Dissertation Proposal

Title: Screening of alpha-synuclein aggregation inhibitors

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Abstract

Alpha-synuclein (α-syn), a predominantly neuronal protein, is localized to pre-synaptic terminal under physiological conditions. Although the nature of α-syn is not clearly known, the structural change of α-syn is related to the development of synucleinopathy including Parkinson's disease (PD). The ability of α-syn to generate a β-sheet structure under certain conditions results in the formation of amyloid-like fibrils with prolonged incubation in solution. This aggregated α-syn is the major component of Lewy body, which is the hallmark of PD. A soluble oligomer is synthesized as an intermediate α-syn, a proto-fibril. The neurotoxic oligomeric form of α-syn is soluble and detected via SDS/polyacrylamide gel electrophoresis. Finally, oligomers aggregate to their final fibrillar condition. Aggregated forms of α-syn are neurotoxic and therefore, inhibition of α-syn aggregation has been investigated as a promising disease-modifying approach for the treatment of PD, which is the most common synucleinopathy and the second most prevalent neurodegenerative disease after Alzheimer's disease (AD). However, structure-based drug design and discovery of novel molecules that can modulate α-syn aggregation have been hindered by the disordered nature of α-syn. Therefore, the analysis of large collections of chemically diverse molecules has been used to identify lead compounds. In this study, a screening strategy was used to identify the chemical compounds, which inhibit the aggregation of α-syn. Further, the ability of inhibitor candidates to disrupt the aggregated α-syn was examined.

AD is the most common neurodegenerative disorder caused by the aggregation of Tau and/or amyloid protein(s). It is already known that α-syn and Tau share striking characteristics suggesting a similar pathophysiological mechanism. It is suggested that α-syn and Tau interact to enhance mutual aggregation. Tau and α-syn are both partially unfolded proteins that form toxic oligomers and abnormal intracellular aggregates under pathological conditions. Therefore, we hypothesized that specific compounds, which inhibit Tau aggregation may have a capability to inhibit α-syn aggregation.

Using a pET-21b plasmid expressed in an *E.coli* pLysS strain, the α-syn protein tagged with N-

terminal-His was expressed and purified using ammonium sulfate precipitation. The time course of α-syn aggregation at various concentrations was analyzed based on thioflavin T (ThT) fluorescence intensity. Sixty-seven chemical compounds, which showed an inhibitory activity on Tau aggregation from a previous study, were screened for the possible candidates inhibiting α-syn aggregation. Five out of sixty- seven compounds were selected as candidate inhibitors after two rounds of screening based on their IC_{50} values. The cytotoxicity of five compounds was evaluated via monitoring the viability of SH-SY5Y cells using MTT assay.

The size of harvested and purified α-syn protein tagged with N-terminal-His was 14kDa. After two rounds of screening eight compounds were selected as candidate inhibitors, which showed less than 50% intensity of ThT fluorescence. Among the eight compounds, five were selected for further analysis. Four candidates displayed sigmoidal growth kinetics based on their IC_{50} values. The selected chemical compounds shared the polyphenol group as a common chemical structure. The final four candidate compounds also exhibited an ability to disrupt pre-existing α-syn aggregates.

This study suggested that the Tau aggregation inhibitor can also inhibit α-syn aggregation even though the identification of the chemical structure responsible for the inhibition of aggregation requires further studies. These novel inhibitors of α-syn aggregation represent promising therapeutic agents for patients with neurodegenerative disorders including PD.

Keywords

α-synuclein, Tau, Aggregation, Parkinson's Disease, Alzheimer's disease

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Introduction

1. Background

Parkinson's disease (PD) is the second most common neurodegenerative disorder following Alzheimer's disease (AD) and accounts for an overwhelming health burden on individuals and society (Weintraub et al., 2008). Until now, PD remains incurable and many drugs available commercially provide only palliative treatment option for symptoms associated with PD (Strecker, 2008). The etiology of PD is not fully understood, but appears to involve a complex combination of environmental and genetic factors (Braak et al, 2003). At the molecular level, alpha-synuclein (α-syn) protein misfolding, accumulation, aggregation and subsequent formation of amyloid deposits are common features in many PD (Lashuel et al., 2013). Alpha-syn, a small size protein of 140 amino acids, is encoded by the *SNCA* gene, although in aqueous solutions it does not have a defined structure (StefanisL, 2012). The native structure and function of α-syn are still poorly defined. The current consensus is that α-syn promotes membrane curvature, thereby contributing to synaptic trafficking and vesicle budding (Chandra et al., 2003; Varkey et al., 2010). Accordingly, it may be associated with presynaptic terminal SNARE complexes (Burre et al., 2010), with a potential role in modulation of dopamine release and regulation of dopamine-producing neurons in the substantia nigra.

Alpha-syn does not have a defined structure, hence is called as "natively unfolded protein". However, α-syn has the ability to generate β-sheets under specific circumstances and forms amyloid-like fibrils upon prolonged incubation in solution (Conway et al., 2000). It is well known that the aggregated α-syn is neurotoxic and the main component of Lewy bodies, which are the hallmark of PD (Spillantini et al., 1997 and Goedert M et al., 2013). Neurons in the substantia nigra and other regions in the brain develop deposits of pre-fibril oligomer and aggregated α-syn (amyloid-like) in PD (Volles and Lansbury, 2002). During the formation of α-synfibril an oligoemer is synthesized as a proto-fibril intermediate. The oligomeric form of α-syn is soluble, but neurotoxic. Finally, oligomers aggregate to form fibrils. The aggregated form of α-syn is neurotoxic and causes PD.

It is clear that α-syn represents a valid therapeutic target in PD. Possible therapeutic strategies include modulation of α-syn transcription, inhibition of pro-aggregating post-translational modification, enhanced degradation of α-syn protofibil, and inhibition of α-syn aggregation. Silencing point mutation of A53T in *SNCA* gene regulates the levels of α-syn (Si et al., 2017). Another therapeutic possibility involves enhancing degradation of α-syn by increasing the proteasomal or lysosomal activity. Among these strategies, inhibiting α-syn aggregation has been an attractive target for drug development. The key therapeutic strategy against PD is regulation of α-syn aggregation during the exponential phase (Jhaet al., 2016). One of the main limitations of this strategy is that monomeric α-syn cannot maintain a well-defined structure in solution, due to its intrinsically disordered nature, which hinders the design of rational inhibitors. Therefore, high-throughput screening protocols have been developed (Silva et al., 2011). Using high-throughput screening, a number of promising small molecules have been discovered, including anle138b (Levin et al., 2014), BIOD303 (Moree et al., 2015), fasudil (Tatenhorst et al., 2016), squalamine (Perni et al., 2017), or SynuClean-D (SCD) (Pujols et al., 2018).

PD and AD show clinically distinct symptoms. However, the high degree of co-morbidity between PD and AD suggests frequent overlap at the structural and molecular levels. At the molecular level, protein self-assembly, aggregation, accumulation and subsequent formation of amyloid deposits are common features in PD and AD (Irwin et al., 2013). This common mechanism suggests that PD and AD likely share a common trigger and the pathology is determined by the type of protein aggregate (α-syn or Tau) and the cellular localization (Moussad et al., 2014).

In addition, α-syn and Tau interact at the cellular level, and their pathological structures represent a template for further misfolding and mutual aggregation (Yan et al., 2020). They also share a number of interacting proteins indicating that network perturbations may contribute to conformational changes of α-syn and Tau (Vanderweyde et al., 2016). Further, the fibrillization and solubility of α-syn and Tau appears to affect each other *in vitro* and *in vivo*. However, the mechanism of this interaction and its effects on neurodegenerative processes are still not fully known and several scenarios are possible.

The overlap and numerous similarities between PD and AD suggest promising therapeutic strategies targeting common pathways of Tau and α-syn aggregation. Therefore, an inhibitor of Tau aggregation that also inhibits α-syn aggregation might be important.

