A strategy for designing inhibitors of α-synuclein aggregation and toxicity as a novel treatment for Parkinson’s disease and related disorders

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ABSTRACT

Convergent biochemical and genetic evidence suggests that the formation of α-synuclein (α-syn) protein deposits is an important and, probably, seminal step in the development of Parkinson’s disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). It has been reported that transgenic animals overexpressing human α-syn develop lesions similar to those found in the brain in PD, together with a progressive loss of dopaminergic cells and associated abnormalities of motor function. Inhibiting and/or reversing α-syn self-aggregation could, therefore, provide a novel approach to treating the underlying cause of these diseases. We synthesized a library of overlapping 7-mer peptides spanning the entire α-syn sequence, and identified amino acid residues 64–100 of α-syn as the binding region responsible for its self-association. Modified short peptides containing α-syn amino acid sequences from part of this binding region (residues 69–72), named α-syn inhibitors (ASI), were found to interact with full-length α-syn and block its assembly into both early oligomers and mature amyloid-like fibrils. We also developed a cell-permeable inhibitor of α-syn aggregation (ASID), using the polyarginine peptide delivery system. This ASID peptide was able to inhibit the DNA damage induced by Fe(II) in neuronal cells transfected with α-syn(A53T), a familial PD-associated mutation. ASI peptides without this delivery system did not reverse levels of Fe(II)-induced DNA damage. Furthermore, the ASID peptide increased ($P<0.0005$) the number of cells stained positive for Bcl-2, while significantly ($P<0.05$) decreasing the percentage of cells stained positive for BAX. These short peptides could serve as lead compounds for the design of peptidomimetic drugs to treat PD and related disorders.

Key words: amyloid fibrils • drug discovery • neurodegeneration • peptide delivery
lesions in the brain known as Lewy bodies (LBs) and Lewy neurites (LNs) constitute the main histopathological features in the brains of patients with Parkinson’s disease (PD) and dementia with Lewy bodies (DLBs). Amyloid-like fibrils composed of a small protein (~14 kDa) named α-synuclein (α-syn) are the main component of LBs and LNs (1, 2). The first indication that α-syn could be involved in neurodegenerative diseases came from work on the non-Aβ component (NAC) isolated from brain amyloid preparations from cases of Alzheimer’s disease (AD) (3). NAC was found to correspond to residues 61–95 of α-syn (3). The pathogenic importance of α-syn was firmly established when two different point mutations in the gene encoding α-syn (resulting in amino acid substitutions A53T and A30P) were found to be the cause of rare, inherited forms of PD (4, 5). α-Syn is also associated with pathological lesions in other neurodegenerative diseases, sometimes involving nonneuronal cells, such as the glial cytoplasmic inclusions (GCIs) found in multiple system atrophy (MSA) (6, 7). The diseases involving α-syn are collectively known as the “synucleinopathies” (8). Recently, it has been reported that lesions similar to those found in the human synucleinopathies can be created in transgenic animals expressing high levels of human wild-type or mutant α-syn. These animals progressively develop a loss of dopaminergic cells in the substantia nigra, together with motor abnormalities consistent with the synucleinopathies (9, 10). These studies provide strong evidence for the central role of α-syn deposition in the pathogenesis of these diseases. The aggregation of α-syn appears to be essential for LB, LN, and GCI formation, since fibrillar aggregates of α-syn are the major component of these inclusions. Protein conformation-dependent neurotoxicity is a common theme in other neurodegenerative disorders such as AD, the “tauopathies”, Huntington’s disease and the prion diseases (11). A strong argument can be made in support of the hypothesis that protein aggregation is a seminal feature of all of these diseases (8, 11, 12). Recent studies support the idea that early “soluble oligomers” (including so-called protofibrils and Aβ-diffusible ligands, or “ADDLs”) are the pathogenic species that drive neurodegeneration and neuronal cell death, rather than mature amyloid fibrils (13–18). Small molecules that can block, slow down, or reverse α-syn aggregation, particularly in its early stages, therefore provide an attractive therapeutic approach for targeting the underlying disease progression of the synucleinopathies. Currently, there is no cure or treatment that significantly retards the progression of these diseases.

The fact that α-syn belongs to the class of natively unfolded proteins, which have little or no ordered structure under normal physiological conditions, makes the rational design of compounds that can stabilize the native, nontoxic conformation of α-syn a challenging task. Previous studies have shown that full-length recombinant α-syn can self-aggregate in vitro in the absence of other proteins to form amyloid-like fibrils with a similar ultrastructural appearance to those observed in LBs, LNs, and GCIs (18–23). Interestingly, the two α-syn mutations associated with inherited forms of PD appear to accelerate the formation of toxic oligomers (18–20). It is believed that the conversion of α-syn into these oligomers, and then into insoluble fibrils, is initiated by conformational changes from random coil to β-sheet structure (18–23).

Recently, it has been reported that modified synthetic peptides based on the native sequences of amyloid peptides or proteins are able to prevent their conversion to β-sheet-rich aggregated structures (24–28). Interestingly, these peptide inhibitors can also block the toxicity of the amyloid aggregates both in vitro in cell culture models and in vivo in animal models (27–31),...
suggesting that these peptide inhibitors could be useful therapeutically. The aim of the work reported here was to design short synthetic peptides that are capable of inhibiting the aggregation of α-syn into toxic oligomers. Our strategy was, first of all, to identify the critical binding region in the α-syn molecule responsible for its self-association and aggregation. It was thought that short synthetic peptides homologous to this region would interact with the same region in the full-length α-syn molecule, and block its self-association into oligomers and amyloid fibrils. To identify the appropriate binding region, we synthesized a library of overlapping short peptides spanning the entire α-syn primary sequence. The binding of these peptides to α-syn was examined by means of an ELISA system. Peptides capable of binding to α-syn were then modified and tested for their ability to inhibit the formation of α-syn oligomers and fibrils. A cell-permeable inhibitor was also tested in a cell culture model of α-syn aggregation and toxicity.

