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Polyphenolic compounds are novel protective agents against lipid membrane damage by α-synuclein aggregates *in vitro*

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ABSTRACT

Cumulative evidence now suggests that the abnormal aggregation of the protein α-synuclein (αS) is a critical factor in triggering neurodegeneration in Parkinson's disease (PD). In particular, a fundamental pathogenetic mechanism appears to involve targeting of neuronal membranes by soluble oligomeric intermediates of αS, leading to their disruption or permeabilisation. Therefore, a model assay was developed in which fluorophore-loaded unilamellar vesicles were permeabilised by soluble oligomers, the latter formed by aggregation of human recombinant αS protein. The αS oligomers induced an impairment of membrane integrity similar to that of the pore-forming bacterial peptide gramicidin. The lipid vesicle permeabilisation assay was then utilised to screen 11 natural polyphenolic compounds, 8 synthetic N′-benzylidene-benzohydrazide compounds and black tea extract for protection against membrane damage by wild-type and mutant (A30P, A53T) synuclein aggregates. A select group of potent inhibitory compounds included apigenin, baicalein, morin, nordihydroguaiaretic acid, and black tea extract. Structure–activity analysis further suggests that a 5,7-dihydroxy-chromen-4-one moiety appears to be favourable for the inhibition reaction. In conclusion, we have identified a group of polyphenols that can effectively hinder membrane damage by αS aggregates. These may serve as a viable source of lead compounds for the development and design of novel therapeutic agents in PD.

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1. Introduction

Alpha-synuclein (αS) is a 14.5 kDa, highly-conserved, neuronal protein that is widely distributed throughout the brain and expressed predominantly in pre-synaptic terminals [1–4]. Increased expression of αS and pathologically altered forms of this protein have been implicated in the pathophysiology of both familial and sporadic Parkinson's disease (PD), culminating in a loss of nigrostriatal dopaminergic neurons [5]. PD is the most common movement disorder and affects approximately 1% of the population over the age of 50 with classical clinical manifestations of bradykinesia, muscle rigidity, resting tremor and postural instability [6].

The role of the various physical forms (i.e. monomers, soluble oligomers, protofibrils or fibrils) of αS in PD pathogenesis remains controversial. Deposits of high-molecular-weight, fibrillar αS aggregates in neurons, termed Lewy bodies, are a ubiquitous pathological feature of PD [7]. Substantial data from *in vitro* and *in vivo* studies, however, supports the hypothesis that soluble αS oligomeric intermediates represent the principal pathogenic species [8–10]. It has been shown that αS oligomers share a common structure with other amyloidogenic proteins, such as amyloid-beta, amylin, insulin, and the cellular prion protein, implying a common mechanism of pathogenesis [11]. Particularly, there is increasing evidence that these oligomers target biological membranes, possibly forming structures with pore-like morphologies that contribute to cytotoxicity in neurodegenerative diseases *via* the disruption of cellular and organelle membranes [12–14]. Lipid-bound oligomers of αS were isolated from brains of transgenic mice as well as from PD patients [15].

Abbreviations: αS, α-synuclein; AFM, atomic force microscopy; Api, apigenin; Baic, baicalein; BTE, black tea extract; EGCG, (−)-epigallocatechin gallate; Gen, genistein; Gink, ginkgolide B; Mor, morin; NBB, N′-benzylidene-benzohydrazide; NDGA, nordihydroguaiaretic acid; ns, not significant; OGB-1, Oregon Green® 488 BAPTA-1; PD, Parkinson's disease; PropylG, propyl gallate; Purp, purpurogallin trimethyl ether; Resv, resveratrol; Scut, scutellarein; SIFT, scanning for intensely fluorescent targets; SUVs, small unilamellar vesicles; ThT, Thioflavin T; WT, wild-type

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The nature of the interaction of αS with biological and artificial membranes is complex [16,17] and dependent on the phospholipid composition of the membranes, the size of the vesicles, as well as the ratio of membrane lipids to protein [18,19]. Thus, αS binds preferentially to small unilamellar vesicles (SUVs) containing acidic phospholipids such as phosphatidic acid, phosphatidylserine and phosphatidylglycerol but less strongly to vesicles with a net neutral charge such as phosphotidylcholine and phosphatidylethanolamine [20,21]. Only the positively-charged N-terminal and hydrophobic central parts of αS (residues 9–90) partake in lipid binding [22] whilst the negatively charged C-terminal region has been proposed to act as a scaffold to recruit additional proteins to the membranes [23].

Oligomeric αS has been observed to induce bilayer disruption upon tight binding to membranes containing anionic lipids [24,25]. The leakage of vesicular contents caused by αS oligomers showed a strong preference for low-molecular-mass molecules, implying a pore-like mechanism for permeabilisation; monomeric αS was determined to be less pore forming [24]. In support of these observations, soluble αS oligomers, but not monomers or fibrils, were able to form pores in planar lipid bilayers [26]. In cellular models, increased calcium influx has been reported using preformed aggregates of αS incubated with Fe^{3+} and organic solvent [27]. Expression of mutant αS (Ala30Pro [A30P] and Ala53Thr [A53T]) is associated with a non-selective ion permeability of the cellular membrane, presumably as a consequence of relatively large pores [28]. Moreover, both A30P and A53T mutants have been shown to form pore-like annular and tubular protofibrillar structures, whilst wild-type (WT) αS formed annular protofibrils only after extended incubation [29]. Pore formation may therefore play a role in the pathophysiology of the aggregated protein by permitting uncontrolled flux of ions into and out of cells. Aside from pore formation, another mechanistic possibility involves thinning of the plasma membrane [30,31]. Membrane thinning was directly observed during aggregation of WT and mutant αS on lipid bilayers; lipid molecules were extracted from the bilayer by the growing aggregates, ultimately resulting in extensive bilayer disruption [32]. A recent landmark study reported a direct link between membrane-bound αS oligomers and severe dopaminergic neuronal loss in a murine model [33]. The most severe neuronal toxicity correlated with those αS variants that formed oligomers; in turn, the more toxic oligomerising mutants exhibited a higher affinity to bind liposomes, implying a stronger reactivity to membranes [33]. Hence, membrane damage by oligomeric αS is considered a likely mechanism of cytotoxicity in PD, which in turn implies that compounds which interfere with disruption and permeabilisation of membranes by αS have a potential role in PD.

