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Substituted cinnamides: characterization of non-toxic inhibitors of alpha-synuclein aggregation

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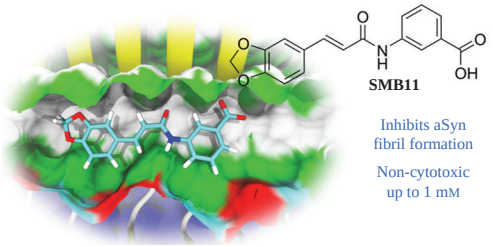
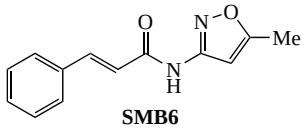
Four substituted cinnamides were studied as potential inhibitors of alpha-synuclein aggregation. (*E*)-3-[3-(Benzo[*d*][1,3]dioxol-5-yl)acrylamido]benzoic acid (SMB11) was shown to inhibit amyloid fibril formation of recombinant human alpha-synuclein according to Congo Red and partial proteolysis by proteinase K assays. This compound and its analog with 3-trifluoromethylphenyl substituent demonstrated no cytotoxicity on human neuroblastoma SH-SY5Y cells.



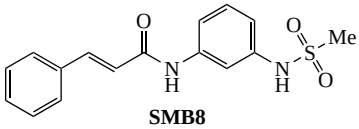
Keywords: alpha-synuclein, amyloid aggregation, cinnamides, surface docking, Parkinson's disease.

Accumulation of alpha-synuclein amyloid aggregates is one of the essential processes involved in the onset and progression of Parkinson's disease.[1] The multistage aggregation of alpha-synuclein implicates the formation of various oligomeric, prefibrillar, and fibrillar species with different toxicity.[2,3] Recent studies have shown that certain cinnamic and hydroxycinnamic acid derivatives can inhibit alpha-synuclein aggregation and reduce its toxicity[4,5] by altering the structure of primary aggregates and preventing the formation of full-length fibrils.[5] Inhibition of alpha-synuclein aggregation has also been shown for curcumin through binding to the monomeric form ultimately leading to inhibition of further fibrillation.[6,7] It is also known that unlike curcumin, natural and synthetic cinnamic acid derivatives possess better solubility and bioavailability and therefore are more promising for prevention and treatment of synucleinopathies.[8]

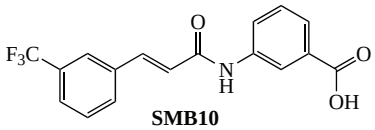
In the present work, we investigated the inhibition of alpha-synuclein aggregation by four synthetic substituted cinnamides **SMB6**, **SMB8**, **SMB10**, and **SMB11** using experimental approaches and molecular docking. The Lead Finder software[9] and a 'surface docking' approach were used to search for potential interaction sites of these small molecule compounds with synuclein fibrils. The synthesis of these compounds was reported previously.[10] Briefly, 150 cube-shaped potential energy grids with 20 Å sides were evenly distributed on the surface of the protein. Ligands were docked for 5 times into each grid, and then the most likely binding region was selected for each ligand.



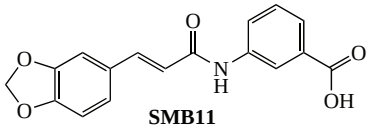
SMB6
N-(5-methylisoxazol-3-yl)cinnamide
M 228.25



SMB8
N-[3-(methanesulfonamido)phenyl]cinnamide
M 316.38



SMB10
(*E*)-3-[3-(3-trifluoromethylphenyl)acrylamido]benzoic acid
M 335.28



SMB11
(*E*)-3-[3-(benzo[*d*][1,3]dioxol-5-yl)acrylamido]benzoic acid
M 311.29

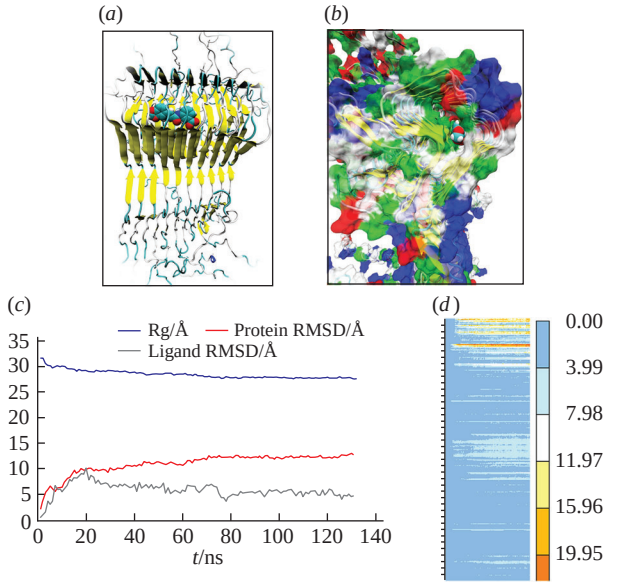


Figure 1 (*a*), (*b*) Probable position of **SMB11** binding to the synuclein fibril (PDB ID 2n0a) in two projections. Trajectory analysis for synuclein/**SMB11** complex: (*c*) radius of gyration, RMSD for protein and ligand; (*d*) RMSF analysis of the protein.