MATERIALS AND METHODS

Peptide library synthesis

All Fmoc protected amino acids were purchased from Advanced Chemtech Europe (Cambridge, UK), Fmoc-6-aminohexanoic acid was purchased from CN Biosciences U.K. (Nottingham, United Kingdom), and d-biotin from Sigma-Aldrich Ltd. (Dublin, Ireland). The PAL support amide resin and N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU) were purchased from PE Applied Biosystems (Cheshire, United Kingdom); piperidine, N,N-diisopropylethylamine (DIEA), dichloromethane (DCM) and N,N-dimethylformamide (DMF) were all from Rathburn Chemicals Ltd. (Walkerburn, Scotland).

Multiple peptide synthesis was carried out at 0.010 mmol scale using an Advanced Chemtech 396 peptide synthesizer (Advanced Chemtech Europe, Cambridge, United Kingdom), using standard Fmoc chemistry. A sixfold excess of amino acid, 6-aminohexanoic acid and d-biotin was used, and coupling was achieved using HATU. The Fmoc-6-aminohexanoic acid was coupled onto the N terminus of the peptide sequence, and after removal of the Fmoc from 6-aminohexanoic acid, the biotin was then coupled onto the aminohexanoic linker using double coupling. Cleavage of the peptides was carried out automatically by the machine over 2 h incubation at room temperature with a mixture of thioanisole, ethanedithiol, triisopropylsilane, water, and trifluoroacetic acid (TFA) (2:1:1:1:95 v/v/v/v/v). Peptide purity was ascertained by reverse phase HPLC using a C5 reverse-phase 250 mm × 4.6 mm column (Phenomenex); peptide identity was verified using a Finnigan LCQ ion trap mass spectrometer. Most of the hydrophobic peptides were not soluble in aqueous solutions; therefore, peptide solutions for the ELISA studies were prepared from stock solutions in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Company Ltd., Dorset, England) at 2.2 mg/ml.

Peptide synthesis

All Fmoc-protected amino acids, Fmoc PAL Support amide resin, Fmoc-L-Val-PEG-PS resin, HATU, DIEA, DMF, N-methylpyrrolidone NMP, Piperidine, and DCM, were purchased from PE Applied Biosystems (Cheshire, United Kingdom).
Peptides were synthesized at 0.1 mmole scale using an Applied Biosystems 433 Peptide Synthesizer using standard Fmoc chemistry as described recently (32). In brief, a 10-fold excess of amino acid was used, and coupling was achieved using HATU. Cleavage of the peptides was carried out over a 2 h incubation at room temperature with a mixture of thioanisole, ethanethiol, triisopropylsilane, water and TFA (2:1:1:1:95 v/v/v/v/v).

Peptide purity was ascertained by reverse-phase HPLC using a C5 reversed-phase 250 mm × 4.6 mm column (Phenomenex); peptide identity was verified using a Finnigan LCQ ion trap mass spectrometer.

**Preparation of α-syn**

Recombinant α-syn from three different sources was used in this study, as follows: (1) Recombinant α-syn was expressed in *Escherichia coli* and purified by Ross Jakes (LMB-MRC, Cambridge, United Kingdom) as described previously (19, 22); (2) Recombinant α-syn was expressed in *E. coli* and purified by J.E.K., F.E.B. and D.A. (full details to be published elsewhere); (3) Recombinant α-syn was purchased from Recombinant Peptide Technologies, (Athens, GA, USA). Similar results were obtained with α-syn from all three sources.

**Peptide binding studies**

Recombinant α-syn (100 pmoles/well in 100 µl) in 200 mM NaHCO3, pH 9.6, was immobilized on Maxisorb microtiter plates (Nunc Maxisorb; Life Technologies, Paisley, Scotland) by incubation overnight at 4°C. The plate was washed 4 times with standard phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 (PBST). 100 µl/well of blocking buffer (PBS containing 2.5% gelatin and 0.05% Tween 20) was added and incubated for 2 h at 37°C. The plate was washed 4 times with PBST and then 100 µl/well of biotinylated test peptide (1000 pmoles/well in PBS containing 0.5% DMSO) was added. The plates were incubated for 2 h at 37°C and washed 4 times with PBST, before the addition of 100 µl/well ExtrAvidin-alkaline phosphatase (Sigma-Aldrich Company Ltd.) diluted 3:5000 in blocking buffer. After incubation for 1 h at 37°C, the plate was washed 4 times in PBST and 100 µl/well of alkaline phosphatase Yellow “pNPP” (Sigma-Aldrich Company Ltd.) was added. After further incubation of the plate for 30 min at room temperature, for color development, absorbance values (at 405 nm) were read on a Victor^2^ 1420 (Wallac) multilabel microtiter plate reader.

