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Research paper

Naturally occurring cinnamic acid derivatives prevent amyloid transformation of alpha-synuclein

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ABSTRACT

In search of the compounds that interfere with amyloid transformation of alpha-synuclein, 9 natural and synthetic cinnamic acid derivatives were studied. They are structurally similar to a half of curcumin, which has pronounced anti-aggregatory and anti-amyloid effects. We have shown that some of these derivatives prevent ovine prion protein amyloidization. Subsequently, thioflavin T binding assay showed that 3 out of 9 studied compounds effectively prevented amyloid transformation of alpha-synuclein with IC50 of 13, 50 and 251 μM. Molecular modeling approach revealed possible binding sites of the three selected ligands with alpha-synuclein fibrils, while monomeric alpha-synuclein does not bind to the ligands according to experimental results. This led us to believe that compounds may act by changing the structure of primary aggregates, preventing the formation of full-length fibrils. The inhibiting effect of the ligands on aggregation of alpha-synuclein was further confirmed by monitoring aggregation via turbidimetry, susceptibility to proteolytic cleavage, changes in beta-sheet content, and scanning ion-conductance microscopy. Studied derivatives were not cytotoxic, and, moreover, two studied compounds (ferulic and 3,4-dimethoxycinnamic acid) are found in plant sources and are natural metabolites present in human blood, so they can be promising candidate drugs for synucleinopathies, including Parkinson's disease.

1. Introduction

Plant polyphenol curcumin is considered to exhibit a wide range of biological activities, including antioxidant, anticarcinogenic and anti-aggregation effects [1–6]. Nevertheless, available literature on the subject provides controversial data since the conclusions drawn *in vitro*, as a rule, cannot be reproduced during the experiments on cell cultures and, moreover, on whole organisms [7], which might be attributed to curcumin poor aqueous solubility [8–11]. This circumstance not only impedes its application but also does not allow determination of its effective concentration in biological systems [7]. There are many ways to increase the solubility and bioavailability of curcumin using detergents, cyclodextrins, and other compounds. It is, however, impossible to prevent its selective accumulation in the nonaqueous phase of biological structures [7,12,13]. Such accumulation, in particular in lipid membranes, may not affect certain functions of curcumin (e.g., antioxidant) and can even be useful [14,15]. However, a decrease in the curcumin concentration in the aqueous phase prevents development of its anti-aggregation and anti-amyloid effects.

Previously, we proposed use of natural polyphenols more water-soluble than curcumin as anti-amyloid compounds [1]. 3,4-dimethoxycinnamic acid (3,4-DMCA), which is structurally similar to a half of curcumin molecule, was proved to prevent amyloid

Abbreviations: 3,4-DMCA, 3,4-dimethoxycinnamic acid; PBS, phosphate buffered saline (10 mM potassium phosphate, 0.137 M NaCl, 2.7 mM KCl); ThT, Thioflavin T; CD, circular dichroism; ITC, isothermal titration calorimetry; SICM, scanning ion-conductance microscopy; IC50, half-maximal inhibitory concentration..

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transformation of the ovine prion protein. This biological activity of 3,4-dimethoxycinnamic acid was due to its interaction with the monomeric form of the prion protein, which probably leads to stabilization of the protein spatial structure. We have suggested that 3,4-dimethoxycinnamic acid and/or its derivatives can prevent pathological transformation of other amyloidogenic proteins [1]. Of particular importance is the analysis of these compounds' effect on amyloid transformation of alpha-synuclein, a soluble presynaptic protein, which in its fibrillar form is the main component of protein aggregates known as Lewy bodies, a hallmark of Parkinson's disease and other synucleinopathies [16,17].

Being an intrinsically disordered protein, alpha-synuclein can adopt different conformations depending on the conditions [18,19]. Genesis and progression of Parkinson's disease are linked with alpha-synuclein fibrillization and factors promoting such aggregation. Some missense mutations in the *SNCA* gene (A53T, A30P, E46K) or duplications/triplications of this gene result in the development of familial forms of Parkinson's disease [20–23]. Altogether, alpha-synuclein aggregation is considered to be a pathogenic process producing multiple conformers varying in size and morphology that are both prefibrillar and fibrillar [24]. Due to the heterogeneity of these structures, inhibition of alpha-synuclein aggregation and stabilization of its non-toxic forms is of considerable interest. Previous studies have highlighted the potential of small molecule compounds to inhibit alpha-synuclein aggregation [25–29]. In the present work, we tested several small molecule compounds which can be effective non-toxic candidates for inhibition of alpha-synuclein fibrillization.

Hydroxycinnamic acid derivatives including 3,4-dimethoxycinnamic acid (3,4-DMCA) are known to be the components of green coffee beans [30,31]. 3,4-DMCA is of special interest because it appears in high abundance (~380 nM at 60 min) in human plasma after coffee intake despite the low concentration in the original coffee extract. Similarly, ferulic acid appears in plasma after ingestion of coffee. Interestingly, content of 3,4-DMCA constitutes only 3% of ferulic acid content in coffee extract, but it reaches 240% in plasma after coffee consumption [31]. 3,4-DMCA in coffee extract occurs as a conjugate with quinic acid, but after coffee intake free 3,4-DMCA was the only form detected in plasma samples [32]. The small intestine was suggested to be an important site for hydrolysis of 3,4-DMCA derivatives by intestinal esterases [33].

The natural origin of cinnamic acid derivatives and their presence in biological fluids and human tissues facilitates both preparation of these compounds and their use as medicinal or preventive drugs in the treatment of Parkinson's disease.

In the present work, we investigate the interaction of human alpha-synuclein with 3,4-dimethoxycinnamic acid and 8 other cinnamic acid derivatives, as well as their impact on the amyloid transformation of alpha-synuclein, using experimental approaches and molecular docking.

2. Materials and methods

In the current work, we used the following chemicals: 3,4-dimethoxycinnamic acid, proteinase K, Thioflavin T, and resazurin (Sigma, USA); inorganic salts (Panreac, Spain) and buffers (Amresco, USA); fetal bovine serum (FBS) (HyClone, USA); GlutaMAX (Gibco, USA); Dulbecco's Modified Eagle Medium (DMEM), trypsin, and penicillin/streptomycin (Paneco, Russia). Human neuroblastoma SH-SY5Y culture was kindly gifted by Dr. Irina Naletova (University of Catania, Italy). Cinnamic acid derivatives were synthesized according to known procedures, then prepared in PBS pH 7.4 as 5 mM stock solutions and stored at +4 °C.

2.1. Molecular docking

Docking of the tested compounds was performed using AutoDockVina software [34]. Structures of alpha-synuclein fibrils with PDB ID 6cu7, 6cu8, and 2n0a were used. The docking was performed separately for these three structures as well as for two additional structures of individual protofilament from 6cu7 and 6cu8.

2.2. Purification of recombinant alpha-synuclein

Full-length alpha-synuclein without additional motifs, wild type and A53T mutant, were expressed in *E. coli* and purified as previously described [35], with minor modifications. Codon encoding Tyr136 was mutated from TAC to TAT, also encoding tyrosine, which prevented cysteine misincorporation in bacterial system [36].

Briefly, cell extract pH was adjusted to 2.8 by addition of 9% HCl, and precipitated proteins were removed by centrifugation (15,000 g, 5 min, 4 °C). The pH value of the supernatant was adjusted to 7.5 using 1 M potassium phosphate solution, pH 11. Alpha-synuclein was salted out by addition of ammonium sulfate to achieve 40% saturation. After precipitate was formed, it was collected by centrifugation, washed twice with 40% ammonium sulfate solution, and stored as suspension at 4 °C. The final protein concentration in the suspension was 2–4 mg/ml.

