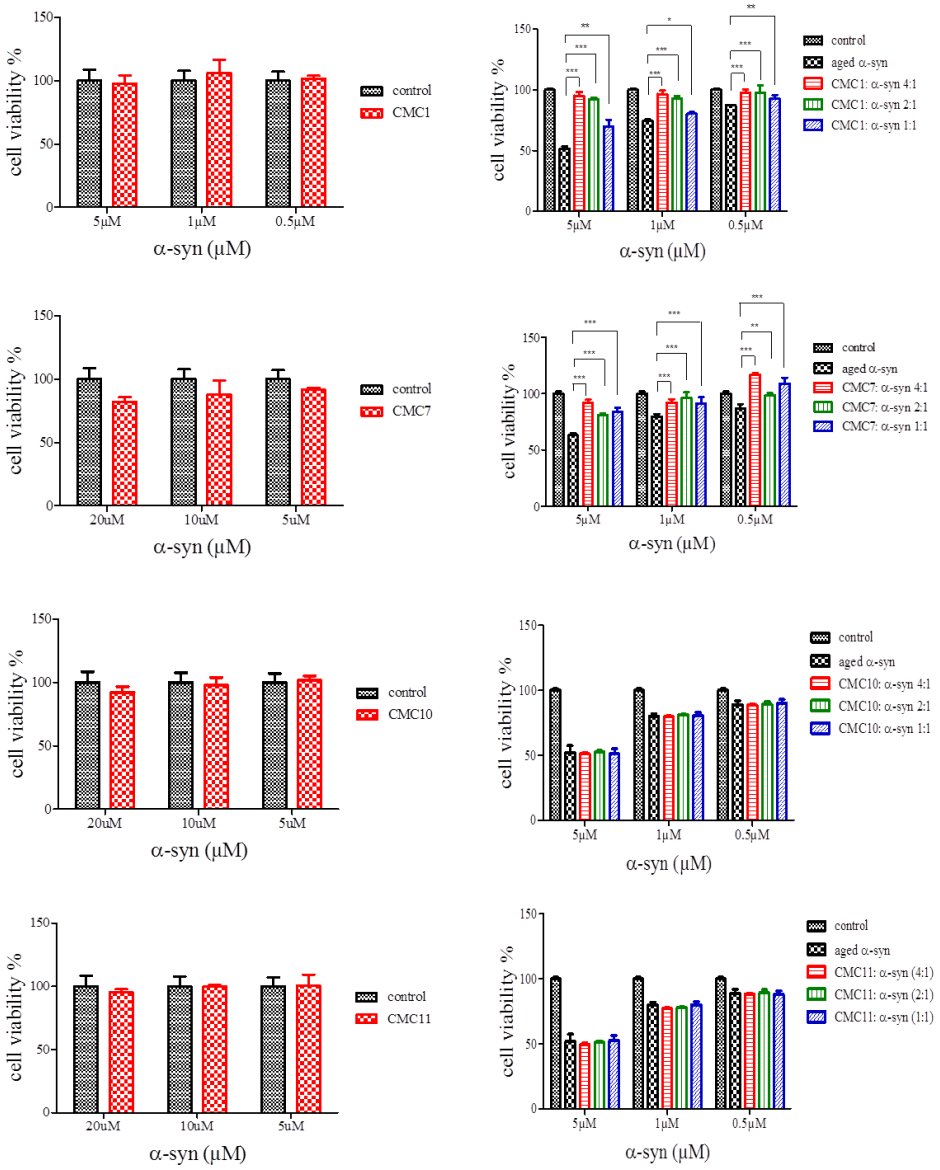


Figure 6.6. The effect of the CMCs on the toxicity induced by the aggregates of α-syn. The viability of BE(2)-M-17 human cells was evaluated by MTT assay. The results are expressed as percentages of the average of the control (i.e. untreated cells). The cells were treated with either α-syn aged with or without the CMC1, CMC7, CMC10, and CMC11 for 48 hours prior to the addition of MTT. The graphs appearing on the left panel illustrate the toxicity of the compounds alone (average of 3 wells ± standard deviation). Statistical analysis was performed using a two-tailed unpaired t-test. ***, $p< 0.001$; **, $p< 0.01$; *, $p< 0.05$.

To confirm the toxicity effect observed in the MTT assay, BE(2)-M-17 neuroblastoma cells were exposed to 5 μM α-syn aged in the presence or absence of the CMC1, CMC7, CMC10, and CMC11 at a molar ratio of 1:4 (α-syn: CMC) and subjected to immunostaining for α-syn. Fluorescence microscopy revealed that, whereas untreated cells were healthy and displayed diffuse cytoplasmic α-syn staining (Fig. 6.7 A), the cells treated with aged α-syn lost their neuronal shape, appearing rounded and unhealthy, with aggregated fibrils being accumulate at the cell membranes (Fig. 6.7 B). This effect was dramatically reversed when the cells were treated with α-syn aged in the presence of CMC1 and CMC7, with no apparent aggregates detected on the cell membranes (Fig. 6.7 C and D respectively). In contrast, the cells treated with the α-syn aged in the presence of CMC10 and CMC11, which were shown above not to inhibit α-syn aggregation, appeared rounded, unhealthy and bearing extracellular, membrane-bound aggregates (Fig. 6.7 E and F).