**Inhibition of α-syn fibril formation**

Recombinant α-syn was dissolved in PBS at 50 µM, either alone or with peptide inhibitor, at molar ratios (α-syn: inhibitor) of 2:1; 1:1, and 1:2. The samples were incubated at 37°C for up to 7 days in an Eppendorf Thermomixer with continuous mixing (1000 rpm). Fibril formation was monitored by fluorescence of the dye Thioflavin T (Th-T) (Sigma-Aldrich Company). A 10 µl sample of each protein/peptide solution was diluted into 190 µl of PBS containing 20 µM Th-T. Fluorescence was measured in black 96-well plates using a Victor^2^ 1420 multilabel microtiter plate reader, with excitation at 440 nm and emission at 490 nm. To allow for background fluorescence, the fluorescence intensity of a blank PBS solution was subtracted from all readings.
Inhibition of α-syn oligomerization

Aggregation of α-syn

Purified samples of α-syn in sterile PBS (pH 7.4) at 50 µM, either alone or with peptide inhibitor at molar ratios (α-syn: inhibitor) of 2:1; 1:1, and 1:2 in parafilm-sealed, 1.5 ml Eppendorf tubes, were incubated at 37°C for 4 days in an Eppendorf Thermomixer with continuous mixing (1000 rpm). Samples were collected at various time points and stored at –80°C until tested by an ELISA for oligomeric α-syn.

An ELISA to measure α-syn oligomers

A 96-well ELISA plate (Nunc Maxisorb; Life Technologies, Paisley, Scotland) was coated by overnight incubation with 1 µg/ml of nonbiotinylated mouse monoclonal antibody (mAb) 211 (100 µl/well), in 200 mM NaHCO₃ (Sigma-Aldrich Company Ltd.), pH 9.6, containing 0.02% (w/v) sodium azide at 4°C, washed 4 times with PBST. Antibody 211 recognizes amino acid residues 121–125 of human α-syn and is from Santa Cruz Biotechnology (Santa Cruz, California, USA). The plate was incubated with 200 µl/well of blocking buffer (see method for peptide binding studies) for 2 h at 37°C. The plate was washed 4 times with PBST, and 100 µl/well of each of the samples to be tested were added to each well (fresh or aged α-syn solutions incubated alone or with peptide inhibitors were diluted to 500 nM in PBS). The plate was incubated at 37°C for 2 h. After washing 4 times with PBST, 100 µl of biotinylated mAb 211 diluted to 1 µg/ml in the blocking buffer was added and incubated at 37°C for 2 h. The wells were washed 4 times with PBST and incubated with 100 µl/well of ExtrAvidin-alkaline phosphatase (Sigma-Aldrich Company Ltd.) diluted 3:5000 in blocking buffer, and incubated for 1 h at 37°C. The wells were then washed 4 times with PBST, before adding the enzyme substrate Yellow “pNPP” (Sigma-Aldrich Company Ltd.) (100 µl/well), and leaving the color to develop for 30 min at room temperature. Absorbance values at 405 nm were determined using a Victor2 1420 (Wallac) plate reader.

Effects of peptides on NAC and Aβ aggregation

The Aβ (1–40) peptide or NAC was dissolved in water and then diluted 1:1 in 2×PBS. The final concentrations of NAC or Aβ (1–40) were 100 µM, either alone or with peptide inhibitors at molar ratios (NAC or Aβ: inhibitor) of 2:1; 1:1, and 1:2. The samples were thereafter incubated at 37°C. Fibril formation was monitored by fluorescence of the dye Th-T (Sigma-Aldrich Chemicals). A 10 µl sample of the peptide solution was diluted into 190 µl of PBS containing 20 µM Th-T. Fluorescence was measured as described above for α-syn aggregation.

An ELISA to measure Aβ oligomers

This assay depends on use of the same anti-Aβ monoclonal antibody for capture and detection (6E10 and biotinylated 6E10, respectively) in a sandwich format (32). Ninety-six-well microtiter plates (Nunc Maxisorb; Life Technologies, Paisley, Scotland) were coated overnight at 4°C with 100 µl/well of 1 µg/ml 6E10 (Signet Pathology Systems Inc., Dedham MA, USA) in PBS and blocked with 200 µl/well of blocking buffer (see method for peptide binding studies) for 2 h at
37°C. The capture plates were washed 4 times with PBST, and 100 µl of each Aβ sample to be tested was added to each well (fresh or aged Aβ solutions incubated alone, or with peptide inhibitors, were diluted to 1 µM in PBS). After 2 h incubation at 37°C, the plates were washed 4 times with PBST and incubated for 2 h at 37°C with biotinylated 6E10 (1 µg/ml in blocking buffer, 100 µl per well). The plates were again washed 4 times with PBST, before the addition of 100 µl/well of ExtrAvidin-alkaline phosphatase (Sigma-Aldrich Company Ltd.) diluted 3:5000 in blocking buffer, and incubated for 1 h at 37°C. The wells were then washed 4 times with PBST, before adding the enzyme substrate Yellow “pNPP” (Sigma-Aldrich Company Ltd.) (100 µl/well), and leaving the color to develop for 30 min at room temperature. Absorbance values, at 405 nm, were determined using a Victor® 1420 (Wallac) plate reader.

Electron microscopy (EM)

Purified samples of α-syn in sterile PBS (pH 7.4) at 50 µM, either alone or with peptide inhibitor at molar ratios (α-syn: inhibitor) of 1:2 in parafilm-sealed, 1.5 ml Eppendorf tubes, were incubated at 37°C for 4 days in an Eppendorf Thermomixer with continuous mixing (1000 rpm). The samples were deposited onto formvar/carbon-coated grids, fixed briefly with 0.5% glutaraldehyde, negatively stained with uranyl acetate, and examined on a JEOL JEM-1010 transmission electron microscope.