Protein concentration of alpha-synuclein solutions was determined spectrophotometrically at 280 nm, using extinction coefficient for 0.1% solution = 0.412.

2.3. Preparation of alpha-synuclein fibrils

The ammonium sulfate precipitate of alpha-synuclein was collected by centrifugation and dialyzed against PBS buffer, pH 4.0. After that, the sample was diluted to a final concentration 0.4 mg/ml (28 μM) with the same buffer, pipetted (by 0.3 ml) into 9-ml glass tubes, and incubated at 37 °C with constant agitation at 300 rpm for 72 h. During the incubation, 5 μl aliquots were taken for Thioflavin T fluorescence analysis to monitor the formation of amyloid aggregates. At the same time, the turbidity of tested solutions was measured as absorption at 400 nm in order to detect the process of protein aggregation.

2.4. Thioflavin T fluorescence assay

Thioflavin T (ThT) fluorescence assay was performed in 96-well FLUOTRAC 200 black immunology plates (Greiner) using a CLARIOstar plate reader (BMG LABTECH GmbH, Germany). Aliquots of 5 μl were taken from the tested samples of growing fibrils and added to 95 μl of 25 μM ThT solution. After 10 min of incubation at 20 °C, fluorescence intensity was measured on the plate reader (440 nm excitation, 490 nm emission). Each point was obtained by three separate measurements of the same protein sample.

2.5. Determination of half-maximal inhibitory concentration (IC50)

3-methoxy-4-acetamidoxycinnamic acid (L7), ferulic acid (L8), and 3,4-DMCA (L9) were added to alpha-synuclein monomers solutions at different concentrations, corresponding to the following protein:ligand molar ratios: 30 μM (1:1), 45 μM (1:1.5), 125 μM (1:5), 250 μM (1:10), 1250 μM (1:50), 2500 μM (1:100), 25000 μM (1:1000). Then alpha-synuclein fibrils were grown for 2 days according to the procedure described earlier. The dependency of ThT fluorescence on the ligand concentration was used to count IC50. The IC50 values were counted using approximation with OriginPro 8.6 (OriginLab).

2.6. Seeding experiments

Alpha-synuclein precipitate was dialyzed against PBS buffer, pH 7.4 and diluted to 0.4 mg/ml (28 μM) with the same buffer. Seeding was performed by the addition of preformed fibril seeds at seed:-monomer molar ratio 1:50 and incubation at 37 °C with constant agitation at 300 rpm for 2–7 days. Tested compounds were added to the mixture at 280 μM concentration, i.e. 10-fold molar excess to alpha-synuclein monomer. Aliquots of 5 μl were taken from the investigated samples in order to monitor fibril formation by ThT fluorescence assay.

2.7. Isothermal titration calorimetry (ITC)

The interaction of alpha-synuclein with the chosen ligands was investigated using VP-ITC instrument (MicroCal Ltd., USA). Titration experiments were performed by 30 sequential 10 μL injections of 0.5 mM 3-methoxy-4-acetamidoxycinnamic acid, ferulic acid or 3,4-DMCA solution, PBS pH 4.0, into the 35 μM (0.5 mg/ml) of alpha-synuclein in the same buffer. All samples were degassed before the experiment. All isotherms of ligands were subtracted from the corresponding experimental isotherms for alpha-synuclein and ligand. The data was statistically processed with the 'one set of sites' model using MicroCal Origin 7.0 software.

2.8. Circular dichroism spectroscopy

Circular dichroism (CD) spectra of alpha-synuclein samples were recorded in the far UV region (190–240 nm) at 20 °C using a 0.1-mm pathlength cuvette on Applied Photophysics Chirascan CD spectrometer (Applied Photophysics, UK). CD spectra of soluble alpha-synuclein (0.4 mg/ml, or 28 μM) without additions or in the presence of 10-fold molar excess of tested compounds were recorded after dialysis against 10 mM potassium phosphate buffer, pH 4.0.

Fibrils and aggregates of alpha-synuclein produced in 0.3 ml of PBS, pH 4.0, either with or without tested compounds (molar ratio compound:monomer 10:1), were collected by centrifugation (13,000 g, 5 min) and resuspended in 0.12 ml of 10 mM potassium phosphate buffer, pH 4.0 to remove the excess of salts. Each spectrum was the average of 5 scans.

2.9. Digestion by proteinase K

The pH value of alpha-synuclein fibrils obtained without additions or in the presence of 10-molar excess of tested compounds were adjusted to pH 7.5 using 1 M potassium phosphate solution, pH 11, and incubated with 0.2 μg/mL proteinase K in the presence of 5 μM Ca^{2+} ions at 37 °C for 30 min total. As described in Ref. [1], proteolysis was stopped by the addition of 4X Laemmli buffer (without beta-mercaptoethanol) to the samples and instant boiling at 95 °C for 5 min. Samples were then loaded onto 16% acrylamide gel and analyzed by SDS-PAGE. To assess the percent of proteolysis we calculated bands by scanning densitometry using ImageJ software (NIH).

2.10. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The samples were analyzed by SDS-PAGE using 16% resolving and 4% stacking polyacrylamide gels according to the standard procedure [37]. Investigated samples were diluted with 4X sample buffer and heated 5 min at 95 °C.

2.11. Resazurin assay

SH-SY5Y cells were cultivated in DMEM with 10% FBS, GlutaMAX, 50 U/ml penicillin and 50 μg/ml streptomycin at 37 °C, 5% CO_2. Day before experiment cells were plated onto 96-well plates at 20000 cells per well. Tested compounds were added directly into culture medium to final concentrations 13.5–1380 μM, as these concentrations were used for the *in vitro* tests, and incubated for 24 h. Buffer used for compounds dilution was added to control wells. Cell viability was assessed by resazurin assay, a dye which is reduced to resorufin by living cells [38]. After changing culture medium, 20 μl of 0.15 mg/ml resazurin dye was added to each well. After 12 h incubation the absorbance at 570 nm (resorufin) and 600 nm (resazurin) was registered (VersaMax microplate reader, Molecular devices, USA). To obtain final values absorbance at 600 nm was subtracted from absorbance at 570 nm. Each data point was replicated three times.

2.12. Scanning ion-conductance microscopy (SICM)

The protein samples (0.3 ml) of alpha-synuclein fibrils and alpha-synuclein fibrils grown in the presence of 3-methoxy-4-acetamidoxycinnamic acid (L7), ferulic acid (L8) or 3,4-DMCA (L9) were pipetted onto the Petri dishes covered with 0.1% poly-L-lysine. The dishes were sealed with Parafilm and left at 4 °C overnight. Before the experiment, the samples were gently washed with PBS buffer, pH 4.0, then ThT solution was added to the final concentration of 0.5 mM. Topography images were obtained by ICAPPIC scanning ion-conductance microscope (ICAPPIC Limited, United Kingdom). Nanopipettes for SICM were pulled from borosilicate glass capillaries (o.d.: 1.0 mm; i.d.: 0.58 mm) (Intrafil, USA). Nanopipettes with typical tip inner radius about 25–40 nm were obtained using two step program [39] with following parameters: heat 310, filament 3, velocity 30, delay 160, pull 0, heat 330, filament 3, velocity 25, delay 160, pul 200 on laser puller P-2000 (Sutter Instruments, USA). Nanopipettes were filled with PBS buffer, pH 4.0. MultiClamp 700B amplifier was used for ion current measurements (Molecular Devices, Wokingham, UK). Typically, a bias potential of 200 mV was used for SICM imaging. The "hopping" mode [40] realized at ICAPPIC scanning software was used for SICM imaging. The fall rate of nanopipette approaching was 200 nm/ms, the hopping amplitude during scanning was 1–2 μm. SICM images were processed with FemtoScan software (Advanced Technologies Center, Russia).