2. Research Objectives

Using a pET-21b plasmid expressed in *E.coli* pLysS strain, we isolated α-syn protein and investigated the aggregation kinetics by performing time course experiments to determine the optimal concentrations of α-syn for aggregation. As the aggregation α-syn and Tau appear to converge and overlap, chemical compounds which have been known to inhibit Tau aggregation were screened to identify the inhibitors of α-syn aggregation. To elucidate the characteristics of α-syn aggregation inhibitor, the IC_{50} values were determined. In addition, the chemical structures of the screened inhibitors were analyzed by comparing to those of the molecules already demonstrated to inhibit α-syn aggregation. Furthermore, the ability of inhibitors to suppress cell death caused by the α-syn aggregate was investigated in SH-SY5Y cells.

In brief, the aim of this research was to screen chemical compounds that inhibit α-syn aggregation and to elucidate the inhibitory mechanism of compounds, which might facilitate the development of drugs against PD.

Materials and methods

1. α-syn purification

The α-syn sequence tagged with N-terminal-His was cloned into pET-21b plasmid. The α-syn protein was expressed in *E.coli* pLysS strain in LB media with ampicillin (0.1 mg/mL) at 37°C until A_{600} reached 0.8. The α-syn was then induced with 1 mM isopropyl-D-thiogalactopyranoside for 2 h at 37°C. The pLysS was then harvested at 6000 rpm for 10 min at 4°C. To maximize the yield of α-syn a non-chromatographic method was performed. Pellets were re-suspended in lysis buffer (50 m MTris, 10 mM EDTA, 150 mM NaCl). Cell lysates were then heated at 95°C for 10 min, and pelleted at the maximum speed for 5 min at 4°C. After removing the supernatant, 10% streptomycin sulfate (136 µl/mL) and glacial acetic acid (228 µl/mL) were added, and then pelleted once again at the maximum speed for 5 min at 4°C. The supernatant was removed and precipitated with 40% ammonium sulfate at 4°C at a 1:1 volume ratio for 30 min. The precipitated proteins were centrifuged and the pellet was washed with 1 mL ammonium sulfate. Pellet was re-suspended with 900 µL of 100 mM ammonium sulfate, followed by the addition of an equal amount of 100% ethanol to the mixture and precipitated once again. The final pellet was collected and re-suspended in 100 mM ammonium acetate and dialyzed overnight (ThermoFisher) in PBS at 4°C. The purified α-syn was analyzed with poly acrylamide gel electrophoresis and stained with Coomassie Brilliant Blue (CBB).

2. Alpha-syn aggregation and screening of chemical compounds inhibiting aggregation

To determine the aggregation ability, the harvested and purified α-syn was dissolved in sterile PBS to final concentrations of 10, 50, and 100 µM, followed by incubation in a constant shaker (FINEPCR,micro mixer) at 37°C for 48 h. The time course of α-syn aggregation was also determined by measuring the thioflavin T (ThT) fluorescence intensity every 12 h until 72 h. After successful evaluation of α-syn aggregation, the initial screening of chemical compounds that inhibited Tau protein aggregation *in vitro* was performed. During the initial screening, α-syn was mixed with PBS (diluted to a final concentration of 100 µM) and 10 µM of chemical compound library (ChemBridge

Corp). These mixtures were incubated in a 96-well plate (SPL) on a constant shaker at 37°C for 48 h. During the second screening, 100 μM of α-syn mixed with PBS was incubated with 10 μM of chemical compounds. The degree of α-syn aggregation was evaluated via ThT fluorescence assay as follows. ThT (5μM) was added to the α-syn itself and α-syn treated with chemical compounds. A multi-label plate reader (Envision, Perkin Elmer) was used to measure the fluorescence of the triplicate samples in a white 96-well plate (SPL) at an excitation wavelength of 440 nm and an emission wavelength of 485nm.

3. IC_{50} values of chemical compounds

The purified α-syn dissolved in sterile PBS to a final concentration of 100 μM was incubated with an increasing concentration gradient of chemical compounds as 0.01, 0.1, 1, 10, 100 μM on a constant shaker (FINEPCR, micro mixer) at 37°C for 48 h. The inhibitory effect was measured via ThT fluorescence intensity as described above. All ThT measurements were normalized by DMSO and each IC_{50} value was calculated.

4. Treatment of the α-syn aggregate with chemical compounds

Purified α-syn was dissolved in sterile PBS to a final concentration of 100 μM and incubated on a constant shaker (FINEPCR, micro mixer) at 37°C for 72 h. After 72 h, the chemical compounds (10 μM) which inhibited α-syn aggregation in the previous screening were incubated with α-syn for more 2 hours. The ThT fluorescence intensity was evaluated 30 min interval. The ThT fluorescence assay was performed as described above.

5. MTT reduction assay

The MTT assay was carried out in a neuroblastoma cell line SH-SY5Y cultured in DMEM with 10%

FBS, in a 5% CO_2 incubator at 37°C. SH-SY5Y cells were cultured at 10,000 cells/well in 48-well plates. The α-syn monomer (10 μM), aggregated α-syn (10 μM), and α-syn aggregate treated with chemical compounds (100 μM) selected after second screening were added to media in each well of the 48-well plates. DMSO was used as a control, followed by the addition of 30 μl of MTT solution (0.5 mg/mL) to each well and incubated for 1 hour.

The cytotoxicity of chemical compounds was also evaluated using MTT-assay. SH-SY5Y cells were treated along with the chemical compounds for 48 h. All measurements were normalized by DMSO.

Results

1. Purification of α-syn and kinetics of α-syn aggregation

His tagged α-syn was expressed in E. *coli* pLysS and purified using the "salting out" method. The cell lysate was heated due to the heat stable characteristics of α-syn before purification and any unstable bacterial proteins were eliminated (Pujols et al., 2017). An α-syn recombinant product expressed in *E. Coli* is a single, non-glycosylated polypeptide chain of 140 amino acids is known to have a molecular mass of 14kDa. The purified α-syn obtained by polyacrylamide gel electrophoresis and with CBB staining also measured 14kDa (Figure 1).

To validate aggregation of α-syn, kinetics experiments of α-syn performed. We changed the concentrations of α-syn to ensure the optimal aggregation concentration. The ThT fluorescence assay was used to determine the α-syn aggregation, The ThT fluorescence assay is a representative method for confirming amyloidosis by reflecting the β-sheet structure formed during aggregation (Younan and Viles, 2015). Low concentrations of α-syn (10 μM) did not show significant difference in fluorescence intensity. Intermediate concentration of α-syn (50 μM) showed less fluorescence intensity than 100 μM concentration of α-syn. After 24 h α-syn reached a plateau phase and the value of fluorescence intensity beyond 48 h showed no significant difference (Figure 2A). We selected 100 μM as the concentration of the α-syn based on time course aggregation. A similar sample of α-syn was also stained with CBB to determine the time course aggregation. After the 24-h period, dimers and tetramers of α-syn were detected suggesting oligomerization of α-syn.