Analysis of α-syn aggregation by immunoblotting

Fresh and aged α-syn solutions were diluted in NuPAGE sample buffer (Invitrogen Ltd., Paisley, UK) and samples (10 ng) were separated on NuPAGE Bis-Tris 4–12%, 1 mm gels (Invitrogen Ltd., Paisley, UK). The separated proteins were transferred to nitrocellulose membranes (0.45 µm; Invitrogen Ltd., Paisley, United Kingdom) at 125 mA for 45 min. The membranes were boiled for 5 min in PBS, and then blocked with 5% marvel dried skimmed milk, dissolved in PBST, for 1 hr. The membranes were probed with mAb 211, overnight at 4°C, and then washed several times with PBST, followed by incubation with HRP-conjugated goat anti-mouse (Dako Ltd., Ely, United Kingdom) for 60 min at room temperature, with gentle agitation. The membranes were extensively washed with PBST for 25 min. The immunoreactive bands were visualized with a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, USA) as described by the manufacturer.

Cell culture

The production of stable cell lines overexpressing mutant α-syn(A53T) from parental BE(2)-M17 (M17-A53T) human dopaminergic neuroblastoma cells has been detailed elsewhere (33, 34). Cells were grown routinely in DMEM (Gibco BRL, Rockville, MD USA) containing (10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 2 mM Glutamine, and 50 µg/ml of Geneticin (G418) (from Roche, Basel, Switzerland) in 75 cm² flasks (Nunc, Life Technologies, Paisley, Scotland) at 37°C in a humidified incubator with 5% CO₂/95% air.
Peptide internalization and visualization

Cells were grown on cover slips (Sarstedt Ltd., Leicester, United Kingdom) placed in a 30 mm dish (3 ml/dish). After the cells were allowed to attain 75% confluence, the culture medium was exchanged. The cells were incubated at room temperature for 10–60 min with the fresh medium (1 ml/dish) containing 1 µM or 10 µM of fluorescein-labeled peptide. Cells were fixed with 4% formaldehyde for 30 min after which they were washed 5 times with 80% ethanol, twice with water and 3 times with PBS. Then the cover slips were mounted on microscope slides using DPX (Sigma-Aldrich Company Ltd.). The distribution of fluorescein-labeled peptide was analyzed on a SP2 AOBS (Leica Microsystems (UK) Ltd, Milton Keynes, United Kingdom) confocal microscope using a 10× or 40× oil immersion lens.

Cell treatments and the ‘Comet’ assay

Alkaline lysis followed by alkaline gel electrophoresis was employed in order to detect DNA single-strand breaks (SSBs) (35–37). Before incorporation into the Comet assay, cultured cells were allowed to attain confluence in 12-well multiwell dishes (35). Peptides, dissolved in sterile distilled H₂O, were added to the cells, at a final concentration of 1 µM, 15 min before treatment with redox metal. Iron(II) chloride (FeCl₂) (Sigma-Aldrich Company Ltd.) stocks were added as freshly prepared solutions in DMSO to give a final concentration of 10 µM, and DMSO was used as a vehicle control: DMSO concentrations did not exceed 1% v/v. Cells were incubated at 37°C for 2 h in the presence or absence of Fe(II), as indicated. Post-treatment, cells were disaggregated with cell dissociation solution (Sigma-Aldrich Company Ltd.) and resuspended in 1 ml PBS. To each 1 ml cell suspension in PBS, 1 ml of warm 1% low melting point (LMP) agarose (made up in PBS) was added and gently vortexed (36). Single-cell suspensions in LMP agarose (150 µl) were then evenly applied to microscope slides and allowed to set on a cold surface for 5 min. The slides were subsequently submerged in cold lysis solution (2.5 M NaCl, 100 mM ethylene diamine tetra-acetic acid (EDTA), 10 mM Tris, 1% Triton X-100, and 10% DMSO), protected from light and stored at 4°C for at least 1 h. Then the slides were transferred to a light-tight container and covered in electrophoresis solution (0.3 M NaOH, 1 mM EDTA, freshly prepared, pH > 13), and stored for 20 min at 4°C to allow DNA unwinding. Finally, slides were transferred to a horizontal electrophoresis tank and covered in fresh electrophoresis solution before electrophoresis at 0.8 V/cm and 300 mA for 24 min. After electrophoresis, slides were neutralized (Tris-HCl, 0.5 M, pH 7.5) and stained with ethidium bromide (20 ng/ml) after which Comet tail length (CTL) (µm) was visualized by epifluorescence using a Leitz Dialux 20 EB microscope. A total of 100 digitized images/data points, 50 from each of two duplicate slides, was measured in each experiment. CTL measurements obtained were compared using the nonparametric Mann-Whitney test.