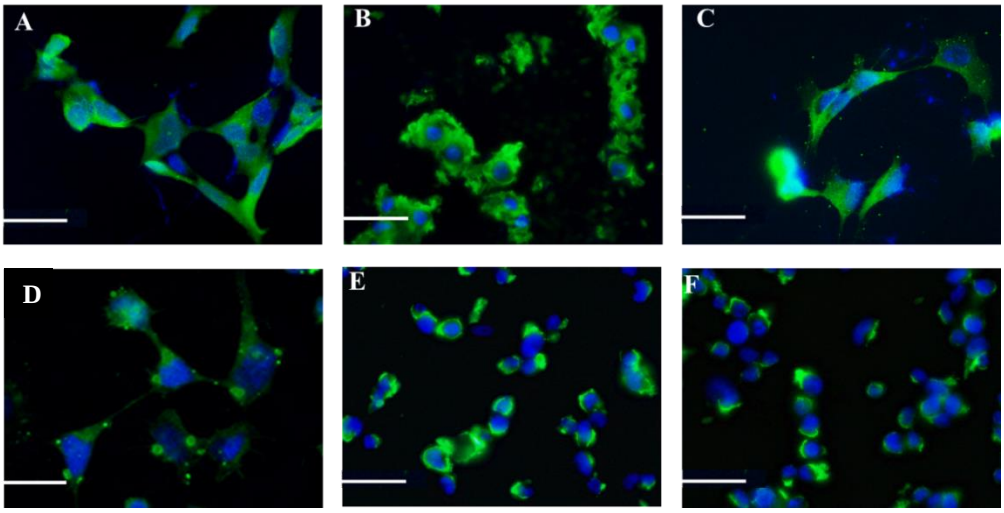


Figure 6.7. Immunocytochemistry against α-syn of BE(2)-M-17 cells. A.The cells were either non-treated or treated for 48 hours with 5 μM of B. aged α-syn alone, or with ginsenoside C. CMC1, D. CMC7, E. CMC10, F. CMC11. At a molar ratio of aged α-syn: compound 1:4. Scale bar 30 μm.

6.3.4 The effect of salvianolic acid B and dihydromyricetin on preformed α-syn amyloid fibrils

Because CMC1 and CMC7 were shown to be effective inhibitors of α-syn fibrillation, we wondered whether they could also reverse fibrillation. Hence, 25 μM of preformed α-syn fibrils was incubated at 37°C in the presence of the above mentioned CMCs at CMC: α-syn molar ratios of 6:1, 4:1 and 2:1 for a period of 48 hours. By measuring the Th-T fluorescence counts (Figs. 6.8 A and B), we estimated the fibril content at the different points. At 0, the Th-T counts were approximately 20,000 for α-syn incubated alone or with CMC1, while in the presence of CMC7, the Th-T count was less at that time point (Fig. 6.8 A and B). The α-syn fibrils that were incubated alone continued to aggregate further, as indicated by the increase in Th-T counts (Fig. 6.8 A and B). However, while α-syn fibrils incubated in the presence of CMC7 disaggregated over time, given the decrease in Th-T counts (Fig. 6.8 B), on

the other hand, CMC1 failed to disaggregate the preformed α-syn fibrils (Fig. 6.8 A). Thus CMC7 disaggregated preformed α-syn fibrils in a dose-dependent fashion.

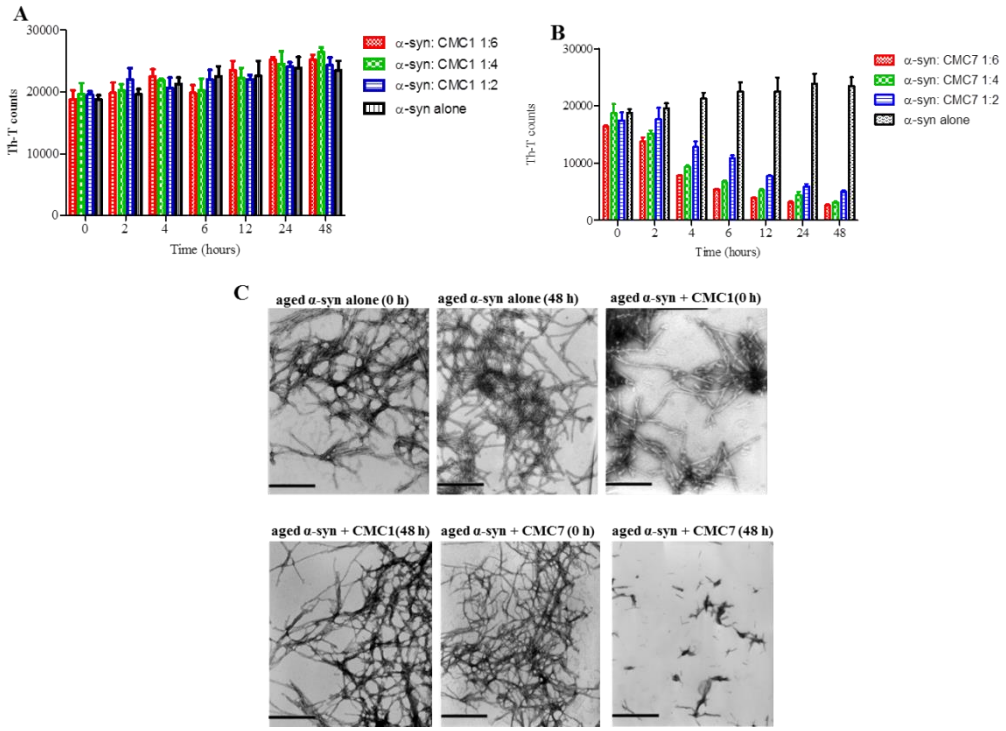


Figure 6.8. Effect of CMC1 and CMC7 on performed α-syn fibrils. A and B. Samples of aggregated α-syn were incubated for 48 hours at 37°C in the absence or presence of various concentrations of CMC1 and CMC7 (CMC: α-syn at 6:1, 4:1, and 2:1). The fibril content was then measured by a Th-T binding assay. The assays were performed in triplicate (average of triplicate measurements ± standard deviations). C. Electron microscopy images of negatively stained samples of aged α-syn incubated with alone or in the presence of CMC1 and CMC7 CMC: α-syn at 6:1) for 0 or 48 hours with continuous shaking at 37°C. *Scale bar*, 500 nm.