Figure 1(*a*),(*b*) schematically shows the most favorable binding position of the **SMB11** molecule to the synuclein fibril (PDB ID 2n0a) in two projections. A synuclein fibril has an internal narrow cylindrical cavity spanning throughout its length, where molecules suitable in shape and electrostatic potential can possibly bind. Based on the calculated binding energy, it is likely that molecules **SMB10** and more preferably **SMB11** bind effectively in this cavity. The stability of the found ligand binding was explored by 130 ns molecular dynamics simulations performed using the Desmond software. The simulation revealed that the ligand is stably bound within the fibril cavity throughout the simulation and does not undergo significant displacement over the timeframe [see Figure 1(*c*),(*d*)]. At the same time, the outermost strands of the fibril have unstable dynamics, and one can assume that both stabilization and destabilization of the growing fibril edge occurs upon binding of small molecule compounds, therefore making synuclein fibrils a druggable target.

The turbidimetry (measured in triplicate as absorbance at 400 nm to detect the process of protein aggregation) results shown in Figure 2 clearly indicate that **SMB11** reduces alpha-synuclein aggregation by about 40%. Upon addition of **SMB6** to the samples a significant turbidity is immediately observed with $OD_{400\ nm}$ of 0.216 in contrast to 0.01–0.04 for the other samples. As the most commonly used method for the detection of amyloid structures (Thioflavin T fluorescence analysis) was not suitable due to the intrinsic fluorescence of the compounds, the Congo Red assay[†] and proteinase K cleavage efficiency monitoring[‡] were used to detect the formation of amyloid aggregates of the obtained recombinant human alpha-synuclein.[§]

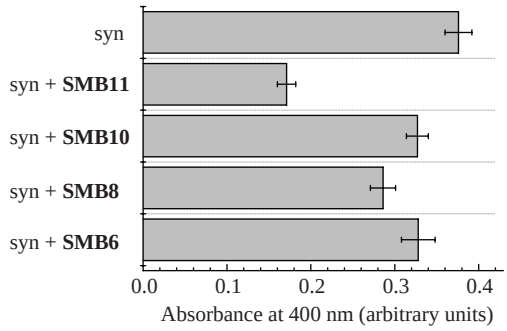


Figure 2 Aggregation of alpha-synuclein fibrillized during 48 h in the presence of **SMB** compounds. Light absorbance at 400 nm of 28 μM alpha-synuclein or 28 μM alpha-synuclein with 140 μM of **SMB** compounds for 48 h is shown after subtracting the initial (0 h).

The partial prevention of amyloid transformation of alpha-synuclein in the presence of **SMB11** is indicated by the shift in the Congo Red dye spectrum shown in Figure 3, as the presence of **SMB11** shifts the dye spectrum towards the spectrum specific

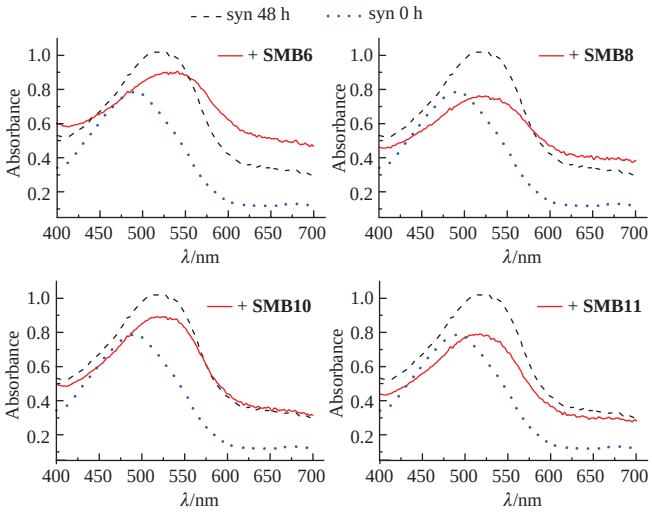


Figure 3 Congo Red absorbance changes of alpha-synuclein fibrillized in the presence of SMB compounds. Congo Red absorbance spectra of 28 μM alpha-synuclein monomer (dotted line), 28 μM alpha-synuclein after 48 h of fibrillation (dashed line), and 28 μM alpha-synuclein with 140 μM of **SMB** compounds after 48 h of fibrillation (solid line).

Table 1 Congo Red absorbance changes of alpha-synuclein fibrillized in the presence of SMB compounds.

Sample	λ_{max}/nm		Δ/nm
	0 h	48 h	
α-syn	494	524	30
α-syn + **SMB6**	488	540	52
α-syn + **SMB8**	491	525	34
α-syn + **SMB10**	497	528	31
α-syn + **SMB11**	496	519	23

[†] Freshly prepared water solution of Congo Red was added to tested samples in PBS buffer, pH 4.0 at a molar ratio of 10 : 1. Congo Red was incubated for 10 min with protein samples before the measurements. Spectra of Congo Red absorption were acquired using Implen NanoPhotometer® NP80 (Germany) at 20 °C in a 3-mm-path-length cuvette in triplicate.