Immunohistochemical staining

Cells were disaggregated and resuspended in complete medium before seeding aliquots (5 ml, ~1×10⁵ cells) into 60 mm petri dishes containing 24 mm glass cover slips (37). After 24 h incubation, cells were treated for 4 h with freshly prepared Fe(II) in DMSO. Peptide inhibitors in sterile distilled H₂O were added as indicated 15 min before Fe(II) treatment. The medium was then aspirated and the cells were washed with PBS before immediate fixation with CytoFixx fixative (CellPath plc, Skelmersdale, Lancashire, United Kingdom). The antibodies were
obtained from DakoCytomation (Ely, Cambridgeshire, United Kingdom) and were diluted in Tris-buffered saline (0.05 M, pH 7.6) (TBS). Bel-2 mouse monoclonal (124, Isotype: IgG1) antibody at a 1:100 dilution and BAX rabbit polyclonal antibody at a 1:50 dilution were used. Fixative was removed by soaking the cover slips in 95% industrial methylated spirits for 30 min. Following a 5 min wash with tap water, cover slips were incubated in 1:5 normal goat sera in TBS for 15 min in a humidified environment. After removal of excess sera, the cover slips were incubated with primary antibody (see above) for 1 h at room temperature. Using the StreptABComplex duet kit (DakoCytomation, Ely, Cambridgeshire, United Kingdom) as recommended by the manufacturer, the cover slips were washed with TBS for 5 min, and then incubated for 30 min with secondary antiseras (goat anti-mouse/rabbit as appropriate) in TBS and then washed with TBS for 5 min. Then the cover slips were incubated with tertiary antiseras (avidin–biotin complex) in TBS for 30 min and washed again with TBS for 5 min. 3,3′-Diaminobenzidine (“DAB”) chromogen in Tris-HCl buffer (0.05 M, pH 7.6) with H₂O₂ (0.1%) was applied to preparations for 15 min followed by another 5 min tap water wash. Finally, the slides were transferred to a rack and stained (1 min) with haematoxylin (50%) rinsed with tap water, blued in Scott’s tap water for 15 s, and rinsed again. The preparations were stained for 1 min with eosin (0.1% in 0.1% CaCl₂.6H₂O), rinsed with water and dehydrated with graded alcohol solutions through to xylene. Cell preparations were then mounted on microscope slides with Pertex (CellPath plc, United Kingdom). The percentage of cells staining positive was determined as the mean ± SD of five separate counts. Treatments were compared using an unpaired t test with Welch’s correction.

RESULTS

Identification of the binding region in the α-syn molecule responsible for its self-association

We synthesized an overlapping library of synthetic 7-mer peptides, corresponding to amino acid residues 1–7, 2–8, 3–9,…, 132–138, 133–139, 134–140 of α-syn. These peptides were tagged with a biotin group, which was attached via a 6-aminohexanoic spacer to the N terminus of each peptide. The biotin tag was used to detect binding of the peptides to full-length α-syn, which was immobilized on a microtiter plate. Because the central NAC region of α-syn has already been shown to bind to α-syn (38), we included biotinylated-NAC (Biot-NAC) as a positive control for the ELISA. The binding of Biot-NAC to full-length α-syn could be detected by adding ExtrAvidin-alkaline phosphatase, followed by an appropriate enzyme substrate to give a colored reaction product. Several preliminary experiments were conducted with Biot-NAC in order to optimize the signal-to-noise ratio of the assay. When we tried two different blocking buffers, we found that a blocking buffer containing 5% BSA gave a high background and low binding of Biot-NAC to α-syn. A decreased background in the ELISA was obtained when PBS containing 2.5% gelatin and 0.05% Tween 20 was used as the blocking buffer and, furthermore, the binding of Biot-NAC to α-syn was increased (data not shown). NAC presumably binds nonspecifically to BSA, which leads to an increase in the background signal measured. We also investigated different concentrations of Biot-NAC and α-syn. A concentration of up to 1000 pmole/well of Biot-NAC, with 100 pmole/well of α-syn, produced the highest binding level, with a low background signal. NAC without any biotin tag gave no signal, which shows that ExtrAvidin-alkaline phosphatase does not bind nonspecifically to the ELISA plates (data not shown). The biotinylated 7-mer peptides from the library were then screened for binding to α-syn, using this
ELISA, and those peptides capable of binding to the full-length molecule were identified. Figure 1A and B show the results for each peptide, with the data expressed as percentage of binding relative to the Biot-NAC control. We also tested 7-mer peptides from the library without any biotin tag, as controls. These gave no signal, which shows again that ExtrAvidin-alkaline phosphatase does not bind nonspecifically to the ELISA plates. Peptides covering the hydrophobic region located in the central part of α-syn (amino acids 64–100) produced the highest binding levels (Fig. 1A). Peptides covering the N-terminal region (residues 1–60) of α-syn had low binding levels (Fig. 1A), whereas peptides covering the C-terminal region (residues 101–140) of α-syn showed no binding at all (Fig. 1B). Interestingly, the peptides which gave the highest level of binding to α-syn were found to precisely cover the N-terminal half of the NAC region (amino acids 64–86 of α-syn).