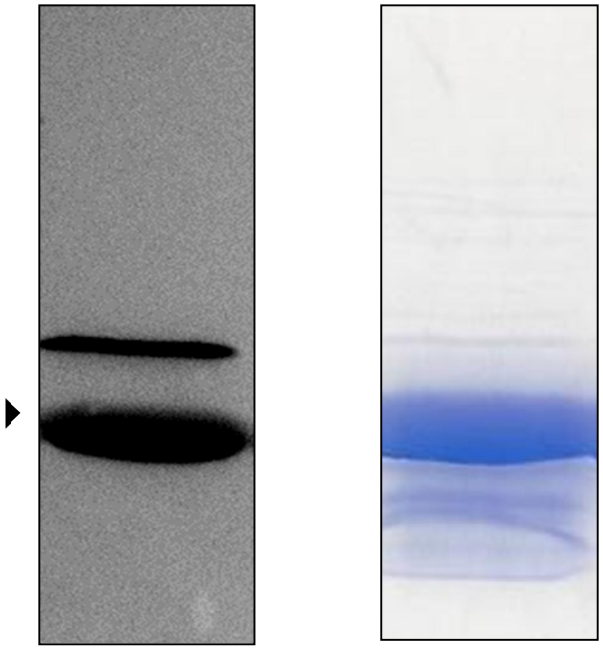
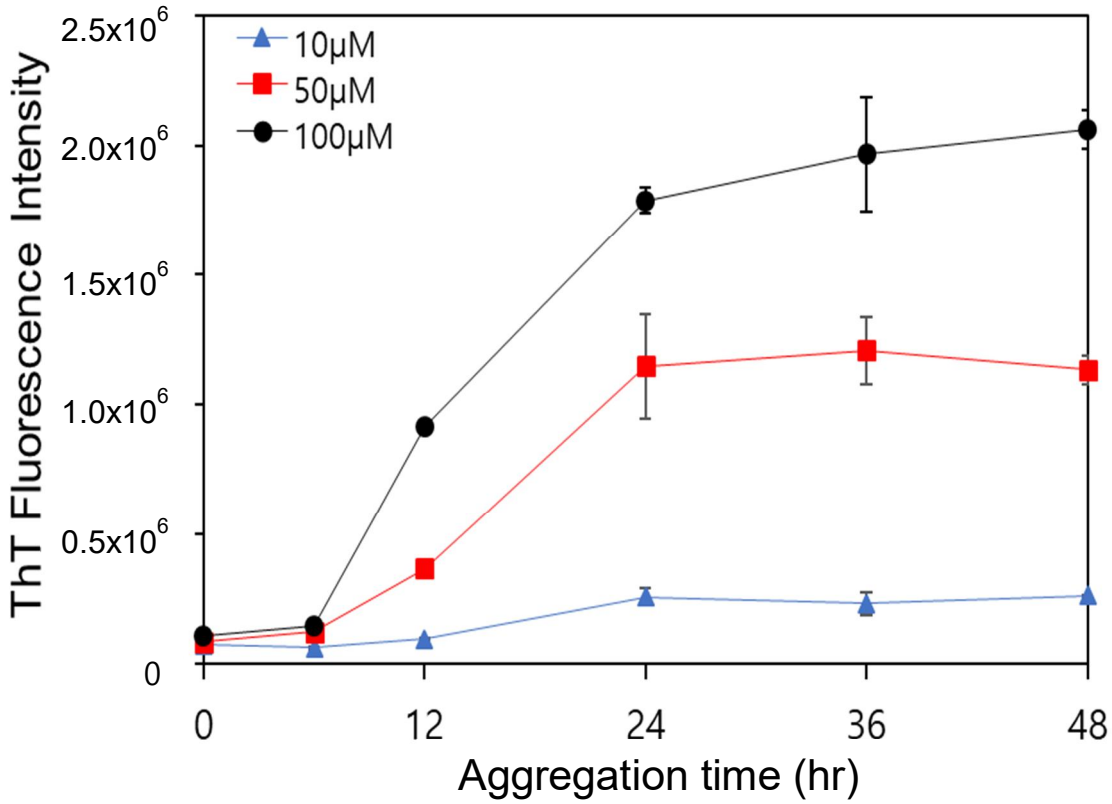

Anti-His
CBB

Figure 1. Expression and purification α-syn

The α-syn sequence tagged with N-terminal-His was cloned into pET-21b plasmid and expressed in *E. coli* pLysS cells and purified using ammonium sulfate precipitation. We used a non-chromatographic method to maximize the yield of α-syn. Immunoblot analysis using anti-His antibody (left) and CBB staining showed the molecular weight 14kDa (right).

(A)


2.5x10^6
2.0x10^6
1.5x10^6
1.0x10^6
0.5x10^6
0
ThT Fluorescence Intensity
10μM
50μM
100μM
0
12
24
36
48
Aggregation time (hr)

(B)

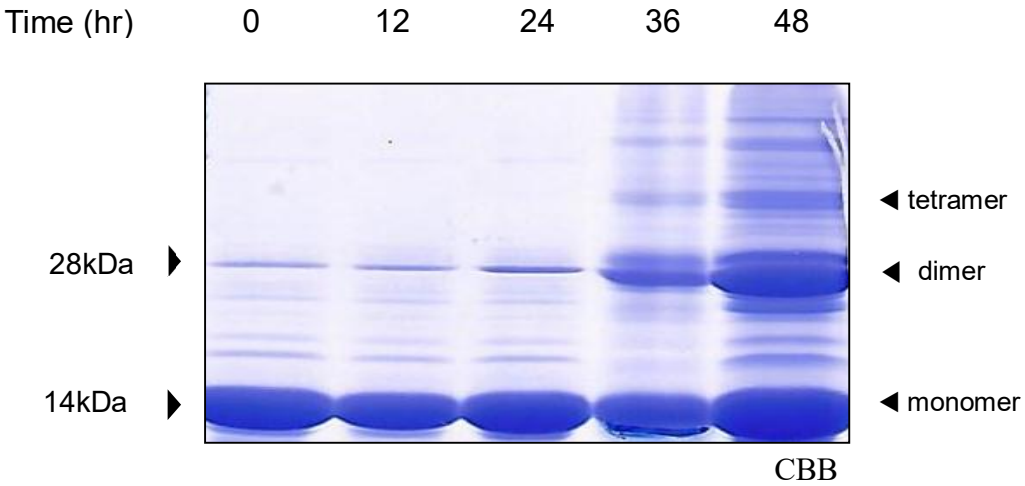

Time (hr)
0
12
24
36
48
28kDa
14kDa
tetramer
dimer
monomer
CBB

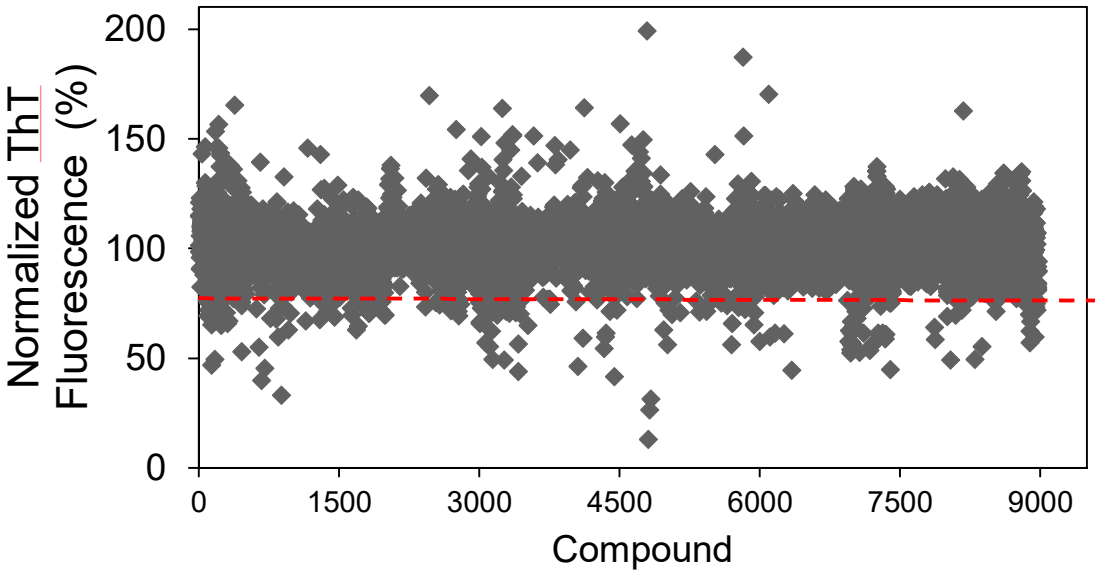
Figure 2. Time course of aggregation of α-syn at various concentrations

(A) To verify the optimal concentration for α-syn to aggregate, the purified α-syn was dissolved in sterile PBS to final concentrations of 10, 50, and 100 μM, followed by incubation. The time course of α-syn aggregation was determined per concentration of α-syn. The ThT fluorescence assay was used to evaluate the degree of the α-syn aggregation. (B) Optimal concentration of α-syn to aggregate was 100 μM. We ensured the aggregation of α-syn (100 μM) in 12-hour interval by CBB staining.

2. Screening inhibitor for α-syn aggregation

Sixty-seven chemical compounds, which showed an inhibitory activity on Tau aggregation from a previous study (70% cutoff), were screened for the possible candidates inhibiting α-syn aggregation using ThT fluorescence assay (Figure 3A). During the initial screening, eleven compounds were identified that showed less than 50% intensity of ThT fluorescence compared to α-syn with DMSO (Figure 3B). A secondary screening was carried out under more stringent conditions to exclude false and week positive with the eleven compounds selected in initial screening. Among eleven chemical compounds, eight (C1, C2, C3, C4, C5, C9, C10, and C11) were selected as they showed less than 50% (C1; 45%, C2; 45%, C3; 35%, C4; 32%, C5; 48%, C9; 43%, C10; 29%, and C11; 36%) intensity of ThT fluorescence compared to α-syn treated with DMSO. However, three chemical compounds (C3, C4, and C11) were excluded from further study because the compounds were no longer synthesized by the manufacturer (Figure 4).