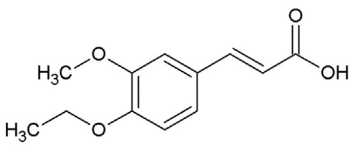
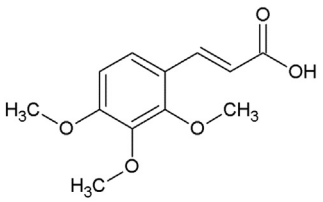
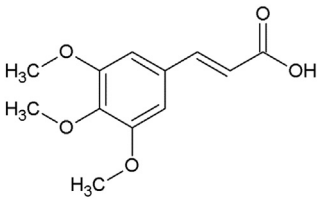
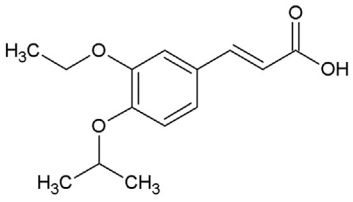
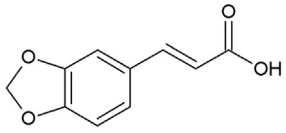
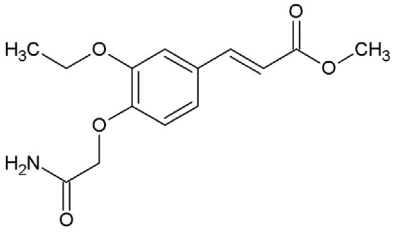
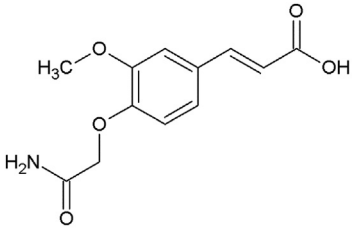
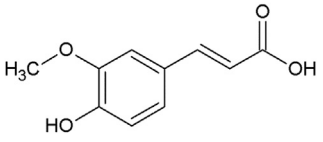
3. Results and discussion

To select the most effective inhibitors of amyloid transformation of alpha-synuclein, we tested nine cinnamic acid derivatives. Chemical structures of these derivatives, similar to a half of curcumin molecule, are presented in Table 1, and serial numbers are used to designate them in the text.

The recombinant alpha-synuclein was isolated from the cell extract of the producer strain by acidification of the original extract with HCl as described in section 2.2. The resulting supernatant (Fig. 1A, lane 4) was neutralized by addition of 1 M potassium phosphate solution, pH 11, and then salted out with ammonium sulfate to 40% saturation. Analysis of the resulting preparation by SDS-PAGE under reducing conditions (in the presence of mercaptoethanol) revealed a single band of 18 kDa (Fig. 1A, right panel) in both cases (wild-type and A53T alpha-synuclein).

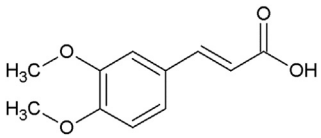
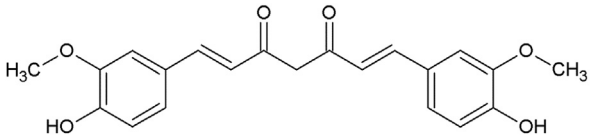
ThT fluorescence intensity change over time in Fig. 1B reveals that only three compounds effectively prevent amyloid transformation of alpha-synuclein: 3-methoxy-4-acetamidoxycinnamic acid (L7), ferulic acid (3-methoxy-4-hydroxycinnamic acid) (L8)

Table 1
Chemical structures of the tested cinnamic acid derivatives.

	Formula	Name
1		3-Methoxy-4-ethoxycinnamic acid
2		2,3,4-Trimethoxycinnamic acid
3		3,4,5-Trimethoxycinnamic acid
4		3-Ethoxy-4-isopropyloxycinnamic acid
5		3,4-Methylenedioxycinnamic acid
6		Methyl-3-ethoxy-4-acetamidoxycinnamate
7		3-Methoxy-4-acetamidoxycinnamic acid
8		Ferulic acid (3-Methoxy-4-hydroxycinnamic acid)

(*continued on next page*)

Table 1 (*continued*)

	Formula	Name
9		3,4-Dimethoxycinnamic acid
		Curcumin

and 3,4-dimethoxycinnamic acid (L9).

It should be noted that among these three compounds 3,4-dimethoxycinnamic acid (L9), which had the most prominent effect in preventing amyloid transformation of the ovine prion protein [1], was less effective than the other two compounds. In further work, we studied the effect of these three ligands on amyloid transformation of alpha-synuclein in more detail.

Firstly, molecular docking of alpha-synuclein interaction with the three ligands that effectively inhibit its fibrillation in ThT assay was carried out to identify the most probable sites of their binding. Three different structures of fibrils were used: structures of rod and twister forms obtained using CryoEM (PDB IDs 6cu7 and 6cu8,

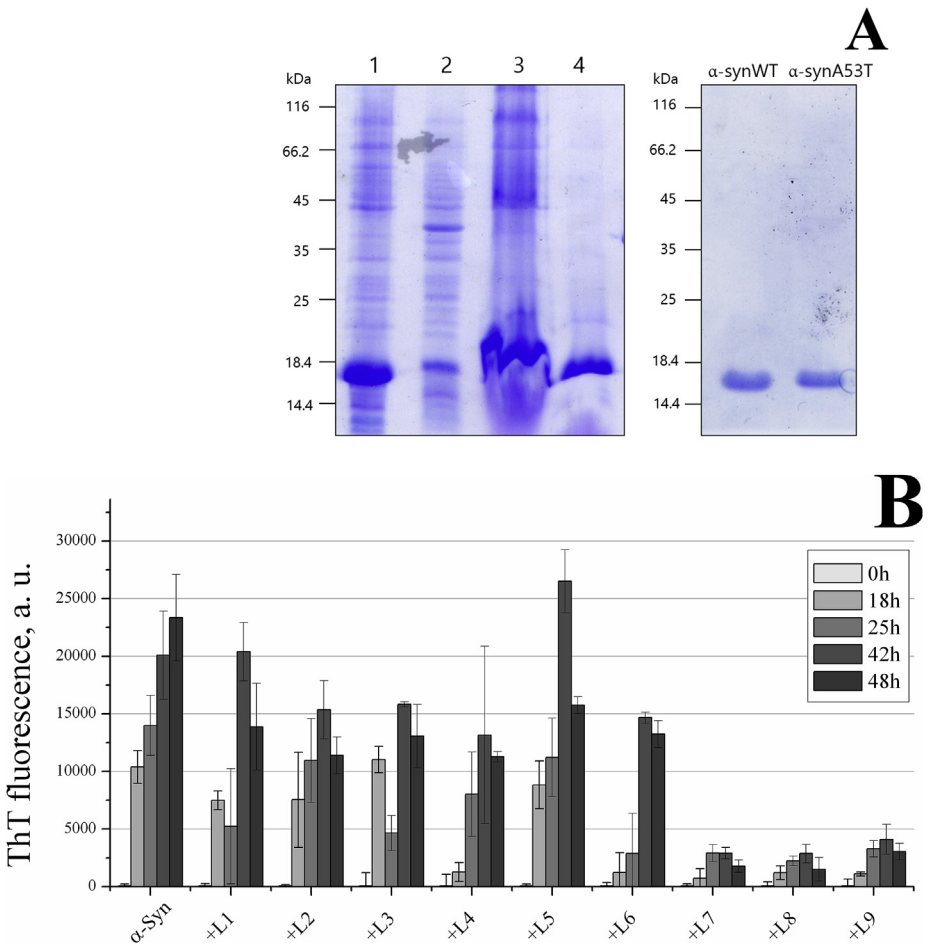
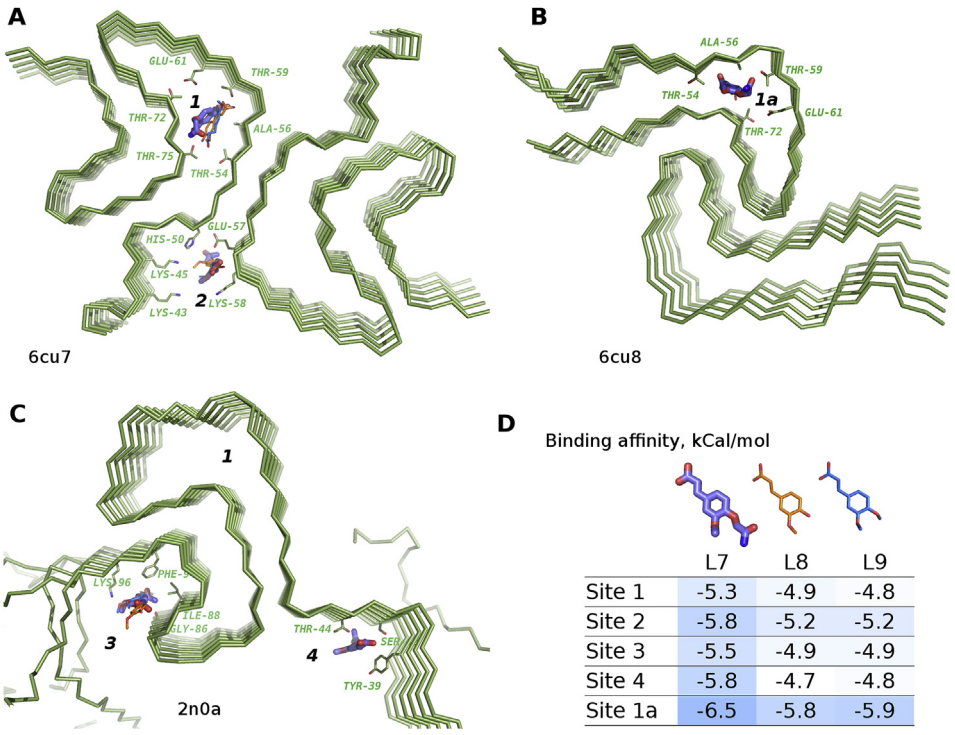