**Designing peptides as inhibitors of α-syn aggregation**

On the basis of the results from screening the peptide library for binding to α-syn, we concluded that the N-terminal region of NAC (residues 64–86 of α-syn) is the main binding region responsible for the self-association and consequent aggregation of α-syn (Fig. 1A). Our strategy to design compounds to inhibit α-syn aggregation was based on small peptides from the binding region (which should interact specifically with full-length α-syn and interrupt its aggregation) linked to further solubilizing amino acid residues. For the binding sequence, we chose the hydrophobic residues 68–72 of α-syn (GAVVT). The strategy that we adopted for the solubilizing component was to place one or two hydrophilic residues at the N- and/or the C-terminus of this GAVVT peptide. We initially chose the cationic arginine (R) residue for this purpose. However, this has the disadvantage that it could decrease the interaction between the peptide and monomeric α-syn, since the targeted binding region in α-syn does not contain these R residues. Glycine (G) is also a hydrophilic residue and is the most conformationally unrestrained amino acid, and so generally breaks secondary structure. Therefore, placing G residues as spacers between the R and the binding residues GAVVT should result in the solubilizing component of the inhibitor peptide, that is, the first two (RG) and/or last two residues (GR), not partaking in binding between the inhibitor and α-syn. Interestingly, the native sequence of α-syn (67–73) already contains a G residue at both the N- and C-terminus. On the basis of these criteria, we designed several peptides, referred to here as α-syn inhibitors (ASI), for evaluation in this study (see Table 1). Because we found that peptides covering the N-terminal region of α-syn had low binding to the α-syn molecule (Fig. 1A), we included some peptides from this region as a control in our study (see Table 1). The N-terminal control (NTC) peptides included the hydrophilic residues 6–10 (KGLSK) (NTC1), the hydrophobic residues 14–18 (GVVAA) (NTC2) and the repetitive amino acid motif (KTKEG) (NTC3). These control peptides also had (RG) and (GR) as additional solubilizing components, similar to the ASI peptides (see Table 1).

**Effect of the peptides on α-syn fibril formation**

Incubation of recombinant α-syn at 50 µM with continuous shaking for 96 h at 37°C in PBS (pH 7.4) led to aggregation of the protein and the formation of amyloid-like fibrils, as confirmed by negative stain EM (see below). Amyloid fibril formation by α-syn was monitored over 96 h by
the Th-T binding assay (Fig. 2A). When α-syn was incubated with the ASI1 (RGAVVTGR-NH₂) peptide at 2:1; 1:1, and 1:2 (α-syn: peptide molar ratio) with a constant α-syn concentration of 50 µM, complete inhibition of amyloid fibril formation was achieved (Fig. 2A). Under the same conditions, NTC peptides from the N-terminal region of α-syn showed no inhibition of α-syn fibrillogenesis (Fig. 2B), and at lower concentrations, these peptides even seemed to accelerate the rate of aggregation (Fig. 2B). Thus, only those peptides designed from the binding region had the potential to act as inhibitors of α-syn aggregation.

**Identification of the shortest peptide capable of inhibiting α-syn fibril formation**

Shorter peptides, truncated at the N- and C-termini of GAVVT, were also synthesized (see Table 1). The RG and GR residues at the N- and C-terminal ends, respectively, of the peptides were again introduced to increase solubility. Before an investigation of the effects of the ASI peptides on α-syn aggregation, they were tested alone to determine if they had any tendency to self-aggregate. We aged all of the peptide solutions, as well as two additional peptide fragments of α-syn corresponding to amino acids 68–75 and 68–78, at 100 µM in PBS (pH 7.4) for 7 days at 37°C in an Eppendorf Thermomixer with continuous mixing (1000 rpm). A Th-T binding assay was carried out on the peptide samples every day to assess the presence of any amyloid aggregates. None of the peptides showed any tendency to aggregate, except for the fragment of α-syn corresponding to residues 68–78 (data not shown). Because the ASI peptides were designed from the region of α-syn covering amino acid residues 68–72, we included the unmodified fragment of α-syn corresponding to residues 68–75, which did not self-aggregate, as a control in our studies on α-syn aggregation. The effects of the ASI peptides on α-syn aggregation over 96 h were then investigated using the Th-T assay (Fig. 2A). The shortest peptide that retained the ability to inhibit α-syn aggregation had the central sequence AVVT, corresponding to α-syn (69–72). The unmodified peptide, α-syn (68–75) fragment, did not show any significant inhibition of α-syn fibrillogenesis (Fig. 2A).

As shown in Fig. 2A, the ASI4 (RGAVVGR) and ASI5 (RGVVTGR) peptides were unable to inhibit α-syn aggregation. Comparing the sequence of these inactive peptides with ASI3 (RGAVVTGR), which is a good inhibitor of α-syn aggregation, the difference is the elimination of two amino acids (alanine and threonine). Elimination of alanine or threonine from ASI3 (RGAVVTGR) renders the peptide unable to block α-syn aggregation, possibly because there are fewer opportunities for hydrogen bond formation between α-syn and the peptide.

Similar results were also obtained when the ASI peptides were tested on NAC aggregation (see below).

**Effect of the ASI peptides on α-syn fibril morphology**

We used EM with uranyl acetate negative staining to examine preparations made from α-syn solutions that were either aged alone or with ASI peptides prepared under the same conditions as those used for the Th-T binding assay. As expected, the α-syn solution incubated alone showed the classic appearance of mature α-syn fibrils, revealed as a dense network of fibrils averaging 10 nm in diameter and extending for several hundred nanometers in length (Fig. 3A). The ultrastructural appearance of these fibrils is similar to that reported previously (18–21). In
contrast, the sample containing peptide ASI1 showed only a thinly scattered distribution of small, irregular, globular aggregates ranging from less than a nanometer, up to a few tens of nanometers in size (Fig. 3B). Samples containing the ASI2 and ASI3 peptides both had a similar appearance, with only a very sparse scattering of irregular aggregates and truncated fibrils, rarely longer than a few tens of nanometers (Fig. 3C, D). The sample containing ASI4 showed a dense network of highly abnormal fibrils, which were larger in diameter and had a more irregular appearance than the control (α-syn alone) fibrils (Fig. 3E). The sample containing peptide ASI5 had fibrils similar in size and appearance to the control (α-syn alone), but with a less dense distribution than was observed in the control (Fig. 3F). The appearance and distribution of fibrils in the sample containing α-syn 68–75 appeared to be very similar to the control (Fig. 3G). A similar distribution of fibrils to the control (α-syn alone) was also observed in the samples containing the NTC peptides designed from the N-terminal region of α-syn (Fig. 3H, I, J). These results are in accord with the Th-T data and confirm that only peptides designed from the binding region were able to inhibit α-syn fibril formation.