6.3.5 The effect of salvianolic acid B and dihydromyricetin on the seeding of α-syn monomers

It has been previously shown that the process of amyloid fibril formation follows a nucleation-dependent polymerization model (Jarrett & Lansbury, 1992). According to this model, soluble species generate via nucleation oligomeric species (nucleation or lag time phase), which in turn polymerize (polymerization or growth

phase) to generate fibrils, thus reaching a final plateau known as the equilibrium phase (Harper, Wong, Lieber, & Lansbury, 1999). Small aggregates or seeds have been shown to accelerate the nucleation phase of amyloid formation *in vitro* and *in vivo* via a process known as seeding (Harper & Lansbury, 1997; Jarrett & Lansbury, 1993; Luk, et al., 2012; Volpicelli-Daley, et al., 2011). Given that CMC1 and CMC7 inhibited α-syn fibrillation and that only CMC7 disaggregated preformed α-syn fibrils, we sought to identify the effect of those compounds on the seeding of α-syn aggregation. More specifically, mature α-syn fibrils were fragmented by sonication to obtain short fibrils, which were employed as 'seeds' (Fig. 6.9 C). These short fibrillar seeds were then added to monomeric α-syn, which was allowed to aggregate as described above. As expected, the addition of the short fibrillar seeds accelerated the fibrillation process of α-syn monomers, as indicated by an increase in Th-T counts. Indeed, with seeding, the extent of α-syn fibrillation after 6 hours of incubation was comparable with the extent of fibrillation of protein incubated for 24 hours without seeding (Figs. 6.9 D, B and Fig. 6.1).

To assess the effect of CMC1 and CMC7 on the seeding of α-syn aggregation, the compounds were added at concentrations of 10 or 50 μM to 100 μM monomeric α-syn containing seeds at a final concentration of 2 μM, and the mixture was then incubated with continuous mixing at 37°C for 6 hours. The tested CMCs at a concentration of 50 μM inhibited the seeding process by approximately 90%, as indicated by the low Th-T counts (Fig. 6.9 A and B). In contrast, at a lower concentration (10 μM), only CMC7 showed an inhibitory effect on the seeding of α-syn monomers, but to a lesser extent in the case of CMC1 (Fig. 6.9 A). These findings (for 50 μM and 6 hours incubation) were confirmed by TEM (Fig. 6.9 C).

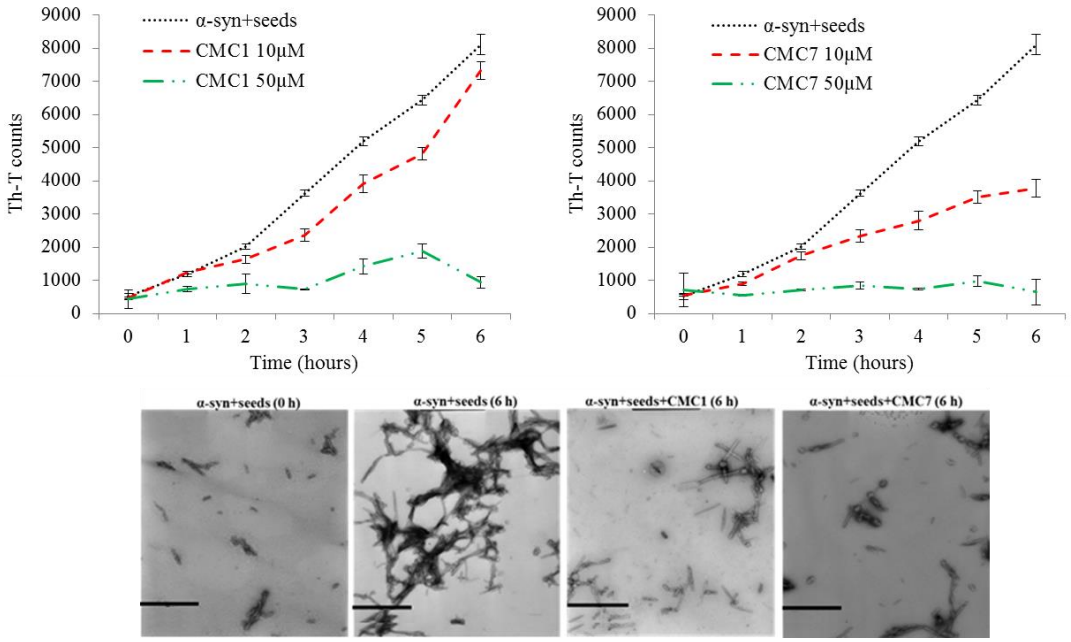


Figure 6.9. Effect of CMC1 and CMC7 on the seeding of α-syn monomers with fibrils. A and B. Samples of α-syn monomers (100 μM) were seeded with 2 μM sonicated α-syn fibrils, which were incubated in the presence or absence of CMC1 and CMC7 at different concentrations (10 and 50 μM) for 6 hours with continuous shaking at 37°C. The extent of fibrillation was estimated by Th-T binding assay. The assays were performed in triplicate (average of triplicate measurements ± standard deviations). C, Electron microscopy images of negatively stained samples of seeds alone and α-syn incubated with seeds alone or in the presence of CMCs (50 μM) for 6 hours with continuous shaking at 37°C. *Scale bar*, 500 nm.

6.3.6 Salvianolic acid B and dihydromyricetin inhibition of α-syn fibrillation is mediated via binding to the intermediate species and forming stable oligomers

Monomeric α-syn (100 μM) was aggregated in the presence of CMC1 and CMC7 and after 5 days of incubation the samples were centrifuged and the supernatant was injected in a superdex 200 SE column. The elution volume for monomeric α-syn was determined by a molecular weight standard (Fig. 4.7 A), and was eluted in a peak corresponding to a column volume of 14-16 mL (Fig. 4.7 B), while oligomeric α-syn eluted in a peak corresponding to column volume of

approximately 7-8 and 12-14 ml for P1 and P2 respectively (Fig. 6.10 A and 6.11 A). The fractions corresponding to the oligomeric and monomeric α-syn peaks were separately pooled together giving rise to P1, P2 and P3 samples (Fig. 6.10 A and 6.11 A), which were concentrated using a speed vac.