Fig. 1. A - Purification steps of wild-type alpha-synuclein.
Lanes: 1 – supernatant after *E. coli* cells sonication, 2 – pellet after *E. coli* cells sonication, 3 – supernatant after incubation with streptomycin, 4 – supernatant after acidic precipitation with 9% HCl to pH 2.8 and restoration of pH back to 7.0–7.5 with 1 M potassium phosphate buffer pH 11.0.
α-synWT, α-synA53T - purified wild-type and A53T alpha-synuclein after ammonium precipitation and subsequent wash with 40% ammonium sulfate solution, 4 μg of protein per lane.
B - Effect of cinnamic acid derivatives on alpha-synuclein amyloid aggregation.
Samples containing 0.4 mg/ml (28 μM) of alpha-synuclein without additions and alpha-synuclein in the presence of tested compounds (10-fold molar excess) were incubated in PBS buffer, pH 4.0, at 37 °C under constant agitation. During the incubation, aliquots of 5 μl were taken for ThT assay (see Sections 2.3 and 2.4 for details). Ligand numbers correspond to their serial number in Table 1. Data are given as mean values of three measurements ± SD.



	L7	L8	L9
Site 1	-5.3	-4.9	-4.8
Site 2	-5.8	-5.2	-5.2
Site 3	-5.5	-4.9	-4.9
Site 4	-5.8	-4.7	-4.8
Site 1a	-6.5	-5.8	-5.9

Fig. 2. Molecular docking of alpha-synuclein interaction with the three selected ligands.
Proposed binding sites of the ligands in structures of alpha-synuclein fibrils: 6cu7 (A), 6cu8 (B) and 2n0a (C), and the corresponding binding affinity values (D). Alpha-synuclein molecules are shown in ribbon representation; ligands L7-L9 are superimposed and shown in sticks representation with different stick radius (see L7-L9 in panel D).

respectively) [41], and the single-filament structure obtained using solid-state NMR (PDB ID 2n0a) [42], which contains full-length protein. In summary, four binding sites were predicted for all three tested ligands (Fig. 2). Positions of the ligands in the sites were the same. The first one (i.e. site 1 or site 1a, Fig. 2) is located inside the fibril "kernel", and the binding of the ligands in this site might affect the fibril formation. In addition, two possible binding sites are located on the side surface of the fibril (sites 3 and 4, Fig. 2C), and one more site is located in the interface of two protofilaments in rod-like form of fibrils (site 2, Fig. 2A). The binding of the ligands to the site 2 might block dimerization of the protofilaments and therefore inhibit formation of rod-type fibrils, but docking of the ligands to individual protofilaments suggested one binding site, namely, site 1. Thus, we suggest that the binding of the ligands to alpha-synuclein pre-fibrillar oligomers or short fibrils affects their regular structure and inhibits fibril growth.

Based on the molecular docking data, two major assumptions can be made: firstly, the selected compounds most likely should not bind to monomeric form of alpha-synuclein, and, secondly, the presence of the cinnamic acid derivatives should prevent the formation of ordered amyloid alpha-synuclein structures due to the interaction with residues involved in this process. Indeed, no change in thermodynamic parameters was found for binding of the three ligands to monomeric alpha-synuclein by isothermal titration calorimetry, which indicates absence of the interaction (data not shown).

The ITC experiment did not reveal a clear effect of binding of the chosen ligands to alpha-synuclein monomers, suggesting that the ligands could interact with oligomeric or fibril forms of alpha-synuclein. This correlates with results obtained by Singh *et al.* [43], where curcumin was shown to bind with oligomers and fibrils of alpha-synuclein and did not bind to its monomeric form.

A more detailed study of amyloid transformation of alpha-synuclein as compared to Fig. 1 confirmed preliminary data on the complete prevention of its fibrillation by the three selected ligands (Fig. 3A). At the same time, alpha-synuclein aggregation assessed by turbidimetric method still occurs in the presence of ligands, although less intensively (Fig. 3B).

Circular dichroism spectra of monomeric and fibrillar forms of alpha-synuclein are shown in Fig. 4, with curve shapes corresponding to literature data [44,45]. Monomeric alpha-synuclein spectrum has a strong negative peak between 195 and 200 nm, characteristic of unfolded protein. The fibrillar form of alpha-synuclein demonstrates a significant reduction in disordered structures content (smaller amplitude of the peak at 195 nm) and an increase in beta-structure content (beta-folds and turns, identified by curve shape at 218–220 nm and 230 nm, respectively) [46,47].

No changes in the CD spectra of monomeric alpha-synuclein in the presence of ligands were observed, although due to the absorption of the compounds in the shortwave region (less than 195 nm), it is difficult to draw an unequivocal conclusion. None of the ligands cause changes in the protein structure, and, probably, do not bind to the protein monomers, which confirms ITC results. That is, the addition of ligands does not lead to changes in the secondary structure of alpha-synuclein monomer. Therefore, the ligands themselves do not induce the process of alpha-synuclein amyloid transformation.