(A)


Normalized ThT Fluorescence (%)
200
150
100
50
0
0
1500
3000
4500
6000
7500
9000
Compound

(B)

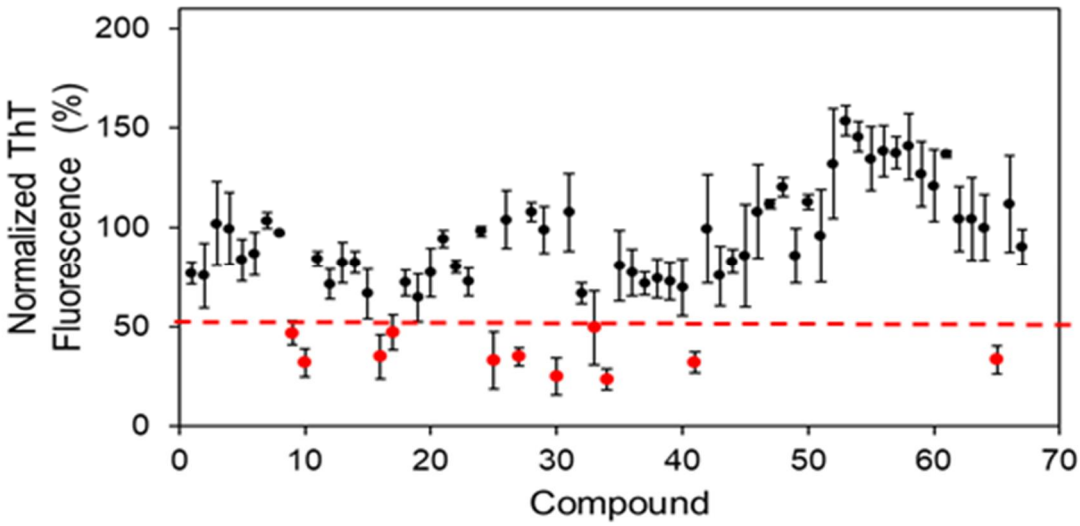

Normalized ThT Fluorescence (%)
200
150
100
50
0
0
10
20
30
40
50
60
70
Compound

Figure 3. Initial screening inhibitors for α-syn aggregation from Tau aggregation inhibitors

(A) Sixty-seven possible inhibitor candidates of α-syn (below the dotted line), which inhibited Tau aggregation during pre-screening were selected for initial screening inhibitor for α-syn aggregation. (B) Each compounds (10 μM) which inhibited Tau aggregation during pre-screening were incubated with α-syn (100 μM) for 48 hours. Alpha-syn aggregation was measured by ThT fluorescence assay. Each ThT fluorescence intensity was normalized by DMSO treated α-syn. Dotted line represented inhibition cutoff indicating 50%. Compounds with value less than 50% cutoff were used as targets of secondary screening.

* adapted from Choi JH., Park SH. (2018). A study on novel inhibitor of Tau aggregation and membrane binding of Tau aggregates. (Ph. D. thesis). Seoul National University, Seoul, Korea.

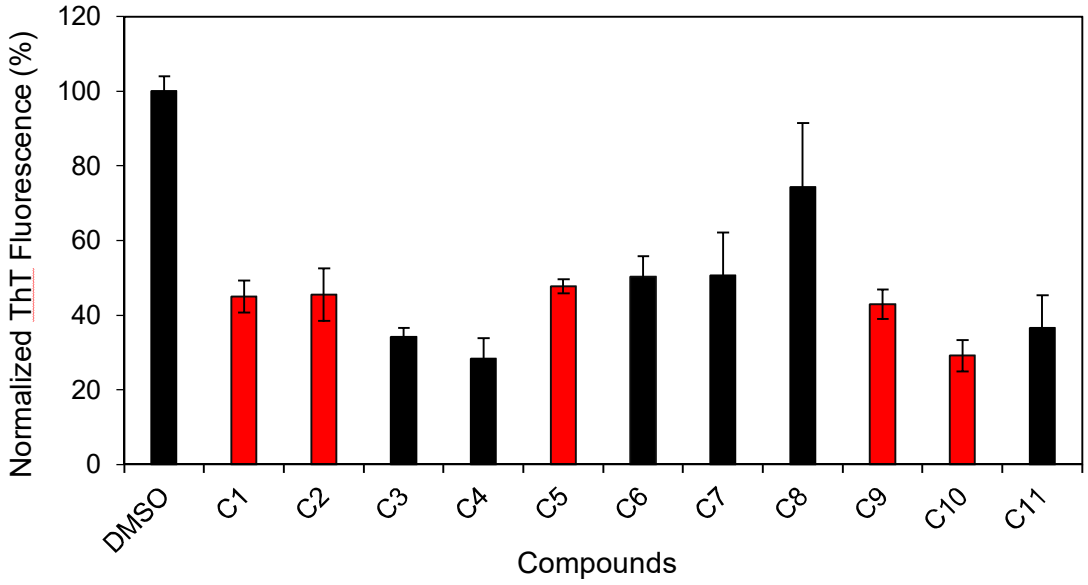

Normalized ThT Fluorescence (%)
120
100
80
60
40
20
0
DMSO
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
Compounds

Figure 4. Selected inhibitors for α-syn aggregation after secondary screening

A secondary screening was performed under more stringent conditions for eleven primary compounds. α -syn (100 μM) and eleven compounds (10 μM) were incubated and α-syn aggregation was analyzed by ThT fluorescence. Each ThT fluorescence intensity was normalized by DMSO treated α-syn. Among eleven, eight compounds showed less than 50% intensity of ThT fluorescence compared to DMSO treated α-syn. Compounds with inhibition lower than the cutoff were selected as the final compounds for the aggregation inhibitors. However, three chemical compounds were excluded from further study because the compounds were no longer available.

3. Characteristics of selected chemical compounds

3.1 Kinetics of inhibition of α-syn aggregation by selected chemical compounds

IC_{50} values of selected chemical compounds indicated that the inhibition of α-syn aggregation by these compounds. To validate this process, the purified α-syn dissolved in sterile PBS to a final concentration of 100 μM was incubated with an increasing concentration gradient of chemical compounds as 0.01, 0.1, 1, 10, 100 μM. ThT assay of four compounds displayed sigmoidal growth kinetics and the IC_{50} revealed 7.9～19.3 μM (C2, 7.9 μM; C5, 19.3 μM; C9, 17.1 μM and C10, 8.0 μM). However, C1 compound revealed undetectable IC_{50} (Figure 5). Four compounds showed the appropriate curve for α-syn aggregation kinetics and values of IC_{50}.

3.2 Disruption of α-syn aggregates by selected chemical compounds

It is known that some compounds disrupt aggregates as well as inhibit the aggregation of amyloid proteins (Ladiwala et al., 2010). Four selected chemical compounds were speculated that would also have such disruption effects. To validate this effect, chemical compounds were treated to the α-syn aggregate. The ThT fluorescence intensity abruptly decrease as 0.3~0.8 x 10^6 (C2, 0.3; C5, 0.3; C9, 0.8; and C10, 0.7 x 10^6) from 2.3 x 10^6 within 1 hour (Figure 6). Compounds efficiently disrupted α-syn aggregates with the disruption efficiency not being significant different from inhibition.

3.3 Selected chemical compounds reducing cytotoxicity of α-syn aggregates

α-syn aggregate is a cytotoxic protein and its cytotoxicity can be reduce by aggregation inhibition. To verify the salvage effect of selected compounds, the toxicity of α-syn aggregates with and without chemical compounds was tested using MTT assay in SH-SY5Y cells. The results of the MTT assay showed that in the presence of α-syn aggregates, the viability of SH-SY5Y cells was approximately 67% suggesting that aggregated α-syn was cytotoxic. However, when α-syn aggregates was pre-incubated with chemical compounds, the viability of SH-SY5Y cells was approximately 66～90%

(C2, 90%; C5, 66%; C9, 82%; and C10, 78%) (Figure 7). Selected compounds saved effectively the cells from aggregated α-syn toxicity except one (C5). The maximum cell viability was observed in case of C2 (cell viability 90%), indicating that this was the most effective compound reducing the toxicity of aggregated α-syn.