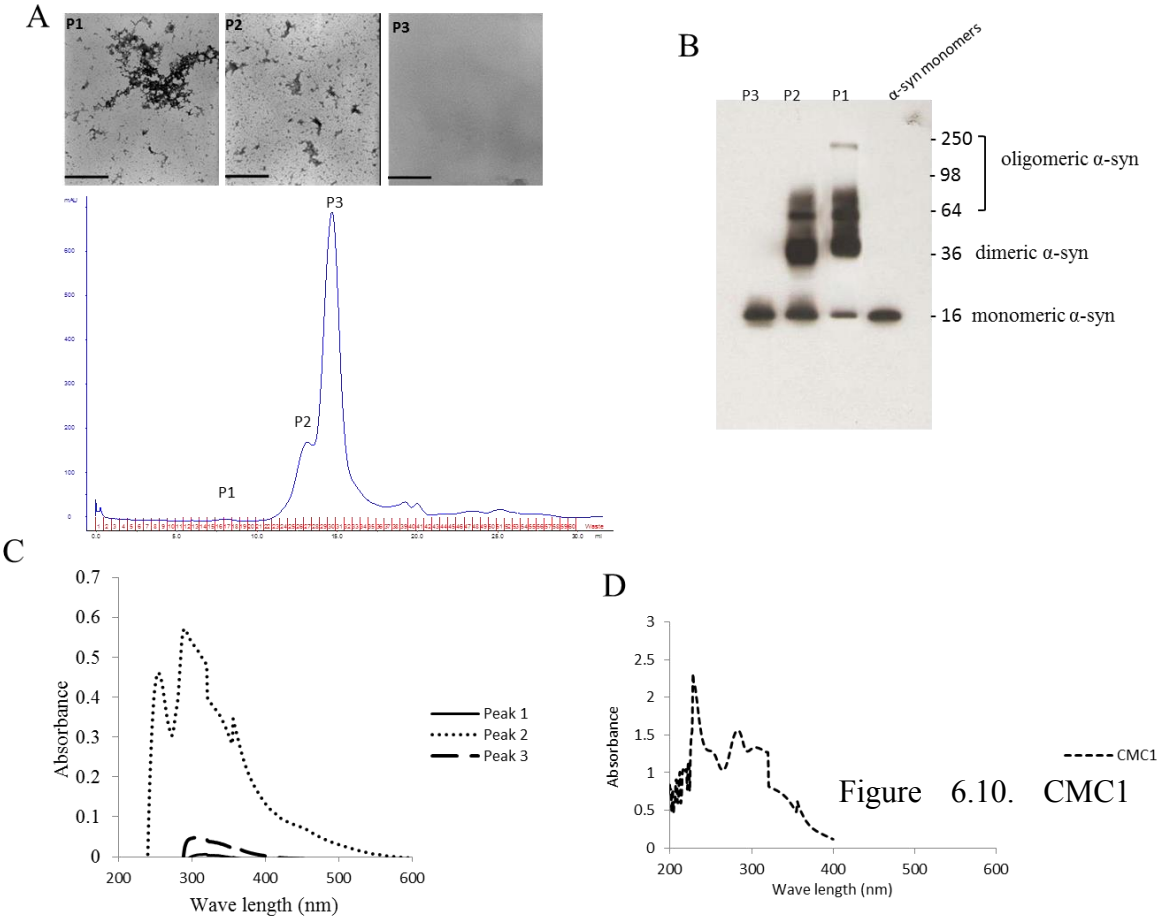


Figure 6.10. CMC1 binding activity to α-syn oligomers (CMC1: α-syn 4:1). A. Gel filtration profile of 100 µM α-syn sample incubated with CMC1 at 4:1 molar ratio (CMC1: α-syn) for 5 days as described before using superdex 200 SE column, P1and P2 represent the oligomeric species while P3 represents the monomeric species, the elution was monitored at absorbance wavelengths of A_{215}.TEM images represent the resulted peaks in the top of A . B. Immunoblot analysis of the pooled fractions represent P, P2 AND P3 resulted from the presence of CMC1 at molar ratios of CMC1: α-syn 4:1 incubated for 5 days with continuous shaking at 37°C were separated by electrophoresis in a 15% SDS-PAGE gel. C. P1, P2 and P3 samples of α-syn resulted from the SE chromatography were placed in a10mm quartz cuvette and the UV absorbance spectra were recorded from 200 nm to 600 nm. D. The UV absorbance spectra of CMC1 alone.

The α-syn species in the samples were characterized by western blotting and EM (Fig. 6.10 and 6.11 A and B). According to the western blotting results, the oligomers generated during the incubation of α-syn with both CMC1 and CMC7 are stable under denaturing conditions (Fig. 6.10 B and 6.11 B). Electron microscopy of the same samples indicates the presence of different species of oligomers in P1 and P2 (Fig. 6.10 A and 6.11 A), in agreement with the imunoblotting results (Fig. 6.10 B and 6.11 B).