At the same time, CD spectra of alpha-synuclein fibrils obtained in the presence of 3-methoxy-4-acetamidoxycinnamic acid (L7) indicate a significant change in their secondary structure (Fig. 4). In the case of alpha-synuclein fibrillation without additions, we observe almost complete disappearance of the disordered structure and an increase in the beta-strands and beta-turns content. Alpha-synuclein fibrillation in the presence of L7 results in almost full preservation of disordered structure while only small amounts of

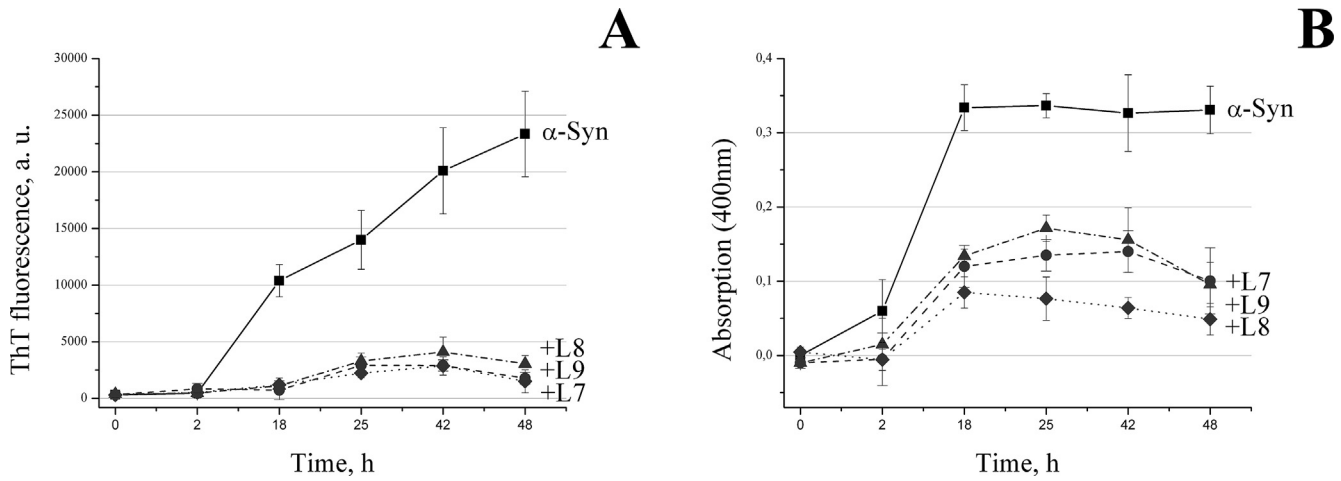


Fig. 3. Effect of cinnamic acid derivatives on the amyloid transformation (A) and aggregation (B) of alpha-synuclein.
Glass tubes containing 28 μM alpha-synuclein (0.3 ml) without additions (■) or in the presence of L7 (●), L8 (◆), L9 (▲) were incubated in PBS, pH 4.0, at 37 °C for 48 h under constant intensive shaking. A) During the incubation, aliquots of 5 μl were taken from the tubes for ThT assay as described in section 2.4. B) The turbidity of the samples was measured at 400 nm in a 0.3-ml quartz cuvette to evaluate the aggregation. Each point is the mean value of three parallel measurements ± SD.

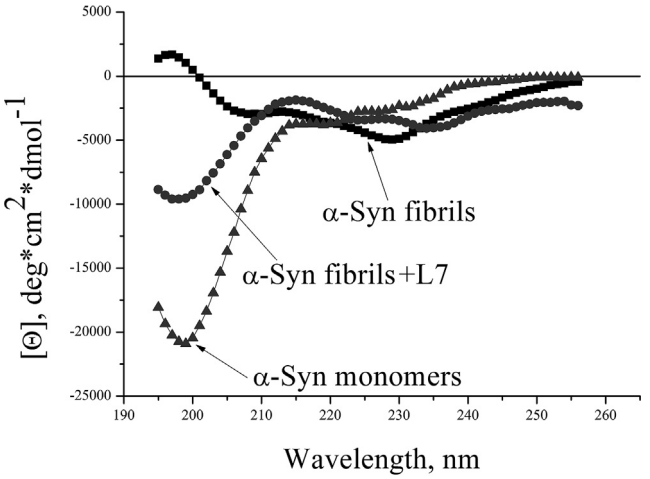


Fig. 4. Influence of cinnamic acid derivatives on CD spectra of alpha-synuclein under fibrillation conditions.
CD spectra of alpha-synuclein before (▲) and after (■) the incubation under fibrillation conditions. CD spectrum after fibrillization in the presence of 3-methoxy-4-acetamidoxycinnamic acid (L7) is shown with circles (●). Solutions containing 0.4 mg/ml (28 μM) of alpha-synuclein without additions or in the presence of L7 were incubated in PBS, pH 4.0, at 37 °C under intensive shaking for 48 h. CD spectra of the investigated samples were recorded after buffer exchange to 10 mM potassium phosphate buffer, pH 4.0. Each spectrum is the average of 5 records (see Section 2.8 for details).

alpha-helices and beta-turns appear. In conclusion, addition of L7 partially prevents alpha-synuclein amyloid transformation.

Unfortunately, it was not possible to obtain reliable CD spectra of the aggregates formed in the presence of L8 and L9, since the amount of aggregates that can be precipitated by centrifugation was miniscule, and spectra recording without buffer exchange was difficult due to the high salt concentration in the fibrillation buffer. However, we assume that L8 and L9 also prevent alpha-synuclein fibrillation in accordance with other experimental results.

To assess the interaction of the three ligands with alpha-synuclein, we determined how their effect on alpha-synuclein amyloid aggregation was dependent on concentration (Fig. 5). The IC50 for 3-methoxy-4-acetamidoxycinnamic acid (L7), ferulic acid (L8) and 3,4-dimethoxycinnamic acid (L9) is 50, 13 and 251 μM, respectively.

The analysis of aggregates formed in the presence of the three studied ligands has also been carried out using scanning ion-conductance microscopy. Images of alpha-synuclein aggregates formed under conditions that stimulate its fibrillation either without any additions (Fig. 6A) or in the presence of the three ligands (Fig. 6B–D) are shown in Fig. 6. In the control experiment, the formation of extended amyloid aggregates more than 10 μm in length and up to 1.6 μm in height was observed (Fig. 6A). Addition of each investigated ligand resulted in the significant decrease of the linear dimensions of the aggregates in the OXY-plane (up to 4 μm in the case of L7). In the presence of L8 and L9, the lengths of the aggregates amounted to 2.2 and 2.6 μm, respectively. Moreover, in case of L7 and L8, the height of the aggregates also decreased (to 0.9 and 0.6 μm, respectively). In case of L9 addition, aggregates were 1.3 μm in height. The volumes of the aggregates formed in the presence of L7, L8, and L9 were also decreased compared to the control. While the average volume of the control alpha-synuclein aggregates was 7.87 μm^3, in the presence of L7, L8, and L9 average volumes of aggregates amounted to 0.6, 0.16 and 0.71 μm^3, respectively. These results confirm the observation that L7 and L8 show a more prominent effect compared to 3,4-dimethoxycinnamic acid (L9).

Fibrillar structures of amyloidogenic proteins are known to be highly resistant to proteolytic enzymes digestion [48,49]. Indeed, in the control samples of fibrils, alpha-synuclein polypeptide chains are hydrolyzed slower (Fig. 7, A and E) than in the alpha-synuclein aggregates obtained in the presence of the studied cinnamic acid derivatives. L8 and L9 accelerate proteolysis more efficiently (Fig. 7 C–E). It can be pointed out that in the control fibril sample after 10 min of incubation there is a significant amount of polypeptide chains with molecular weight less than 14 kDa, completely absent in the remaining samples (Fig. 7. A compared to B-C). Alpha-synuclein fibrils probably undergo limited proteolysis in which only fragments of protein molecules “packaged” into amyloid structures but still exposed into the solution are cleaved off. Alpha-synuclein molecules in less structured aggregates, obtained in the presence of the ligands, undergo complete hydrolysis faster. The reasons for the L7 ligand being less effective than L8 and L9 in promoting proteolysis require further investigation.