3.4 Cytotoxicity of selected chemical compounds

Extracellular aggregates such as α-syn, β-amyloid, and Tau displayed cytotoxicity by reducing mitochondrial activity and increasing ROS of cells (Kamp et al., 2010). The cellular response to selected chemical compounds was validated. The viability of SH-SY5Y cells with compounds was determined by the MTT assay. It showed that in the presence of α-syn monomer, the viability of SH-SY5Y cells was approximately 96%, whereas in the presence of α-syn aggregate, the viability was approximately 68%. SH-SY5Y cells incubated with only inhibitor candidates showed a cell viability of approximately 65～83% (C2, 83%; C5, 65%; C9, 80%; and C10, 80%) (Figure 8). Four selected chemical compounds showed less cytotoxicity than aggregated α-syn. However, C5 was similar to α-syn aggregate. Cytotoxicity of selected chemical compounds might influence on the reducing cytotoxicity effect of chemical compounds

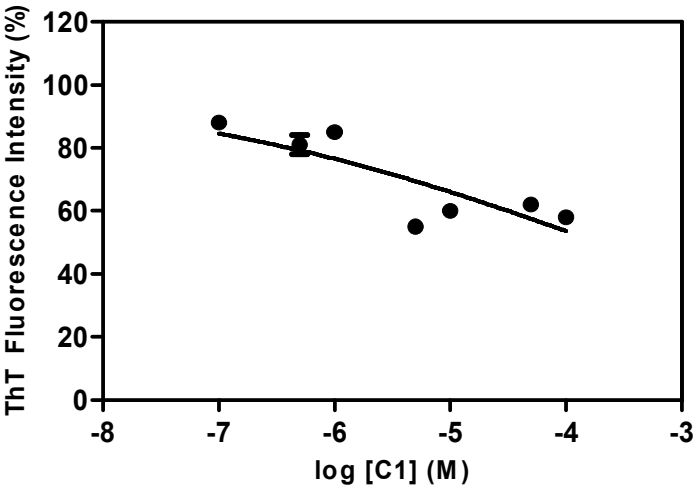
3.5 Chemical structure of selected chemical compounds

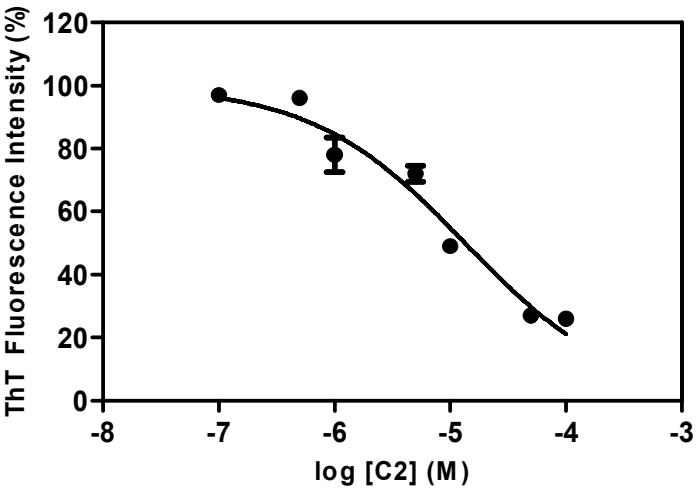
One of the main obstacles to design proper inhibitors of α-syn aggregation is that α-syn cannot maintain a well-defined structure in solution. Therefore, using high-throughput screening, a number of promising small molecules have been discovered. Among them, chemical compounds which have polyphenol have been known to exert neuro-protective effects by targeting multiple mechanisms including prevention of α-syn formation from the misfolded aggregates and other mechanism (Kujawska and Jodynis-Liebert, 2018). The chemical structures of the five inhibitors were analyzed by comparing to those of the molecules already demonstrated to inhibit α-syn aggregation. We

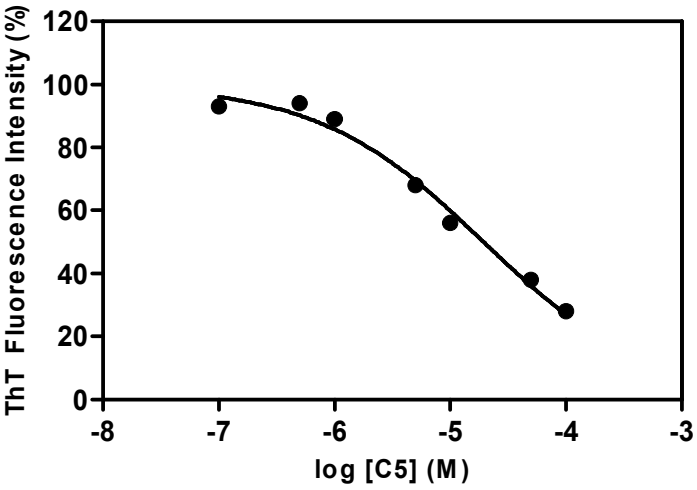
noticed that every inhibitor candidate shared polyphenol in the chemical structure, which suggested that these candidates prevent the formation of misfolded aggregates of α-syn due to the polyphenol structure.

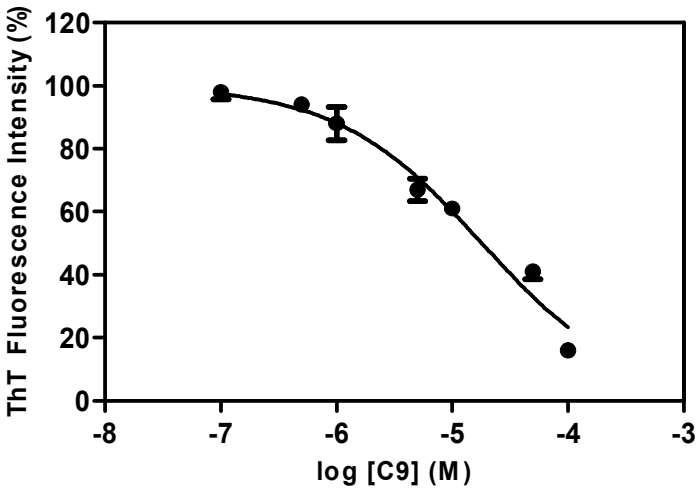
3.6 Summary of characteristics of candidate chemical compounds

In summary, among the five chemical compounds identified, the compound C1 was not considered eligible as a candidate inhibitor against α-syn aggregation as the IC_{50} was not available. The compound C5 showed low recovery percentage from α-syn aggregate toxicity in our experiment, but a dose adjustment is necessary due to higher toxicity. Based on this study, C2, C9, and C10 represent appropriate compounds for further investigation. C2 appears to be the most effective compound in reducing α-syn aggregate toxicity (Table 1).