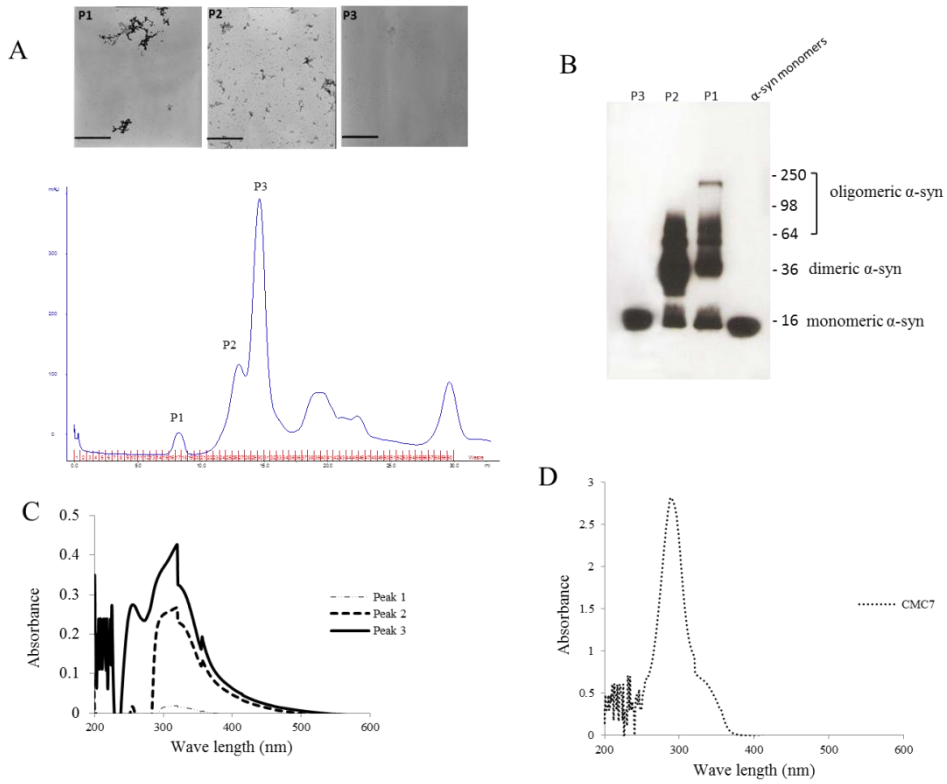


Figure 6.11. CMC7 binding activity to α-syn oligomers (CMC7: α-syn 4:1)**.** A. Gel filtration profile of 100 µM α-syn sample incubated with CMC7 at 4:1 molar ratio (CMC7: α-syn) for 5 days as described before using superdex 200 SE column, P1, P2 represent the oligomeric species and P3 represent the monomeric species, the elution was monitored at absorbance wavelengths of A_{215}. TEM images represent the resulted peaks in the top of A. B. Immunoblot analysis of the pooled fractions represent P1, P2 and P3 resulted from the presence of CMC7 at molar ratios of CMC7: α-syn 4:1 incubated for 5 days with continuous shaking at 37°C were separated by electrophoresis in a15% SDS-PAGE gel. C. P1, P2 and P3 samples of α-syn resulted from the SE chromatography were placed in a 10mm quartz cuvette and the UV absorbance spectra were recorded from 200 nm to 600 nm. D. The UV absorbance spectra of CMC7 alone.

In order to detect the incorporated CMC1 and CMC7 in the P1, P2 and P3 samples, we exploited the property of both compounds to produce UV absorbance spectra with three notable peaks (Fig. 6.10 D and 6.11 D). In the sample containing CMC1: α-syn at 4:1 molar ratio, we detected CMC1 only in the oligomeric P2 samples (Fig. 6.10 C) while in the sample containing CMC7: α-syn at 4:1 we detected CMC7 in both P2 and P3 which represent the oligomeric and monomeric species respectively.

To further evaluate whether CMC1 and CMC7 interact with α-syn monomers, we monitored a titration of both compounds into a solution of monomeric α-syn using two-dimensional NMR spectroscopy, which provides signals covering the entire amino acid sequence of α-syn (Fig. 6.12 and Fig. 6.13 respectively). At stoichiometries of up to 6:1 CMC1: α-syn, and 10:1 CMC7: α-syn, we observed no significant chemical shifts or resonance intensity changes (Fig. 6.12 A and Fig. 6.13 A respectively), and the peak intensity ratio plots of (wt α-syn + CMC1 or CMC7) (Fig. 6.12 B and Fig. 6.13 B respectively) indicate that there is no sign of interaction between α-syn and CMC1 or CMC7, confirming that CMC1 and CMC7, does not interact significantly with monomeric α-syn.