Inhibition of α-syn oligomerization (formation of early aggregates)

We also tested the effects of the ASI peptides on α-syn oligomerization and the formation of ‘early aggregates’. The same fresh and aged samples of α-syn solutions alone or with inhibitor peptides were also tested by a novel ELISA developed in our laboratory. This ELISA method recognizes only oligomeric forms of α-syn (El-Agnaf, et al., manuscript submitted for publication). The ELISA is based on a conventional sandwich system with capture of α-syn by an anti-α-syn monoclonal antibody (mAb 211), followed by detection with a biotinylated form of the same mAb 211. The biotinylated mAb is subsequently detected with ExtrAvidin-alkaline phosphatase, followed by a colorimetric enzyme substrate. Monomeric α-syn cannot give a signal in this assay because the capture mAb occupies the only antibody binding site available on the protein, but, in the case of oligomeric forms of α-syn, multiple mAb binding sites are available, permitting both capture and detection.

Interestingly, all of the peptides that inhibited the formation of “late” aggregates of α-syn (as demonstrated by EM and Th-T) also inhibited the formation of “early” α-syn aggregates, as detected by the ELISA (Fig. 4). Furthermore, we also investigated the effects of the peptide inhibitors on α-syn aggregation as detected by immunoblot analysis. The majority of freshly dissolved α-syn migrated as a band at ~16 kDa, corresponding to the monomer (Fig. 5, lane 1), whereas α-syn that had been aged for 4 days showed a much less intense “monomer” band and larger amounts of higher molecular weight bands, namely a dimer at ~34 kDa and a high-molecular weight species that did not enter the separating gel, suggesting the formation of large aggregates (Fig. 5, lane 2). However, the samples of α-syn aged in the presence of the ASI1 and ASI2 peptides contained predominantly the monomeric and dimeric species (Fig. 5, lanes 3 and 4), with no high molecular weight aggregates. These findings confirm the results obtained by the Th-T binding, EM and ELISA assays (Figs. 2, 3, and 4).

Inhibition of NAC fibril formation

The peptide inhibitors are designed from the α-syn (68–72) sequence, which is part of the N-terminal region of the NAC peptide, corresponding to amino acid residues 61–95 of α-syn. We
hypothesized that the inhibition of α-syn aggregation by the peptides is due to the specific binding of the peptides to their homologous native sequence in the α-syn molecule. If our hypothesis is correct, then these peptide inhibitors should also inhibit NAC aggregation. Therefore, we investigated the effect of some of the peptides on NAC fibril formation, using the Th-T binding assay. Interestingly, the peptides that were active inhibitors of α-syn aggregation also inhibited NAC fibrillogenesis (Fig. 6A, B), and, conversely, the inactive peptide failed to inhibit NAC fibrillogenesis (Fig. 6C, D). These results support the idea that the inhibition of α-syn aggregation by the active peptides is due to the binding of these peptides to their homologous native sequence (amino acid residues 68–72) in the α-syn molecule (Fig. 6D).

Effect of ASI peptides on Aβ aggregation

To investigate the inhibition specificity of the ASI peptides, we tested their effects on the aggregation of another amyloid protein, namely β-amyloid (Aβ), which is a 39- to 42-residue peptide that plays a central role in the development of AD (reviewed by Soto (11)). It has been shown that solutions of Aβ peptide form fibrillar aggregates, in a similar fashion to α-syn (reviewed by El-Agnaf (12)). Therefore, we investigated the effects of three active inhibitors (ASI1, ASI2, and ASI3) and one nonactive inhibitor (ASI4) of α-syn aggregation on Aβ (1–40). Using the Th-T binding assay, we found that these inhibitors delayed the appearance of amyloid fibrils from Aβ (1–40), but did not prevent their formation over extended incubation times (data not shown). The presence of amyloid fibrils in samples of Aβ (1–40) incubated for extended time periods with these inhibitors was also confirmed by EM (data not shown). We also investigated the effects of these same ASI peptides on Aβ oligomerization during 9 days of incubation using an ELISA specific for Aβ oligomers (32). All of the ASI peptides tested showed concentration-dependent inhibition during the first 6 days of incubation. However, they showed no inhibition over extended incubation times. This is illustrated by the data in Fig. 7, and is in agreement with the Th-T and EM data for these peptides.

Effect of ASI peptides on Fe(II)-induced DNA damage in BE-M17 cells expressing mutant α-syn(A53T)

Recently, it has been reported by us and others, that neuronal cells transfected with mutant forms of α-syn responsible for familial PD (A30P or A53T), which are associated with increased α-syn oligomerization (18–20), exhibit an enhanced susceptibility to apoptotic cell death by various toxic insults, including Fe(II) exposure (36, 39–42). Such increased susceptibility may be due to protein misfolding of the mutant forms of α-syn (18, 19, 36, 43). This could abolish the antiapoptotic physiological function of α-syn and/or lead to the formation of toxic aggregates within cells (36, 39–43). If this is the case, then inhibitors of α-syn aggregation should decrease the susceptibility of neuronal cells transfected with mutant α-syn to these toxic insults. To this end, we have determined if active ASI peptides can protect BE(2)-M17 cells expressing α-syn(A53T) (M17-A53T) from Fe(II)-induced DNA damage.