ThT Fluorescence Intensity (%)
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
-3
log [C1] (M)


ThT Fluorescence Intensity (%)
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
-3
log [C2] (M)


ThT Fluorescence Intensity (%)
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
-3
log [C5] (M)


ThT Fluorescence Intensity (%)
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
-3
log [C9] (M)

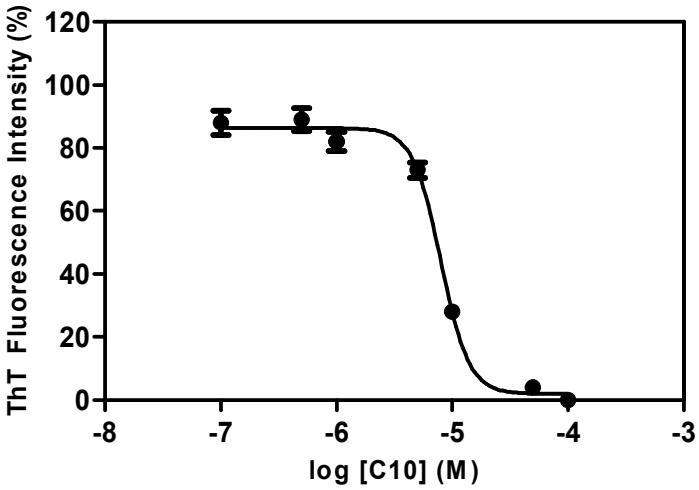
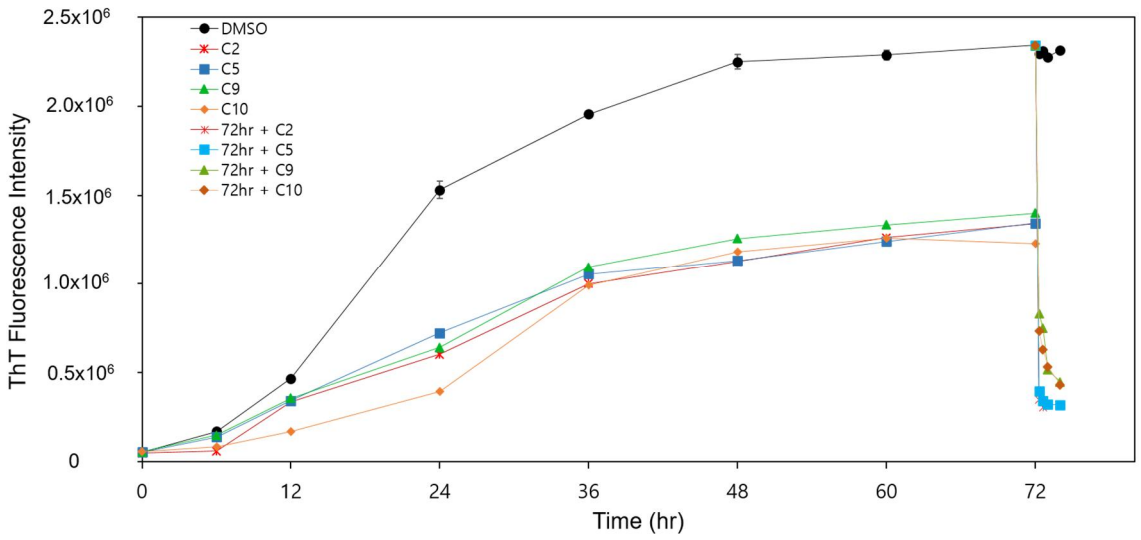

ThT Fluorescence Intensity (%)
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
-3
log [C10] (M)

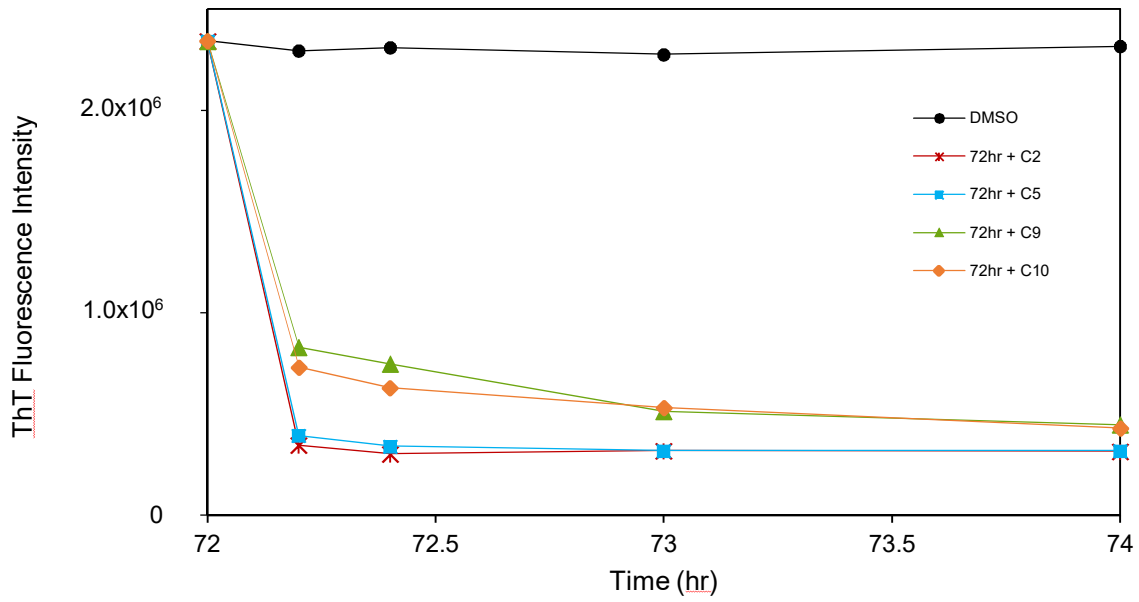
Figure 5. Determination of IC_{50} values of selected chemical compounds

α-syn (100 μM) were incubated with various concentration of selected chemical compounds (0.01, 0.1, 1, 10, 100 μM) and the inhibition effect was measured by ThT fluorescence intensity. All ThT measurements were normalized by DMSO.

(A)


2.5x10⁶
2.0x10⁶
1.5x10⁶
1.0x10⁶
0.5x10⁶
0
ThT Fluorescence Intensity
DMSO
C2
C5
C9
C10
72hr + C2
72hr + C5
72hr + C9
72hr + C10
0
12
24
36
48
60
72
Time (hr)

(B)


2.0x10⁶
1.0x10⁶
0
ThT Fluorescence Intensity
DMSO
72hr + C2
72hr + C5
72hr + C9
72hr + C10
72
72.5
73
73.5
74
Time (hr)

(C)

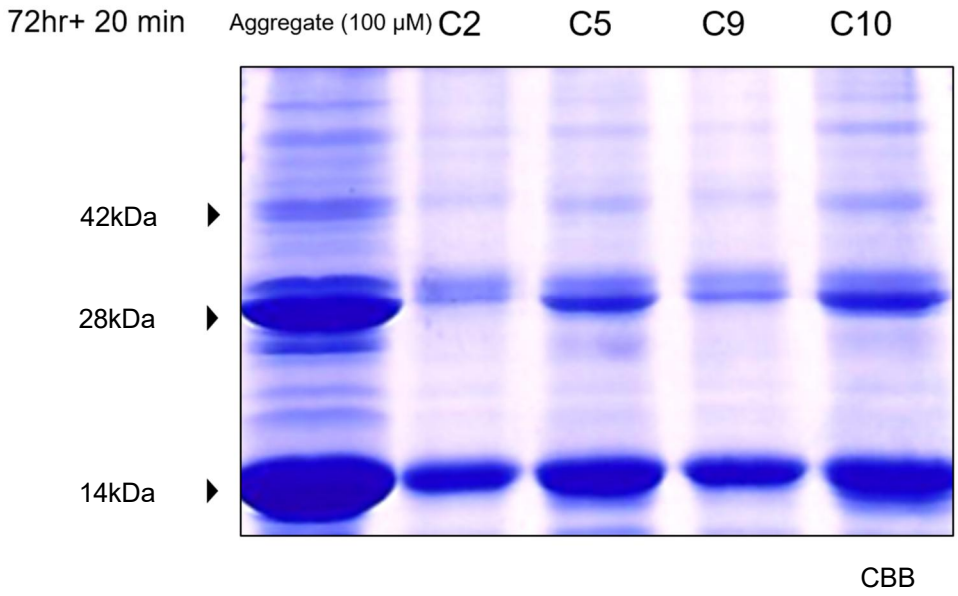

72hr+ 20 min
Aggregate (100 μM)
C2
C5
C9
C10
42kDa
28kDa
14kDa
CBB

Figure 6. Disruption of pre-aggregated α-syn by selected chemical compounds

(A), (B) Purified α-syn was dissolved in sterile PBS to a final concentration of 100 μM and incubated for 72 h. After 72 h, the chemical compounds (10 μM) which inhibited α-syn aggregation in the previous screening were incubated with α-syn aggregates for additional 2 hours. The ThT fluorescence intensity was evaluated 30 min interval. Measured fluorescence intensity was normalized by DMSO treated measured α-syn. (C) The pre-existing α-syn aggregates were disrupted by adding compounds in CBB staining gel.