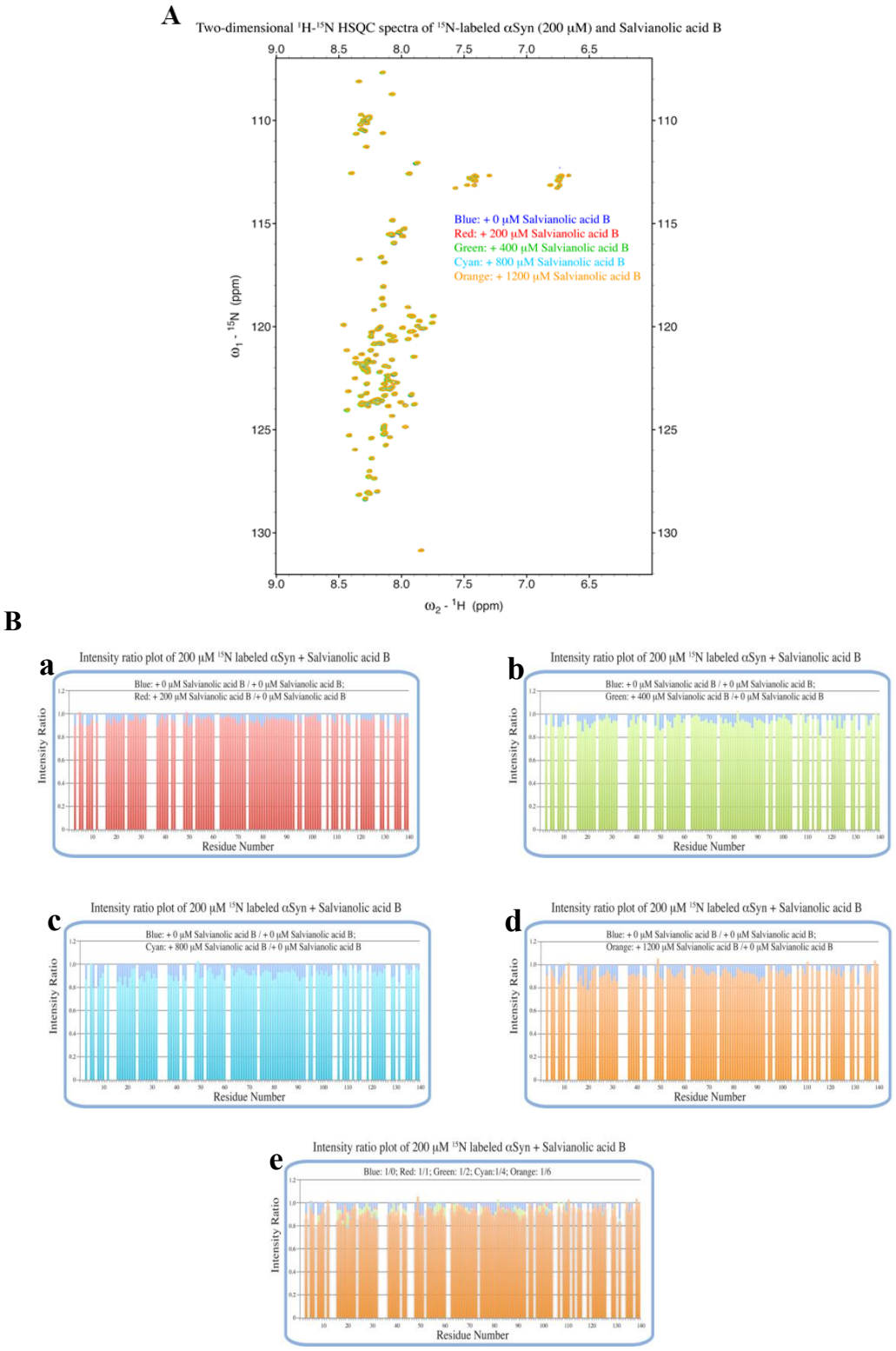


Figure 6.12. Analysis of CMC1 binding to monomeric α-syn by NMR spectroscopy. A. Proton-Nitrogen correlation (HSQC) spectra of monomeric α-syn in the presence of increasing ratios of Salvianolic acid B CMC1: α-syn. B. Peak intensity ratio plot of 200 µM ^{15}N-labeled wt α-syn FL+ CMC1 (CMC1: α-syn 1:1, 2:1, 4:1 and 6:1). From the HSQC spectra and the intensity plot, there might be interaction between α-syn monomer and CMC1. A. 1:1/0:1 and 0:1/0:1; B. 2:1/0:1 and 0:1/0:1; C. 4:1/0:1 and 0:1/0:1; D. 6:1/0:1 and 0:1/0:1; E. All ratios.

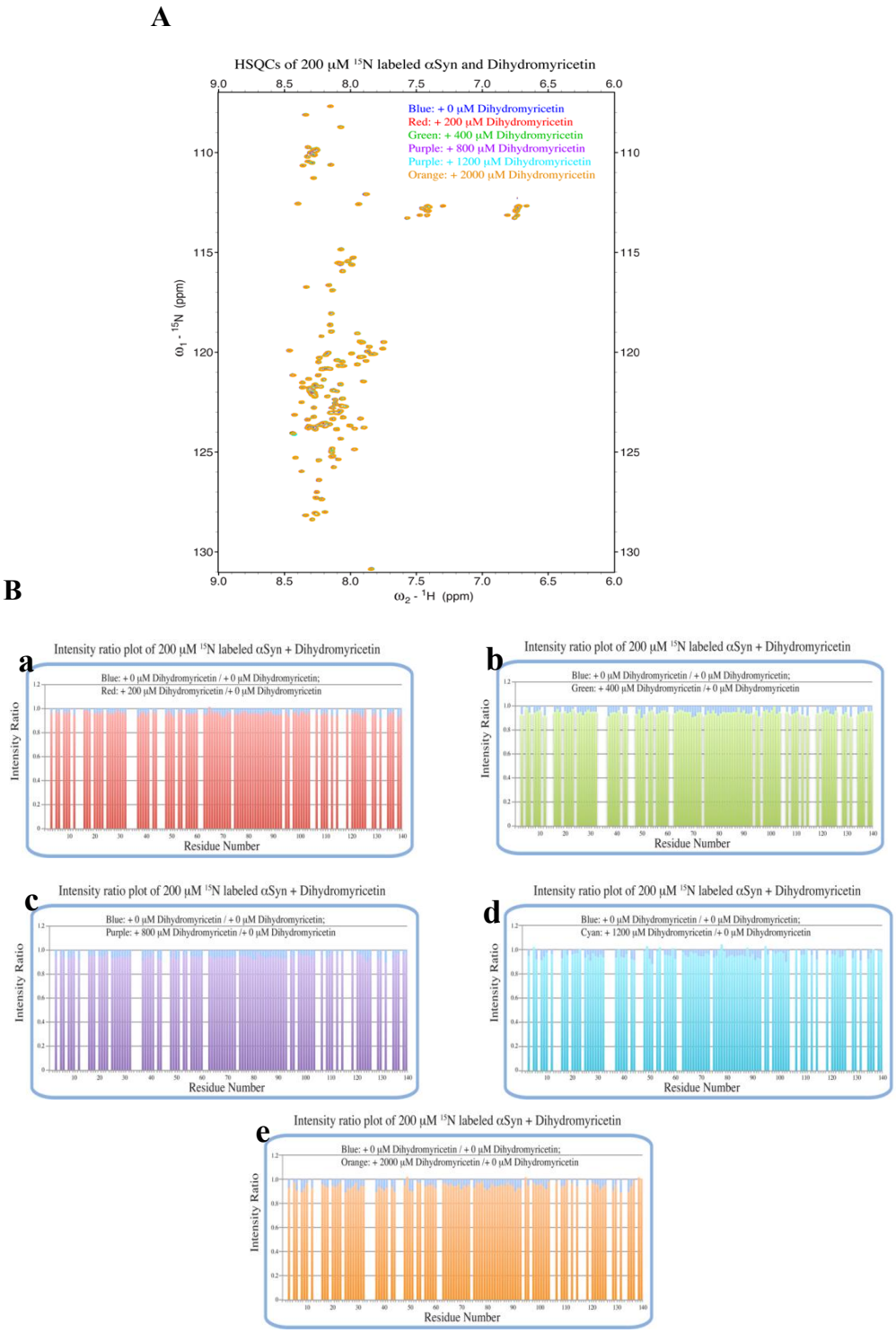


Figure 6.13. Analysis of CMC7 binding to monomeric α-syn by NMR spectroscopy. A. Proton-Nitrogen correlation (HSQC) spectra of monomeric α-syn in the presence of increasing ratios of Dihydromeryricetin CMC7. B. Peak intensity ratio plot of 200 µM 15N-labeled wt α-syn FL+ CMC7 (CMC7: α-syn 1:1, 2:1, 4:1, 6:1 and 10:1). From the HSQC spectra and the intensity plot, there is no interaction between α-syn monomer and CMC7. a. 1:1/0:1 and 0:1/0:1; b. 2:1/0:1 and 0:1/0:1; c. 4:1/0:1 and 0:1/0:1; d. 6:1/0:1 and 0:1/0:1; e. 10:1/0:1 and 0:1/0:1

6.4 Discussion

In this study, a total of eleven CMCs were assessed for their effect on the formation of early aggregates (soluble oligomers) and late aggregates (fibrils). Among the compounds screened, salvianolic acid B and dihydromyricetin (CMC7) were shown to be the most potent inhibitors of α-syn oligomerization and fibrillation. These compounds were also able to protect neuroblastoma cells from the toxic effects of aged α-syn.

