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Polyphenols in combination with β-cyclodextrin can inhibit and disaggregate α-synuclein amyloids under cell mimicking conditions: A promising therapeutic alternative

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ABSTRACT

Parkinson's disease is characterized by the presence of insoluble and neurotoxic aggregates (amyloid fibrils) of an intrinsically disordered protein α-synuclein. In this study we have examined the effects of four naturally occurring polyphenols in combination with β-cyclodextrin (β-CD) on the aggregation of α-synuclein in the presence of macromolecular crowding agents. Our results reveal that even at sub-stoichiometric concentrations of the individual components, the polyphenol–β-CD combination(s) not only inhibited the aggregation of the proteins but was also effective in disaggregating preformed fibrils. Curcumin was found to be the most efficient, followed by baicalein with (−)-epigallocatechin gallate and resveratrol coming in next, the latter two exhibiting very similar effects. Our results suggest that the efficiency of curcumin results from a balanced composition of the phenolic —OH groups, benzene rings and flexibility. The latter ensures proper positioning of the functional groups to maximize the underlying interactions with both the monomeric form of α-synuclein and its aggregates. The uniqueness of β-CD was reinforced by the observation that none of the other cyclodextrin variants [α-CD and HP-β-CD] used was as effective, in spite of these possessing better water solubility. Moreover, the fact that the combinations remained effective under conditions of macromolecular crowding suggests that these have the potential to be developed into viable drug compositions in the near future. MTT assays on cell viability independently confirmed this hypothesis wherein these combinations (and the polyphenols alone too) appreciably impeded the toxicity of the prefibrillar α-synuclein aggregates on the mouse neuroblastoma cell lines (N2a cells).

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1. Introduction

A number of human pathological diseases such as Alzheimer's disease, type 2 diabetes, Parkinson's disease, Amyotrophic lateral sclerosis (ALS), the prion diseases, and Huntington disease arise from the unwanted misfolding, oligomerization and aggregation of proteins [1]. Most of these diseases are characterized by the deposition of amyloid fibrils in body tissues. Among them Parkinson's disease is one of the most common diseases with 7–10 million people worldwide suffering from it. Parkinson's disease and dementia (due to synucleinopathies) are characterized by the presence of macroscopic amyloid deposits of the protein α-synuclein known as Lewy neuritis and Lewy bodies in brain tissues [2].

α-Synuclein is copiously present in human brain tissues and also in some other tissues such as red blood cells [2]. It is an intrinsically disordered protein, composed of 140 amino acid residues that can be structurally divided into three distinct segments (Fig. S1, Supporting Information) namely: (a) N-terminal amphipathic segment (1–60 amino acid residues), (b) a hydrophobic central region (61–95 residues) and (c) C-terminal acidic region (96–140 residues) [3]. The N-terminal region shares homology with lipoproteins (with amphipathic α-helices) and contains a characteristic consensus hexameric sequence (KTKEGV) which is repeated about four times. The central hydrophobic region is known to be responsible for the aggregation of α-synuclein, without which the protein loses its propensity to form amyloids. The carboxy terminus region is highly acidic in nature and has been found to interfere in the formation of aggregates due to its role as an

Abbreviations: β-CD, β-cyclodextrin; CR, congo red; EGCG, (−)-epigallocatechin gallate; HP-β-CD, (2-Hydroxypropyl)-β-cyclodextrin; IPTG, isopropyl β-D-thiogalactopyranoside; SEC, size exclusion chromatography; TEM, transmission electron microscopy; ThT, Thioflavin T.

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intramolecular chaperone [4]. Three tyrosine residues, which are highly conserved, are also present in this C-terminal region. *In vivo*, higher expression level of α-synuclein, increases its tendency to aggregate [5]. The actual function of this protein is ambiguous with its role in various cellular activities having been reported, that includes interaction with cell membranes/lipids, release of neurotransmitters and maintenance, storage and transport of vesicles across cells [2,6]. In presence of alcohols, α-synuclein is known to form ordered structures such as α-helix and β-sheet with low concentrations of ethyl alcohol enhancing its aggregation [7]. Soluble oligomers apart from fibrils of α-synuclein have also been implicated in neurotoxicity during Parkinson's disease [2].

Numerous small molecule inhibitors (e.g. flavonoids, dopamine, carbohydrates, antibiotics, synthetic chemical compounds, natural plant products etc.) of amyloid formation by α-synuclein have been reported in literature [8,9]. Polyphenols also constitute an important class of molecules that have been shown to inhibit and in some cases even disaggregate amyloids formed by α-synuclein [10–20] and other proteins [21,22]. Moreover, polyphenols are known to have anti-cancer, anti-oxidative and anti-microbial properties thereby providing several additional medicinal benefits. They are also reported to prevent cardiovascular diseases and control cell death [23]. Recent studies on the mechanism of inhibition and disaggregation of α-synuclein amyloids by polyphenolic compounds have shown that the presence of two or more 'vicinal polyhydroxyphenyl groups' is more important rather than the total number of hydroxyl groups [10,13]. Molecular dynamics studies have also pointed towards the role of these —OH groups in inhibition and disaggregation of amyloids [21,24]. However, curcumin, a naturally occurring polyphenol derived from turmeric (*Curcuma longa*), is a unique molecule with only monohydroxyphenyl rings but still being a highly potent aggregation inhibitor [25–28]. It has been hypothesized that curcumin binds to the aliphatic residues present in the middle hydrophobic stretch of native monomeric α-synuclein [29]. After this initial interaction, the aromatic rings of curcumin bind to nearby hydrophobic residues of α-synuclein. The same study proposed that curcumin strongly prevents the formation of oligomers and fibrils of α-synuclein by accelerating the intramolecular diffusion of the protein. This leads to an increase in the reconfiguration rate of α-synuclein thereby limiting the exposure time of the hydrophobic patches. Another study has shown that curcumin reduces the toxicity of α-synuclein by binding to it and minimizing the availability of hydrophobic surface [11].