Currently, no preventive therapy is available for PD [34]. Over the past decade, intensive research has gone into identifying small organic molecules that can inhibit and/or disaggregate αS oligomer formation *in vitro* [27,35]. Among the most studied are the natural polyphenolic compounds, characterised by the presence of multiple hydroxyl groups on aromatic rings [36–40]. Polyphenols either form soluble, non-toxic, oligomeric complexes with the αS protein or disaggregate mature multimeric structures into smaller, non-toxic aggregates [41–44]. Polyphenols can also interact with and permeate phospholipid membranes [45]. Both polar and nonpolar forces were shown to have a significant impact on flavonoid–biomembrane interactions [46]. Results of a study in which alterations in membrane fluidity in a phospholipid bilayer were monitored, suggest that flavonoids and isoflavonoids, similar to cholesterol and alpha-tocopherol, partition into the hydrophobic core of the membrane and cause a dramatic decrease in lipid fluidity [47].

In summary, it is highly desirable to develop compounds that can interfere with membrane damage by oligomeric αS since they could offer powerful therapeutical potential. A liposome permeabilisation assay, in which synthetic lipid vesicles were exposed to pre-aggregated αS, was used as a robust screening method to identify such compounds. We thereby tested a select group of natural polyphenols and N′-benzylidene-benzohydrazide compound derivatives, that we had previously identified as potent inhibitors of αS oligomer formation [27,40].

2. Materials and methods

2.1. Materials

Compounds tested in this work were obtained as follows: apigenin (Api), baicalein (Baic), black tea extract (BTE; >80% theaflavins), (−)-epigallocatechin gallate (EGCG), genistein (Gen), ginkgolide B (Gink), morin (Mor), nordihydroguaiaretic acid (NDGA), propyl gallate (PropylG), purpurogallin trimethyl ether (Purp) and resveratrol (Resv) were all purchased from Sigma-Aldrich (Munich, Germany); scutellarein (Scut) was obtained from Pharmasciences Laboratories (Cour-bevoie, France); N′-benzylidene-benzohydrazide (NBB) compounds were obtained from Chembridge Corp. (San Diego, CA, USA). In all cases, the purity of the compounds was >98%. Polyphenols, NBB compounds and BTE were prepared as stock solutions in DMSO and stored at −20 °C. During the experiments, compounds were protected from light and used immediately after thawing. Unless otherwise stated, all other chemicals indicated in the protocols below were purchased from Sigma.

2.2. Expression and purification of α-synuclein

Wild-type (WT) or mutant (A30P, A53T) human recombinant αS-containing pET-5a plasmid was transfected into *Eschericia coli* BL21 (DE3) cells (Novagen, Madison, WI, USA). Expression was induced with isopropyl-D-thiogalactopyranose (Promega, Mannheim, Germany) and the proteins purified as described previously [48]. Protein concentration was determined using a bicinchoninic acid protein-quantification kit (Pierce, Rockford, IL, USA). Aliquots of purified recombinant synucleins (0.5–1.0 mg/l) were stored at −80 °C.

2.3. Fluorescent labelling of α-synuclein

Fluorescent labelling of αS with Alexa Fluor-488-O-succinimidylester (green) and Alexa Fluor-647-O-succinimidylester (red) (Invitrogen, Eugene, OR, USA) was performed as previously described [27,48]. Quality control of labelled αS was done by fluorescence correlation spectroscopy measurements on an Insight Reader (Evotec-Technologies, Hamburg, Germany). Aliquots of purified recombinant fluorescently-labelled monomeric synuclein were stored at −80 °C.

2.4. Preparation of wild-type and mutant α-synuclein aggregates

The aggregation assay was performed as described [48]. Briefly, 7 μM monomeric αS with 1% (v/v) DMSO and 10 μM $FeCl_3$ in sterile phosphate-buffered saline (PBS) buffer (pH 7.4) was incubated for 4 h at 25 °C. Concentrations given for aggregated αS refer to the respective equivalent monomer concentration.

2.5. Confocal single particle analysis

Fluorescence correlation spectroscopy (FCS), fluorescence intensity distribution assay (FIDA) and scanning for intensely fluorescent target (SIFT) measurements were carried out on an Insight Reader (Evotec-Technologies, Hamburg, Germany) according to established protocols [48]. In general, fluorescence emitted from dual colour excitation at 488 and 633 nm was recorded simultaneously with two single-photon detectors. Photons were summed over time intervals of constant length (bins) using a bin length of 40 μs. The frequency of specific combinations of “green” and “red” photon counts, derived from monomeric control or aggregation measurements respectively,

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