We have also analyzed the effect of the three ligands on amyloidogenic transformation of alpha-synuclein under conditions

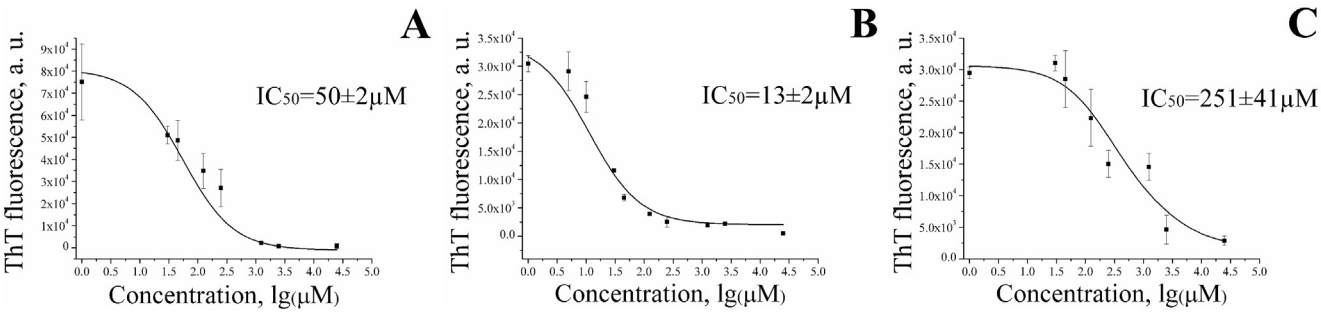


Fig. 5. The dose-dependent inhibition effect of L7 (A), L8 (B), L9 (C) on alpha-synuclein amyloid aggregation.
The horizontal axis represents the common logarithm of different cinnamic acid derivatives concentrations added to the alpha-synuclein samples (see Sections 2.4 and 2.5 for details). The IC50 values were calculated using approximation with OriginPro 8.6 software (OriginLab).

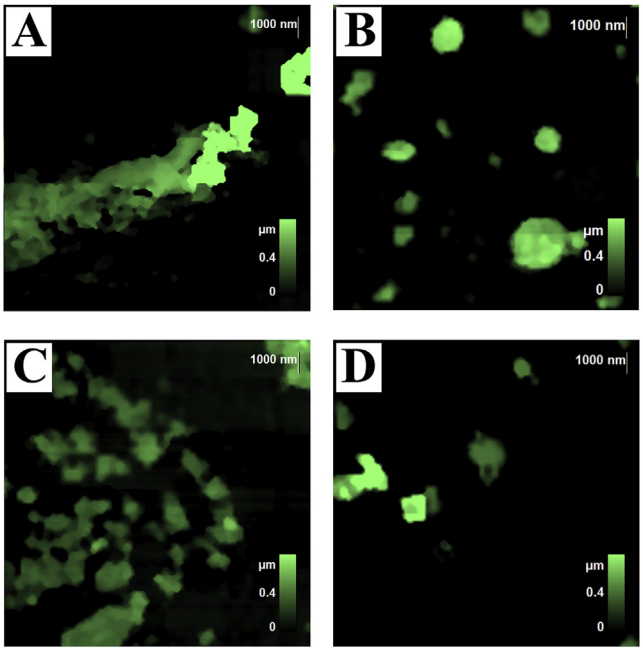


Fig. 6. Scanning ion-conductance microscopy of alpha-synuclein aggregates produced under fibrillation conditions without any additions (A) or in the presence of L7 (B), L8 (C), L9 (D).
Alpha-synuclein fibrils, grown in PBS buffer, pH 4.0, at 37 °C for 2 days, were pipetted onto the Petri dishes covered with 0.1% poly-L-lysine and left at 4 °C overnight. Then 2–3 ml of 0,5 mM ThT solution were added before the surface scanning with SICM. The color gradient represents the dimensions of alpha-synuclein fibrils relative to the axes.

closer to those *in vivo*. In the previous experiments, we carried out fibrillation at pH 4.0 and investigated only wild-type recombinant alpha-synuclein. Fig. 8 presents data on amyloid transformation of alpha-synuclein at pH 7.4 using preformed protein fibrils as seeds to stimulate the process. Before the experiment, fibrils were obtained using wild-type alpha-synuclein or mutant A53T prone to pathological transformation and associated with the development of Parkinson's disease [50]. In both cases, fibril seeds were obtained by incubation in an acidic medium, as was done in the previous experiments. Comparison of the graphs in Fig. 8 A and B shows that the fibrillation of A53T alpha-synuclein already begins after 28 h of incubation, which is several days earlier than in the case of wild-type alpha-synuclein (after 70 h). Amyloid transformation in both cases does not occur without fibril seed. At the same time, the resulting Thioflavin T fluorescence intensity for a mutant protein is 3-4-fold higher than for a wild-type alpha-synuclein. The three studied cinnamic acid derivatives completely prevent amyloid transformation of both mutant and wild-type protein. There were no differences in the effect of the analogs in these experiments, since they were added in amounts equal to or greater than their IC50.

To check further potential and possibility of using cinnamic acid derivatives as compounds preventing amyloid transformation of alpha-synuclein in principle, their cytotoxicity was tested on neuroblastoma cells. No effect of the three ligands on cell viability was shown by resazurin assay up to the concentrations of 1.38 mM (the highest concentration used for the previously described *in vitro* assays) which many times exceeds the IC50 even for the least effective ligand - 3,4-dimethoxycinnamic acid (L9) (Fig. 9).

Thus, we have found that three out of nine studied cinnamic acid derivatives are able to prevent alpha-synuclein amyloid transformation, namely, 3-methoxy-4-acetamidoxycinnamic acid (L7), ferulic acid (3-methoxy-4-hydroxycinnamic acid) (L8) and 3,4-dimethoxycinnamic acid (L9). Using known structures of alpha-synuclein fibrils, specific binding sites were predicted by molecular docking method for each of these compounds including sites formed by amino acid residues belonging to different protofibrils within the same fibril, as well as in the "inner" part of the protofibrils. Presumably, the binding of ligands with protofibrils or short fibrils may impede the formation of "full-length" fibrils. Simulation of ligands and the monomeric form of alpha-synuclein interaction was impeded due to disordered structure of the protein.

No interaction of the ligands and monomeric alpha-synuclein was detected using isothermal titration calorimetry, as well as there were no changes in the CD spectra of alpha-synuclein monomers in the presence of the ligands. These results differ from those obtained earlier for the ovine prion protein, whose partially ordered structure allowed to perform molecular modeling experiment of its binding with 3,4-dimethoxycinnamic acid (L9) and isothermal titration calorimetry to determine the binding parameters [1]. It was shown that in an acidic pH binding of 3,4-DMCA with the prion protein is characterized by protein:ligand ratio 1:0.35 with binding constant $K = (2.47 \pm 0.45) \times 10^6\ M^{-1}$ (corresponding to a dissociation constant of 405 nM). Consequently, the experimentally determined stoichiometry of protein–ligand complex of 3 to 1 suggests that 3,4-DMCA might bind anti-cooperatively to the prion protein, or, alternatively, stabilize naturally non-observed prion trimers that do not aggregate further under the same conditions [1]. It can be assumed that the mechanisms for preventing amyloid aggregation by cinnamic acid derivatives in the case of alpha-synuclein and prion protein are different.