Salvianolic acid B (CMC1) is one of the most abundant compounds extracted from Radix *Salvia miltiorrhiza* (Danshen). For centuries, traditional Chinese medicine has exploited the medicinal properties of danshen, employing it as a remedy against cardiovascular disorders. The medicinal properties of salvianolic acid B rely on its anti-oxidative ability, which is attributed to it by its polyphenolic structure. Dihydromyricetin (CMC7) is a flavonoid that can be extracted from the Japanese raisin tree (*Hovenia dulcis*) and is employed as a hangover cure.

Our results show for the first time that salvianolic acid B and dihydromyricetin disrupt the oligomer and fibril formation of α-syn *in vitro.* Salvianolic acid B and dihydromyricetin blocked α-syn oligomerization and fibrillation in a concentration-dependent manner. Salvianolic acid B also protected neuroblastoma cells against α-syn toxicity *in vitro*, demonstrating a marked increase in cell survival, while dihydromyricetin exhibited weak neuroprotection at the highest concentration. Electron microscopy of α-syn aged solutions with salvianolic acid B and dihydromyricetin showed that the protein forms small non-toxic, sheared, rod-like fibrils that fail to elongate.

These results are in accordance with previous reports that described salvianolic acid B as an anti-fibrillogenic and neuroprotective compound. Indeed, salvianolic acid B has been previously reported to inhibit the fibril formation of certain amyloidogenic compounds [β-amyloid (Aβ), human islet amyloid polypeptide (hIAPP)] and to protect cells from their toxic effects. In fact, salvianolic acid B not only blocks the fibrillation of Aβ (Tang & Zhang, 2001) but also disaggregates preformed Aβ fibrils (Durairajan et al., 2008). Furthermore, salvianolic acid B was shown to have neurotrophic properties, protecting cells from the toxic effects of $A\beta_{42}$ (Durairajan, et al., 2008) and $A\beta_{25\text{-}35}$ (Lin et al., 2006; Tang & Zhang, 2001). Recent reports indicate that the neurotrophic properties of salvianolic acid B also extend to animals (D. H. Kim, et al., 2011; Y. W. Lee et al., 2013). A recent study also demonstrated that salvianolic acid B inhibits the fibrillation of hIAPP, disaggregates preformed hIAPP fibrils and protects pancreatic INS-1 cells against the toxic effects of hIAPP (Cheng et al., 2013).

Many phenolic compounds, including baicalein (Caruana, et al., 2011; Zhu, et al., 2004), epigallocatechin gallate (EGCG) (D. E. Ehrnhoefer, et al., 2008), rosmarinic acid (Caruana, et al., 2011; Rao, Dua, & Ulmer, 2008), tannic acid (Caruana, et al., 2011; Ono & Yamada, 2006) and myricetin (Caruana, et al., 2011; Ono & Yamada, 2006), have been previously shown to inhibit the oligomerization and fibrillation of α-syn, which is in accordance with our findings. Furthermore, several lines of evidence indicate that polyphenolic compounds inhibit the elongation phase of amyloid aggregation (Ono et al., 2003; Zhu, et al., 2004), which may explain why salvianolic acid B and dihydromyricetin inhibited α-syn fibrillation (Fig. 6.1 and Fig. 6.2) and generated small aggregates with a sheared appearance and not resistant to PK digestion (Fig. 6.3 and Fig. 6.4). Salvianolic acid B (CMC1) and

dihydromyricetin (CMC7) are phenolic compounds containing four and two phenyl rings in their structures, respectively. The mechanism by which phenolic compounds inhibit α-syn aggregation is believed to be mediated by interactions between the phenolic compounds and the aromatic residues of the amyloids, directing the former towards the amyloidogenic core and thus interfering with the π-stacking of the rings of the aromatic residues that promote amyloid aggregation (Porat, et al., 2006). In addition, the hydroxyl moieties attached to the phenyl rings of the phenolic compounds are considered to play an essential role in the inhibition of the β-sheet structure by competitive hydrogen bonding (Porat, et al., 2006).. Interestingly, CMC7 was able to disaggregate the preformed fibrils (Fig. 6.8 B), whereas CMC1 failed to do so (Fig. 6.8 A). Moreover, both CMC1 and CMC7 were shown to block the seeding of α-syn monomers with preformed fibrils, and CMC7 was capable of inhibition at both tested concentrations (Fig. 6.9 B), while CMC1 had this effect only at 50 μM (Fig. 6.9 A). In this study, we showed also that CMC1 has the ability to bind to oligomers (Fig. 6.10 C), while CMC7 was found to bind to both monomers and oligomers (Fig. 6.11 C), which could be a starting point to investigate the mechanism behind the activity of these two compounds, to inhibit the fibril formation of α-syn.

Regarding the neuroprotective effect that salvianolic acid B exerted on neuroblastoma cells, we assumed that it is due to the anti-aggregating activity that both compounds demonstrated, but we cannot exclude the possibility that the anti-oxidant properties of salvianolic acid B (Lin, et al., 2006) also contribute to this effect. Membrane permeabilization and alteration in calcium homeostasis, which can cause cytotoxicity, resulted from the binding of the rich β-sheet amyloid fibrils formed by aged α-syn (El-Agnaf, Jakes, Curran, Middleton, et al., 1998; Pieri, et al.,