A recent study from our group has demonstrated that curcumin in combination with β-cyclodextrin (β-CD) not only inhibited but also disaggregated α-synuclein amyloids *in vitro* [30]. We hypothesized that curcumin, with its hydrophobic region and —OH groups, interacts with the hydrophobic middle region and hydrophilic residues of α-synuclein, respectively. β-CD then further helps by binding to aromatic residues such as phenylalanine present in α-synuclein. β-CD is a cyclic polysaccharide with seven glucose units. It is a bucket shaped molecule with a hydrophobic internal cavity and hydrophilic exterior [31]. β-CD itself is a biologically active molecule and has been used in various applications such as protein folding as a pseudochaperone [32], catalysis [33], drug delivery [34], lowering of cholesterol [35] and inhibiting protein aggregation [36], to name a few. β-CD interacts with polyphenols and helps in overcoming their limitations such as low bioavailability, insolubility, instability [37] and also increase their activity, efficiency [30], anti-inflammatory and anti-proliferative properties [38].

Polyphenolic compounds are not always effective *in vivo* due to various reasons [39]. One of the major factors that add to the intracellular complexity is the congested cellular interior. The intracellular environment is highly crowded with the concentration of macromolecules ranging from 50 g/L to as high as 400 g/L [40]. Such an environment has been shown to affect protein folding, structure, function, aggregation and other thermodynamic properties primarily via the excluded volume effect [41]. Hence, the experiments carried out in dilute buffer solutions may or may not always provide a reliable picture of the *in vivo* environment. To address this issue, researchers have used both synthetic and protein based crowders in an attempt to mimic the physiological milieu [40,41]. In the current study, we have tried to extend the use of the combination of polyphenols and β-CD to such cell mimicking conditions. Based on our observations, we have also attempted to provide molecular insights into the underlying mechanism and role of —OH groups present in these polyphenols with regards to their ability to modulate protein aggregation. We have used four different naturally occurring polyphenols namely, resveratrol (from red grapes), baicalein (from the Chinese herbal medicinal plant *Scutellaria baicalensis*) and (−)-epigallocatechin gallate (EGCG) (from green tea) along with curcumin which is obtained from turmeric (*C. longa*) (Fig. 1). The macromolecular crowding agents used in this study are: Dextran 6, Dextran 40 and Ficoll 70 with an average molecular weight of 6 kDa, 40 kDa and 70 kDa, respectively. Our results reveal that these polyphenols in an optimized combination with β-CD not only precluded formation of aggregates but also disaggregated amyloids formed by α-synuclein with varying efficiencies in the presence of the macromolecular crowding agents. Moreover, the pronounced toxicities displayed by the α-synuclein aggregates were significantly diminished when the cells were treated with these polyphenol–β-CD combinations. Taken together our data suggest that these synergistic combinations may facilitate the long awaited expedition of such polyphenols to drug molecules, though views against the same have been put forward recently [42].

2. Materials and methods

2.1. Materials

Curcumin, resveratrol, baicalein, EGCG, thioflavin T (ThT) and congo red (CR) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Ethanol was procured from Merck (Mumbai, India). Ampicillin, LB medium for bacterial growth and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Himedia chemicals (Mumbai, India). All other chemicals used were of highest purity available/analytical grade.

2.2. Expression, isolation and purification of human α-synuclein

Human α-synuclein was expressed, isolated and purified with a similar process as described earlier [30,43]. Plasmid pT7-7 containing the gene for human α-synuclein was first transformed into *E. coli* BL21 (DE3) cells using calcium chloride. Then a single colony was picked and inoculated into 100 mL LB medium enriched with 100 mg/mL ampicillin. The culture was incubated at 37 °C and the absorbance at 600 nm was recorded at regular intervals. Induction was carried out by adding IPTG (1 mM final concentrations) when absorbance reached 0.7 to 1.0. The cells were harvested and resuspended in 0.75 mL of buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 150 mM NaCl) and frozen at −80 °C. Tubes with frozen cells were placed in a boiling water bath for 7 min and the supernatant was then collected after centrifugation at maximum speed for 5 min. Streptomycin sulfate (136 μL/mL of 10% solution per mL of supernatant) and glacial acetic acid (228 μL/mL of supernatant) were added and centrifuged for 2 min. Again the supernatant was recovered and precipitated with ammonium sulfate (saturated ammonium sulfate at 4 °C was used 1:1, v/v, with supernatant). The protein was collected as a precipitate by centrifugation and washed once with 1 mL of ammonium sulfate solution (4 °C, 1:1, v/v, saturated ammonium sulfate and water). The washed pellet was resuspended in 900 μL of 100 mM ammonium acetate (to form a cloudy solution) and precipitated by adding an equal volume of ethanol at room temperature. Precipitation with ethanol was repeated once more. The pellet was resuspended in 100 mM ammonium acetate and extensively dialyzed against 10 mM Tris-HCl buffer, pH 7.4.

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