In our work, we did not find any strong interactions between the monomeric form of alpha-synuclein and the 3 studied compounds, whereas 3,4-DMCA binds sufficiently strong to the monomeric form of prion protein both according to the results of molecular

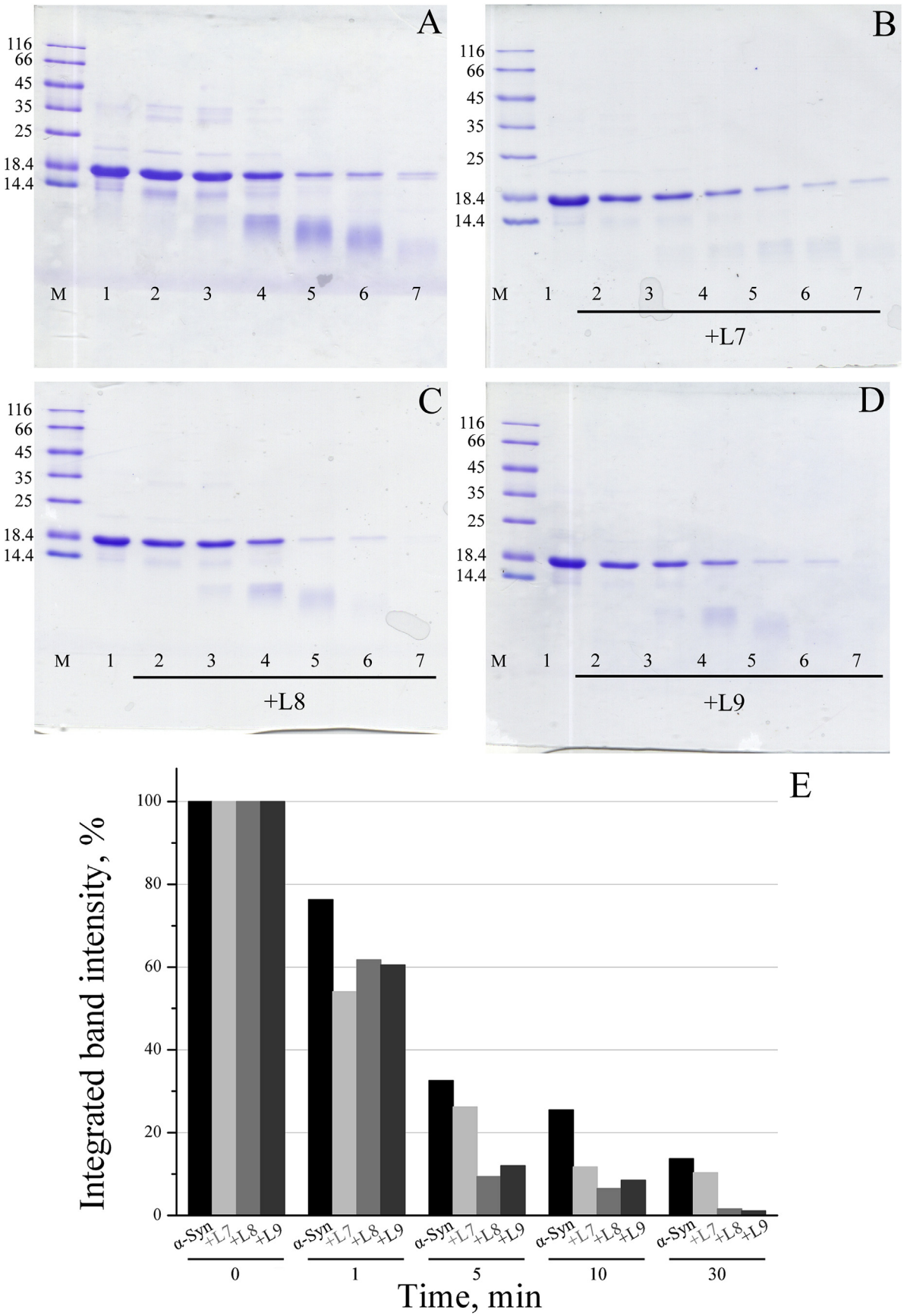


Fig. 7. The effect of cinnamic acid derivatives on alpha-synuclein fibrils susceptibility to digestion by proteinase K.
Alpha-synuclein fibrils grown without any additions (A) or in the presence of 3-methoxy-4-acetomidoxycinnamic acid (L7) (B); ferulic acid (L8) (C); 3,4-DMCA (L9) (D). E –

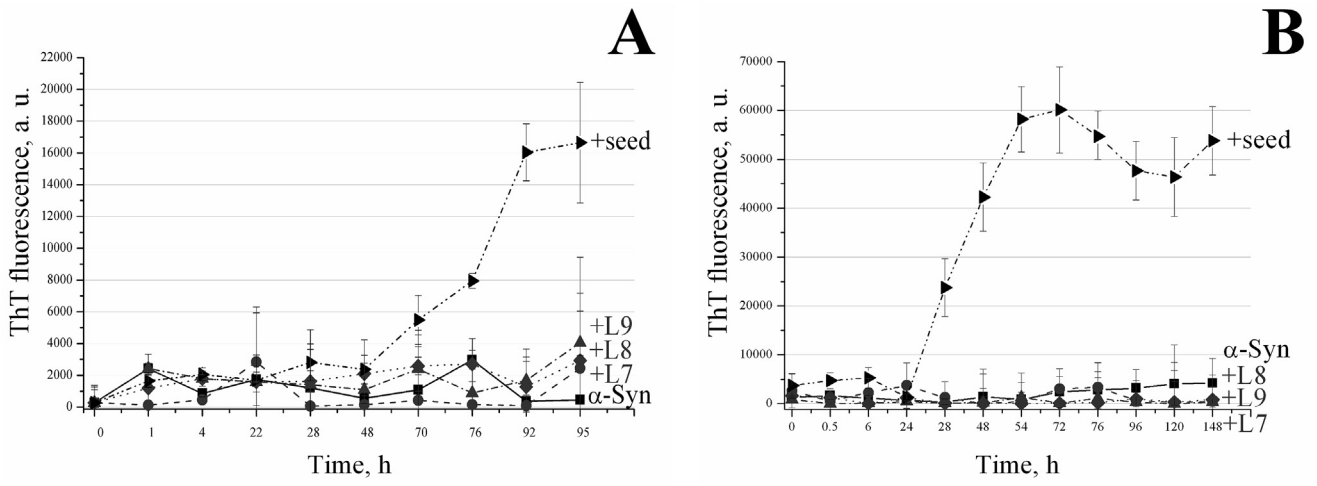


Fig. 8. The effect of cinnamic acid derivatives on seeding of wild-type (A) and A53T mutant (B) alpha-synuclein.
Seeds of wild-type (A) and A53T mutant (B) alpha-synuclein fibrils at 1:50 (seed:monomer) molar ratio were added to samples containing 0.4 mg/ml (28 μM) monomer of wild-type (A) or A53T mutant (B) alpha-synuclein, respectively, in PBS buffer, pH 7.4, without additions or in the presence of ligands 7–9. Tested compounds were added at 10:1 ligand:protein molar ratio. Samples were incubated at 37 °C under constant agitation. During the incubation, aliquots of 5 μl were taken for ThT assay (see Sections 2.3 and 2.4 for details). Data are given as mean values of three measurements ± SD. Seeding of wild-type (A) or A53T mutant (B) alpha-synuclein without additions is shown with triangles (►); seeding with 3-methoxy-4-acetamidoxycinnamic acid (L7) - with circles (●); seeding with ferulic acid (3-methoxy-4-hydroxycinnamic acid, L8) – with rhombi (◆); seeding with 3,4-DMCA (L9) - with triangles (▲). Alpha-synuclein WT without any additions is shown with squares (■).

modeling and experimental data. This is probably due to the fact that the prion protein has regions of an ordered structure, whereas in alpha-synuclein such regions are formed only during oligomerization and further fibrillation. Comparison of 3 cinnamic acid derivatives structures shows that ferulic acid (L8), the structure of which is completely identical to half of the curcumin molecule, has the maximum effect. Any side-chain substitutions reduce (in cases of L7 and L9) or completely prevent the antiaggregatory effect of cinnamic acid derivatives. From the look of it, the presence of a carbonyl group in position 4 and methoxy group in position 3 are necessary. Interestingly, 3,4-DMCA (L9) has a greater effect on the prion protein rather than ferulic acid (L8), probably due to the participation of the additional methyl group in binding to the monomeric form of the prion protein.