2012; Reynolds, et al., 2011). The fluorescence pattern of α-syn aggregates that we observed in cultured BE(2)-M-17 cells treated with aggregated α-syn alone is indicative of the localization of these aggregates to the plasma membrane, while the cells lost their characteristic neuronal shape as they appeared rounded and unhealthy (Fig. 6.7 B) While treatment of the cells with α-syn aged in the presence of CMC1 and CMC7, which did not result in α-syn fibrillation, appeared healthy without the accumulation of α-syn at the cell membranes (Fig. 6.7 C and D), whereas exposure to α-syn aged in the presence of CMC10 or CMC11, which were poor inhibitors of α-syn fibrillation, led to the formation of membrane-bound aggregates where the cells appeared unhealthy and rounded (Fig. 6.7 E and F). This observation is in accordance with the results obtained from the MTT assay, wherein cells treated with aged α-syn alone or in the presence of CMC10 or CMC11 displayed cytotoxicity, which was not observed when the cells were exposed to α-syn aged in the presence of CMC1 or CMC7. Since both CMC1 and CMC7 were found to inhibit α-syn fibrillation and stabilize soluble, non-toxic oligomers without a β-sheet content (Fig. 6.1), we hypothesize that the neuroprotective role of CMC1 and CMC7 stems from its ability to inhibit the formation of mature amyloid fibrils containing β-sheet structures and thereby prevent plasma membrane disruption in neuroblastoma cells.

Our findings using both NMR and SEC combined with UV spectroscopy, suggested that salvianolic acid B inhibits α-syn fibrillation not by interacting with the monomeric α-syn and preventing it from fibrillation, but rather by stabilizing the structure of soluble oligomeric α-syn without any β-sheet content (Fig. 6.10 A, and Fig. 6.12), which appears to be non-toxic (Fig. 6.6 A and Fig. 6.7 C). While dihydromyricetin binds to both oligomers and monomers as shown in the FPLC analysis (Fig. 6.11 D), whereas the NMR analysis didn't show the binding of this

compound with monomeric α-syn (Fig. 6.13). Since some interactions between α-syn and binding partners are not revealed in chemical shift changes, we also analyzed for changes to resonance intensities, which can sometimes reveal interactions that are otherwise overlooked. In this case, neither chemical shifts nor intensities changed. Nevertheless, it remains possible that a very weak interaction occurs that is missed by both measures, as indeed was the case in the analysis of Hsp70 binding by Dedmon et al. (Dedmon, et al., 2005). The NMR data suggests that if any interaction is present with monomeric α-syn, it is a very weak and transient one. When combined with the size exclusion data, we conclude that the predominant interaction of dihydromyricetin is with oligomeric α-syn rather than with the monomeric protein. These findings are in accordance with previous studies indicating that some polyphenolic compounds, such as baicalein, curcumin and epigallocatechin gallate induce the formation of soluble, non-toxic oligomers of α-syn or Aβ (Porat, et al., 2006).

In conclusion, two compounds from a small library of eleven CMCs isolated from widely used CHMs, salvianolic acid B and dihydromyricetin, were shown for the first time to inhibit α-syn aggregation and toxicity *in vitro.* The next step is to elucidate the mechanism by which these compounds inhibit α-syn aggregation and toxicity and to assess their effect on PD animal models. The compounds we identified in this study could represent the starting point for designing new molecules that may be used as drugs for the treatment of PD and related disorders.

7 Chapter 7: Conclusion

7.1 Conclusion

Several studies supporting the hypothesis that the aggregation of native protein is directly seeded by exogenous aggregates. Overall, these studies support the notion that amyloid toxicity is mediated by a nucleation-dependent polymerization process. In agreement with those studies, our in vitro data also showed that the a-syn amyloid toxicity is mediated by the ongoing nucleation-dependent polymerization 'seeding'. In contrast with wt-α-syn, p-S129 α-syn found to inhibit the seeded aggregation of wt-α-syn, and failed to aggregate and form amyloid fibrils. These finding prompted us to screen compounds that can inhibit the fibril formation and the seeding of α-syn aggregation. From the compounds screened, only gallic acid, ginsenoside Rb1, salvialonic acid B and dihydromyricetine found to inhibit the seeding aggregation of α-syn and also displayed an inhibitory effect on α-syn fibril formation by stabilizing non-toxic soluble oligomers with no β-sheet content, while only dihydromyricetine was found to bind with both monomeric and oligomeric forms of α-syn. Those compounds may represent the starting point for designing new molecules that could be used for the treatment of PD and related disorders.

7.2 Future work

There is an immediate need to further investigate the correlation between the aggregation and toxicity of α-syn and the phosphorylation at S129, especially in the light of recent findings supporting the idea that α-syn mediated toxicity is caused by the nucleation-dependent polymerization process.

Based on our *in vitro* screening, together with previous findings, those potent compounds need to be tested *in vivo* to investigate their ability to inhibit the seeded aggregation of α-syn and their effect on the motor behavior and pathology of PD animal models.

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Lu, J. H., Ardah, M. T., Durairajan, S. S., Liu, L. F., Xie, L. X., Fong, W. F., Hasan, M. Y., Huang, J. D., El-Agnaf, O. M. and Li, M. (2011) 'Baicalein inhibits formation of α-synuclein oligomers within living cells and prevents Aβ peptide fibrillation and oligomerisation', *Chembiochem,* 12(4), 615-24.

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Conference Posters

Gallic Acid Inhibits Alpha-Synuclein Aggregation and Toxicity.

Mustafa Taleb Ardah, Salema Begum, Jia-Hong Lu, Min Li and Omar M. A. El-Agnaf

Small Compounds Extracted From Chinese Herbal Medicine Inhibit The Toxicity and The Formation of Early and Late Aggregates of Alpha-Synuclein.

Mustafa Taleb Ardah, Salema Begum, Jia-Hong Lu, Min Li and Omar M. A. El-Agnaf

Presented in the international conference “Alpha-Synuclein in Parkinson’s disease and Related Neurodegenerative Diseases: From Mechanisms to Therapeutic Strategies, Dubai, UAE (March 01 – 03, 2013).

Screening for Inhibitors of Alpha-synuclein Aggregation and Toxicity as a Potential Novel Drug for Parkinson's Disease.

Mustafa Taleb Ardah, Omar M.A. El-Agnaf

Presented in “The 10th International Conference on Alzheimer’s and Parkinson’s Diseases AD/PD”. Barcelona, Spain, (March 9-13, 2011).

Baicalein Inhibits Alpha-Synuclein Agregation And Toxicity.

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