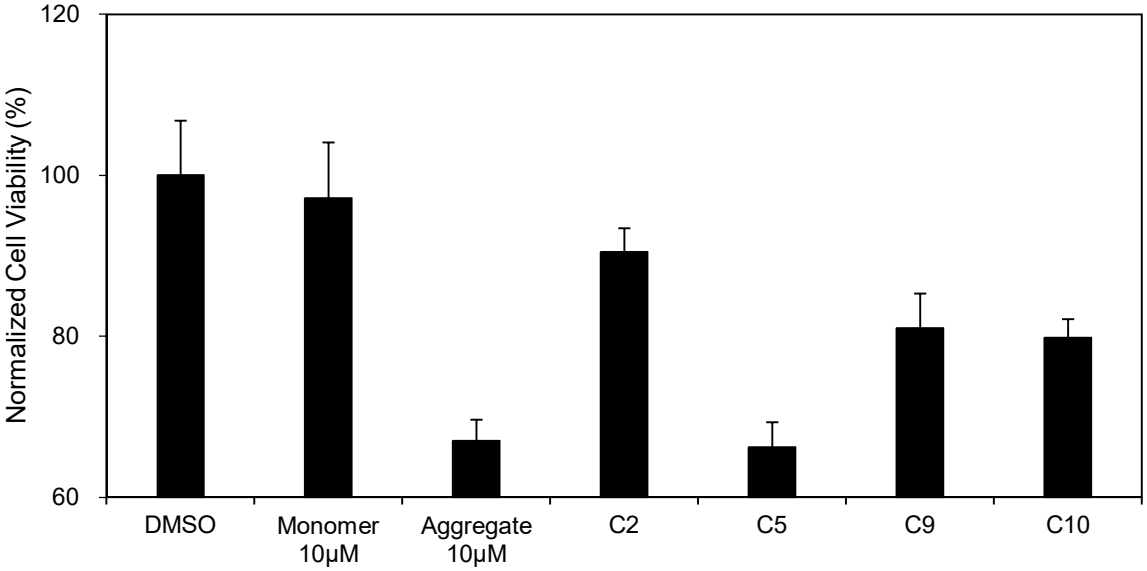

120
100
80
60
Normalized Cell Viability (%)
DMSO
Monomer 10μM
Aggregate 10μM
C2
C5
C9
C10

Figure 7. Cell viability of selected chemical compounds

Cell viability assay by MTT reduction with SH-SY5Y cells cultured in DMEN with 10% FBS was performed. The α-syn monomer (10 μM), α-syn aggregate (10 μM), and α-syn aggregates treated with chemical compounds (10 μM) selected after second screening were added to media and cultured for 48 hours. Then added 30 μl of MTT solution (0.5 mg/mL) to each well and incubated for 1 hour and cell viability was measured.

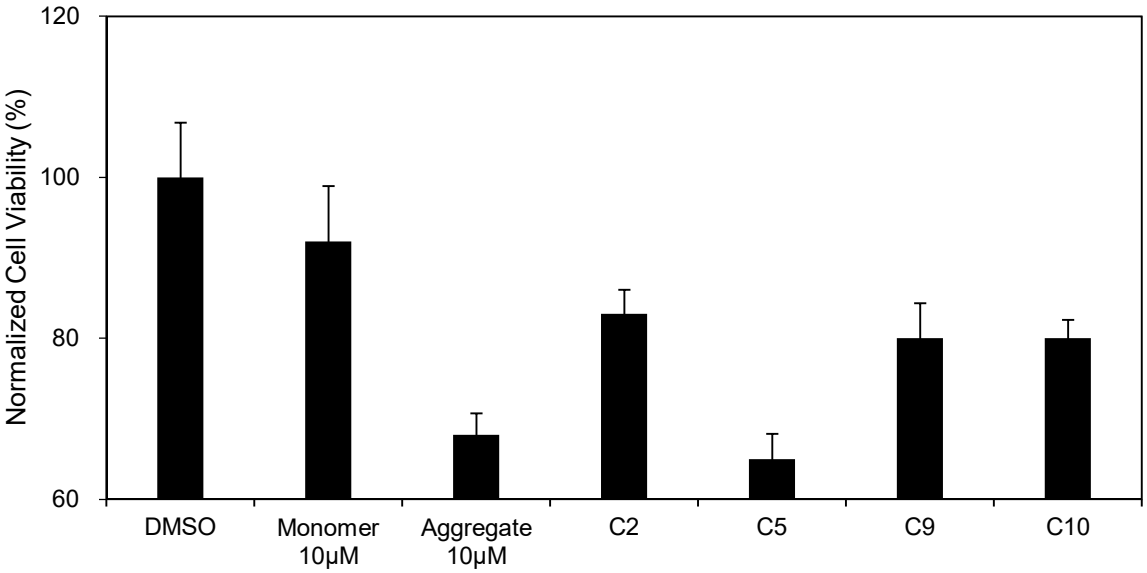

120
100
80
60
Normalized Cell Viability (%)
DMSO
Monomer 10μM
Aggregate 10μM
C2
C5
C9
C10

Figure 8. Cytotoxicity of selected chemical compounds

The α-syn (10 μM), and chemical compounds (100 μM) selected after second screening were added to media containing SH-SY5Y cells in each well of the 48-well plates. Treated SH-SY5Y cells were cultured in DMEM with 10% FBS at 10,000 cells/well in 48-well plates for 48 hours. Then added 30 μl of MTT solution (0.5 mg/mL) to each well and incubated for 1 hour. Using DMSO as control cell viability was measured.

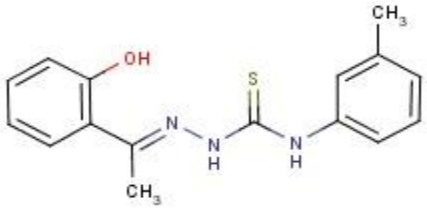
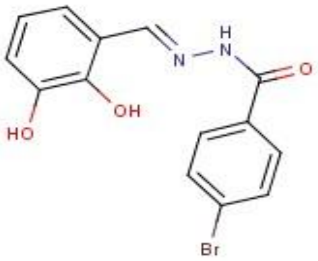
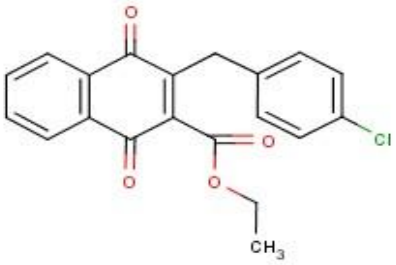
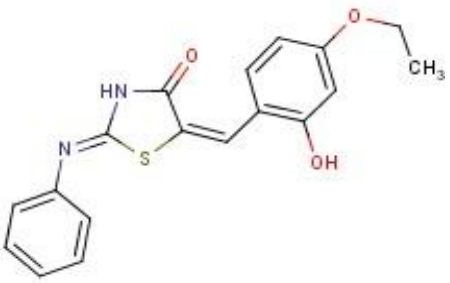
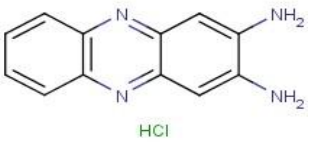
Candidate inhibitor of α-syn aggregation	Chemical Structure
C1	
C2	
C5	
C9	
C10	

Figure 9. Chemical structure of candidate inhibitors of α-syn aggregation

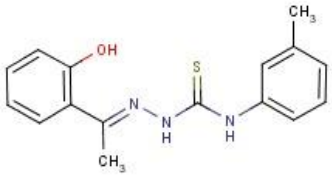
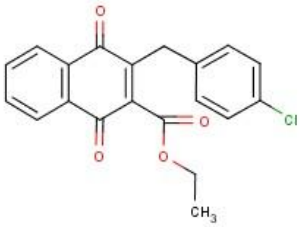
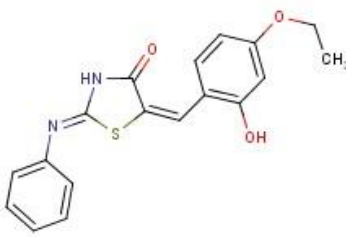
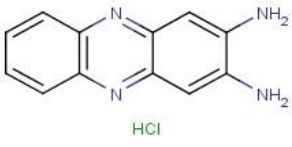
Chemical compound	Normalized ThT fluorescence (%)	IC_{50} (µM)	Recovery percent of cell viability (%)	Disruption of aggregated α-syn from $2.3x10^6$ ($x 10^6$)	Cytotoxicity by own toxicity (%)	Chemical structure
C1	45	ND	ND	ND	ND	
C2	45	7.9	90	0.3	17	
C5	48	19.3	66	0.3	35	
C9	43	17.1	82	0.8	20	
C10	29	8.0	78	0.7	20	