For prion protein, the binding of these ligands to the monomeric protein form suppresses its further aggregation and transformation. In the case of alpha-synuclein, cinnamic acid derivatives may change the structure of the primary aggregates, preventing the formation of full-length amyloid fibrils.

The studied ligands have different effects on aggregation and amyloid transformation of alpha-synuclein. 3,4-dimethoxycinnamic acid (L9) was less effective than the other two ligands since achievement of visible effect requires the addition of higher ligand concentrations (IC50 value of 251 μM). 3-methoxy-4-acetamidoxycinnamic acid (L7) and ferulic acid (L8) prevent alpha-synuclein amyloid transformation more effectively (with IC50 of 50 μM and 13 μM, respectively). Nevertheless, only for 3-methoxy-4-acetamidoxycinnamic acid (L7) it was possible to obtain CD spectra due to the formation of a sufficient quantity of aggregates. The same compound (L7) is the least effective in promoting proteolytic cleavage of alpha-synuclein fibrils. The reason for such an effect may be in the increased accumulation of aggregated but not amyloid forms of alpha-synuclein, which are less susceptible to proteolysis.

All studied ligands do not possess cytotoxicity towards neuroblastoma cell culture, which allows further study of the possibility of their use as antiaggregants. The most promising compound for the prevention and possibly treatment of synucleinopathies, especially Parkinson's disease, is ferulic acid (L8). Contrary to synthetic 3-methoxy-4-acetamidoxycinnamic acid (L7), ferulic acid can be obtained from raw plant materials (such as *Angelica sinensis*, *Cimicifuga heracleifolia,* and *Lignsticum chuangxiong*) or used in the form of enriched plant extracts [51]. Of particular note is the fact that ferulic acid is a natural metabolite, especially for humans, and it can be found in human blood at concentrations up to 140 nM after coffee consumption [52]. Implementation of 3,4-dimethoxycinnamic acid (L9) for the prevention of synucleinopathies can also be useful, despite its lower efficacy, because this compound is also present in human blood and its concentration also significantly increases after coffee ingestion.

4. Conclusions

Among the 9 studied cinnamic acid derivatives 3 compounds were found to reduce amyloid transformation of alpha-synuclein, their effectiveness decreasing in the following order: ferulic acid, 3-methoxy-4-acetamidoxycinnamic acid and 3,4-dimethoxycinnamic acid (with IC50 of 13, 50 and 251 μM, respectively).

Monomeric alpha-synuclein probably does not bind to the selected ligands, since no thermal effects are observed after their addition to protein monomers during isothermal titration calorimetry. The absence of changes in the circular dichroism spectra of alpha-synuclein monomers in the presence of the three ligands also confirms this assumption.

The molecular docking method revealed possible binding sites

Quantification of alpha-synuclein fibrils digestion rate by proteinase K depending on time. M – markers (116 – 14.4 kDa); 1- alpha-synuclein monomer; 2- alpha-synuclein fibrils (grown either with or without the cinnamic acid derivatives); 3- alpha-synuclein fibrils right after adding proteinase K into the sample; alpha-synuclein fibrils after 1 min (4), 5 min (5), 10 min (6) or 30 min (7) of proteolysis. The intensity at the start of incubation was taken as 100%. Samples containing 0.4 mg/ml (28 μM) of alpha-synuclein without additions and alpha-synuclein in the presence of tested compounds (10-fold molar excess) were incubated in PBS buffer, pH 4.0, at 37 °C under constant agitation (see Section 2.3 for details). After 2 days, the probes were incubated with proteinase K at 37 °C for 30 min total, analyzed by SDS-PAGE, and quantified by densitometric analysis (see Section 2.9 for details).

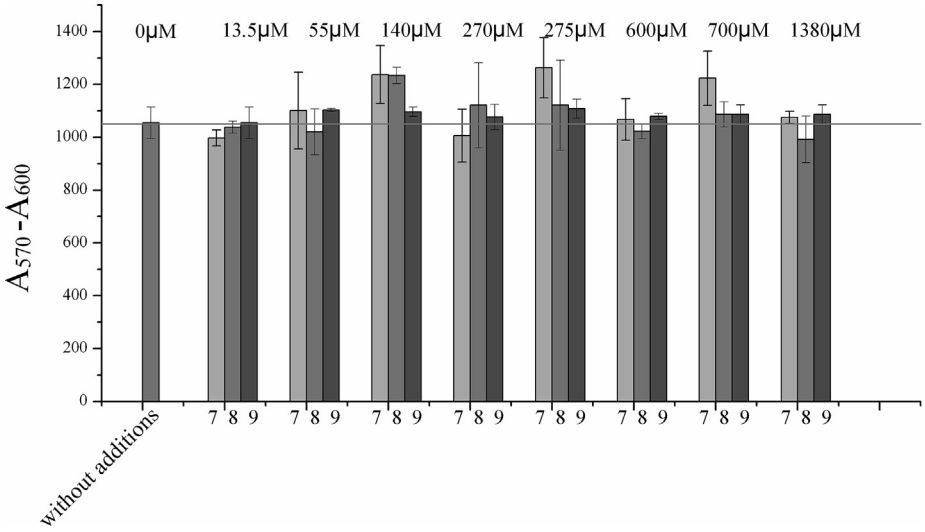


Fig. 9. The effect of the selected cinnamic acid derivatives (L7-L9) on neuroblastoma SH-SY5Y viability measured by resazurin assay.
Tested compounds were added directly into the culture medium to final concentrations 13.5–1380 μM, and incubated for 24 h. After incubation with resazurin, the absorbance at 570 nm (resorufin) and 600 nm (resazurin) was registered (see Section 2.11 for details). Each data point was replicated three times.

for the three selected ligands with fibrillar forms of alpha-synuclein.

The effect of ferulic acid, 3-methoxy-4-acetamidoxycinnamic acid and 3,4-dimethoxycinnamic acid on the formation of alpha-synuclein amyloid aggregates was confirmed by the use of fluorescence spectroscopy in the presence of Thioflavin T, measuring the turbidity of the samples, scanning ion-conductance microscopy, proteolytic cleavage and, in the case of 3-methoxy-4-acetamidoxycinnamic acid, circular dichroism spectroscopy.

Three selected compounds at the concentrations up to 1.38 mM do not possess cytotoxicity towards human neuroblastoma cell line, which indicates the possibility to use them for the prevention and treatment of synucleinopathies, including Parkinson's disease.

Given the natural origin of ferulic and 3,4-dimethoxycinnamic acid, which are contained in plant sources (cereals and coffee beans, respectively) and are natural metabolites present in human blood, they can be the most promising therapeutical for synucleinopathies' treatment.

Author contributions

P. Semenyuk designed, performed and analyzed molecular modeling experiments. Ion-conductance microscopy experiments were designed, performed and analyzed by V. Kolmogorov, P. Gorelkin, and A. Erofeev. K. Barinova, A. Melnikova and V. Muronetz conceived and designed all other experiments, which were performed by M. Medvedeva and A. Melnikova. V. Muronetz, M. Medvedeva and K. Barinova wrote the manuscript. All authors approved the final version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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