[‡] Protein samples were incubated with 0.2 μg ml^{-1} proteinase K in the presence of 5 μM Ca^{2+} at 37 °C. Proteolysis was stopped by the addition of Laemmli buffer (without β-mercaptoethanol) to the samples and by the instant boiling of the sample for 5 min. Then 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and consequent staining of gel with Coomassie blue R-250 were performed.

[§] Full-length wild type alpha-synuclein without additional motifs was expressed in *E. coli* and purified as described (ref. 11) with minor modifications. In order to avoid erroneous cysteine incorporation in bacterial system (refs. 11, 12), codon encoding of Tyr136 was mutated from TAC to TAT, also encoding tyrosine. The ammonium sulfate precipitate of alpha-synuclein was dialyzed against PBS buffer, pH 4.0, and was diluted to a final concentration of 0.4 mg ml^{-1} (28 μM) with or without 10-fold molar excess of tested compounds prepared in DMSO stock solutions. Growing fibrils pipetted (by 0.3 ml) into glass tubes, and incubated at 37 °C with constant agitation at 600 rpm for 52 h. During the incubation, the turbidity of samples was measured as light absorption at 400 nm in order to detect the process of protein aggregation.

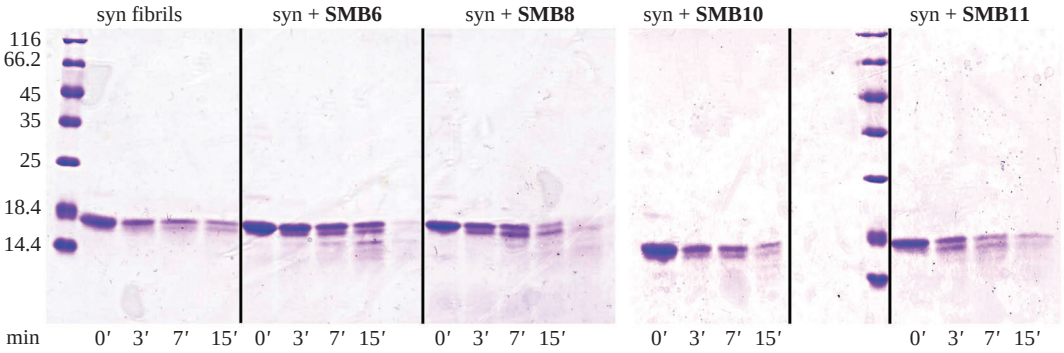


Figure 4 SDS-PAGE analysis of samples after proteinase K cleavage.

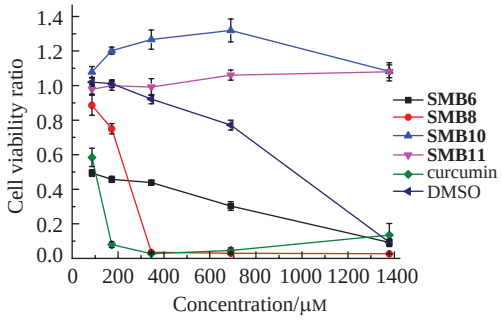


Figure 5 Cytotoxicity of compounds on human SH-SY5Y neuroblastoma cells.

for the monomeric form of alpha-synuclein (Table 1). In addition, the presence of **SMB6** leads to a strong increase in amyloid transformation in the Congo Red assay.

The resistance of the fibrils to proteolysis by proteinase K was assessed by SDS-PAGE analysis of the proteolysis products, followed by calculation of the area of the stained protein bands. Within 3 min of incubation, synuclein fibrillated in the presence of **SMB11** cleaved 15% more efficiently than untreated fibrils (Figure 4). At the same time, alpha-synuclein protein fibrils obtained in the presence of SMB6 were twice more resistant to 15 min proteolysis compared to control fibrils.

Additionally, we studied the cytotoxicity of the compounds on human SH-SY5Y neuroblastoma cells using the MTT test.[¶] Curcumin, a known natural inhibitor of alpha-synuclein amyloid aggregation,[6,7,13] was used as a control compound. As can be seen from Figure 5, both **SMB10** and **SMB11** are non-toxic up to millimolar concentrations, and can even alleviate the decrease in cell viability caused by relatively high concentrations of DMSO.

[¶] Cells SH-SY5Y were placed in 96-well plates (3000 cells in 0.1 ml DMEM supplemented with 2% FBS) and treated with 86–1380 μM concentrations of inhibitors. Each concentration was tested fourfold. Cytotoxicity was measured using a standard MTT assay after drug exposure for 24 h. The results were quantified using a Universal Microplate Reader (Bio-Rad) at a 570 nm wavelength.

In conclusion, compound **SMB11** showed the ability to inhibit amyloid alpha-synuclein aggregation and is non-toxic to neuroblastoma SH-SY5Y cells in relatively high concentrations, *i.e.*, it appears most promising for further research as an anti-Parkinsonian agent.

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