Table 1. Summary of chemical compounds which inhibit α-syn aggregation

Discussion

PD, which accounts for approximately 15% of all dementia cases, is the second most prevalent neurodegenerative disorder after AD (Dauer and Przedborski, 2003). The cause of PD is largely attributed to the death of dopaminergic neurons in the substantia nigra pars compacta, located in the basal ganglia of the brain (Meade et al., 2019). However, the underlying cause of this overwhelming disease is not clear and the development of therapeutic agents that prevent or cure PD is not satisfactory. At the molecular level, α-syn is suspected to mediate the pathogenesis of PD. The main functions of α-syn are still not clearly known, but are apparently associated with the control of dopamine release, the neurotransmitter that is critical in PD (Tsika et al., 2010). In addition, despite considerable effort, the precise native structure of α-syn is not fully known. α -syn is an intrinsically disordered protein and forms amyloid-like fibrils via aggregation upon prolonged incubation in solution (Conway et al., 2000). The aggregated α-syn, especially oligomeric form, is neurotoxic and contributes to PD pathology (Bengoa-Vergniory et al., 2017; Winner et al., 2011). As a possible therapeutic strategy, inhibiting and reducing α-syn aggregation has been an attractive target for drug development. Meanwhile, a similar pattern of aggregation and suspected interaction between α-syn and Tau (neurodegenerative protein that causes AD) has been reported. Based on these findings, it was hypothesized that a chemical compound that inhibits Tau aggregation could also inhibit α-syn.

We screened the chemical compounds that act against both Tau and α-syn using chemical compounds with confirmed potential for Tau aggregation in previous studies in our laboratory (Choi, 2018). In this study, we successfully obtained α-syn with a molecular weight of 14KDa using recombinant bacterial expression protocols. It aggregated spontaneously after 24 h of incubation at a concentration of 100 μM, which might be attributed to the product of recombinant bacterial expression. It was reported that α-syn synthesized using recombinant bacterial expression protocols *in vitro* and via over-expression, sample heating and/or denaturing gels in cell culture and tissue studies aggregate upon prolonged incubation in solution. In contrast, endogenous α-syn isolated and analyzed

under non-denaturing conditions from neuronal and non-neuronal cell lines, brain tissues and living human cells occurs largely as a folded tetramer of ～58 kDa. Whereas recombinantly expressed monomers readily aggregated into amyloid-like fibrils *in vitro*, native human tetramers underwent little or no amyloid-like aggregation (Barteles et al., 2011). Following the initial screening of sixty-seven compounds inhibiting Tau aggregation, eleven were primarily selected based on the 50% or less reduction in ThT intensity of α-syn. After the secondary screening, five compounds were selected. The IC_{50} value of four candidates measured by ThT assay showed a satisfactory curve. We noticed that every inhibitor candidate shared polyphenol in the chemical structure. Polyphenol exerts neuro-protective effects by targeting multiple mechanisms including prevention of α-synformation from the misfolded aggregates and reduced mitochondrial dysfunction-induced oxidative stress and inflammatory responses (Kujawska and Jodynis-Liebert, 2018). This finding suggested that these candidates prevent the formation of misfolded aggregates of α-syn due to the polyphenol structure. Selected chemical compounds showed a SH-SY5Y cell recovery of 66～90% from aggregated α-syn cytotoxicity. Incubation with SH-SY5Y cells leads to the death of 17～35% cells. These data indicated that the candidates' toxicity influenced the efficiency of reducing the cytotoxicity by α-syn aggregate.

α-syn aggregates are composed of protein fibrils with a dominant β-sheet structure. They are water-insoluble, and mediate the pathogenesis of PD. Accordingly, the design and development of agents for PD should focus on the suppression as well as dissolution of such aggregates. Some trials have been performed to identify agents with potential ability to dissolve amyloid deposits (Takekiyo and Yoshimura, 2018). In this study, four chemical compounds disrupted already aggregated α-syn. Although further studies are needed, these compounds could be used to disassemble water-insoluble α-syn fibrils with a dominant β-sheet structure.

In conclusion, among the sixty-seven chemical compounds, which showed an inhibitory activity on Tau aggregation from previous study, three candidates were identified. This study demonstrated that three candidates sufficiently prevented α-syn from aggregation, on the other hand could disrupt the α-

syn aggregate. These also rescued cells from the cytotoxicity of α-syn aggregate. Further, the polyphenol structure of the inhibitors may lead to the synthesis of compounds with enhanced therapeutic effect. Based on the final inhibitors additional *in vivo* experiments using animal model are needed, followed by studies involving patients diagnosed with PD to corroborate the effect of these compounds.

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국문 초록

알파시뉴클린은 140개의 아미노산으로 이루어진 특정한 구조를 갖고 있지 않는 단백질로 신경전 말단에 다량이 밀집되어 분포한다. 이 단백질은 정상 상태에서는 단량제로 존재하지만 병리적인 환경에서는 단량체들 간에 응집이 일어나 섬유화가 되어 아밀로이드 양상의 섬유소가 형성된다. 이렇게 만들어진 알파시뉴클린 응집체는 파킨스병의 특징적인 병리학적 소견인 루이 소체의 주성분을 이룬다. 알파시뉴클린은 신경전달물질인 도파민의 분비에 관여하는 것으로 여겨지고 있으며 이 물질이 응집되어 섬유화되어 아밀로이드 양상을 띄게 되면 도파민 분비에 장해가 초래되어 파킨스병이 발병하는 것으로 추측하고 있지만 그 정확한 기전에 대해서는 이해가 부족한 실정이다. 알파시뉴클은 생리적인 상태에서 분리하면 접힌 나선 사성체를 이루며 쉽게 응집이 되지 않으나 재조합 세균 표현 방법으로 얻어진 알파시뉴클린은 장시간 배양을 하면 응집이 되는 것으로 알려져 있다. 본 연구에서도 재조합 세균 표현 방법으로 재조합 세균 표현 방법으로 얻어진 알파시뉴클린을 수획할 수 있었는데 24시간 이상 배양 시 응집체를 형성하였다.

한편, 퇴행성신경질환 중 가장 흔한 알츠하이머병은 파킨슨병과 임상양상이 겹치는 부분이 많고 알츠하이머병의 원인 물질로 여겨지는 타우 단백질도 알파시뉴클린과 마찬가지로 응집체를 형성하고 섬유화가 되어 발병하는 것으로 알려져 있다. 또한 타우 단백질과 알파시뉴클린은 체내에서 상호작용을 하여 서로 응집되는 과정에 영향을 미치는 것으로 알려져

있다. 본 연구는 타우의 응집을 방해하는 화학복합체가 알파시뉴클린의 응집도 방해할 수 있을 것이라는 가설 하에 시행되었다. 타우의 응집을 방해하는 화학복합체 67개를 두 번의 선별시험을 통해 알파시뉴클린의 응집도 방해하는 5 종류의 화학복합체를 찾을 수 있었다. 이들 물질 중 3 종류는 IC_{50} 수치를 얻을 수 있고 자체 독성도 낮았다. 한편, 응집 알파시뉴클린의 세포 독성 효과도 감소시킬 수 있었다. 또한, 이미 형성된 응집 알파시뉴클린을 분해할 수 있었다. 이들 물질은 폴리페놀 화학구조를 모두 갖고 있었는데, 이들은 알파시뉴클린의 응집을 방해하는 천연물질에 포함되어 있는 것으로 알려져 있다. 이들 3 종류의 화학복합체는 알파시큐클린의 응고를 방해하는 기전을 통해 앞으로 파킨스병 치료제로 개발될 수 있기를 기대한다. 한편, 타우 단백질의 응집을 방해하는 화학복합체가 알파시뉴클린의 응집도 방해할 수 있다는 것을 확인했다.

Keywords: 알파시뉴클린, 타우, 응집, 파킨슨병, 알츠하이머병