Using the Comet assay we also demonstrated that DNA single-strand breaks (SSBs) could be detected in BE(2)-M17 cells expressing various forms of α-syn following 2 h treatments with Fe(II). Such damage was quantified by measuring comet tail length (CTL) (µm) (36). Fe(II) treatment, at concentrations as low as 0.01 µM, induced significant increases in CTL in cells
transfected with mutant α-syn (A30P or A53T), whereas untransfected cells, cells transfected with vector only, or cells transfected with wild-type α-syn were less susceptible (36). In the light of these previous results, we examined the effect of ASI peptides on DNA damage induction in M17-A53T cells treated for 2 h with 10 µM Fe(II).

The addition of ASI peptides (which inhibit α-syn aggregation in vitro) to the media of M17-A53T-cultured cells did not reverse Fe(II)-induced DNA damage, probably because these peptides lack a delivery system to facilitate its transport into cells (Fig. 8A–F). However, methods have been developed recently to allow the delivery of exogenous proteins into living cells. These methods employ membrane-permeable carrier peptides such as HIV-1 Tat, consisting of amino acid residues (49–57), Antennapedia (43–58), and arginine-rich peptides (see references (44–46)). Therefore, we synthesized ASI1 peptide fused to an arginine-rich carrier peptide (see Table 1). This new ASI1 peptide (ASI1D), containing the delivery peptide consisting of 6 Arg residues at the C-terminal end, was found to retain its activity as an inhibitor of α-syn aggregation in vitro. Figure 9 shows that ASI1D was capable of inhibiting both early oligomers and late fibrillar aggregates of α-syn. We then examined whether this ASI1D peptide would be able to cross cell membranes. An extra lysine amide was incorporated into the C terminus of ASI1D to allow fluorescent labeling. Internalization of the peptide was monitored by confocal microscopy following 10–60 min incubation of the peptide with M17-A53T cells at 37°C. Thirty minutes after incubation, fluorescent-labeled ASI1D was observed as a fluorescent signal in all living cells and was found to be distributed throughout the cells (Fig. 10). A similar level of ASI1D internalization was observed in the human dopaminergic neuroblastoma SHSY-5Y cell line (data not shown). Fluorescence was observed in cells as early as 5 min after the addition of ASI1D (1–10 µM) to the medium. The cytotoxicity of the ASI peptides was also investigated using an MTT assay. ASI1D did not exhibit any significant cytotoxicity toward M17-A53T or SHSY-5Y cells following treatment with 0.01–10 µM of the peptide for up to 24 h (data not shown).

Figure 8 shows that significant increases in comet-forming activity were observed following the treatment of M17-A53T cells with 10 µM Fe(II). In vehicle control cells, a median CTL of 35.41 µm was observed and in the presence of 10 µM Fe(II) treatment, an increase in median CTL to 57.60 µm (P<0.0001) was induced (Fig. 8A, B). However, in M17-A53T cells exposed to 10 µM Fe(II) in the presence of 1 µM ASI1D, the levels of DNA damage induction were reversed, giving rise to a median CTL of 31.15 µm (Fig. 8H); this was in comparison with an ASI1D control median level of 39.04 µm (Fig. 8G). In contrast to these protective effects, ASI1 and ASI2 peptides without the delivery system did not reverse levels of Fe(II)-induced DNA damage, as measured in the Comet assay (Fig. 8C–F).

Effect of ASI1D peptide on the expression levels of the apoptosis-related proteins, Bcl-2 and BAX, in M17-A53T cells treated with Fe(II)

In the light of the above results, we examined the effects of Fe(II), in the presence or absence of ASI1D peptide, on the percentage of M17-A53T cells staining positive for pro- and antiapoptotic proteins. Only cells exhibiting distinct perinuclear staining were scored as staining positive for these two proteins (37). Bcl-2 proteins are considered to be crucial regulators of programmed cell death or apoptosis. Bcl-2 itself is believed to possess antiapoptotic function and antagonize
p53-induced apoptosis (47). While functionally expressed in mitochondria, it may also act as an antioxidant regulating intracellular reactive oxygen species (48). Translocation of cytosolic BAX into mitochondria induces cytochrome c release, thus activating caspase-3. Bcl-2 expression may inhibit BAX expression or its proapoptotic conformational changes, thus blocking apoptosis. However, the cytotoxic effects of the aggregated α-syn may also be elicited through the Bcl-2 family proteins (49). Figure 11 showed that treatment with 10 µM Fe(II) for 4 h up-regulated the expression of both Bcl-2 (P<0.005) and BAX (P<0.05) in M17-A53T cells as compared with vehicle control cells. However, Fe(II) treatment in the presence of ASI1D peptide resulted in a further increase in Bcl-2 positive cells (P<0.0005) and a decrease in BAX positive cells (P<0.05) as compared with cells treated with Fe(II) alone (Fig. 11). These results suggest that inhibition of formation of toxic α-syn aggregates in M17-A53T cells by the ASI1D peptide may lead to activation of protective mechanisms via Bcl-2 related proteins, thus, facilitating cell survival and diminishing cytotoxicity.

DISCUSSION

There is now substantial evidence from molecular genetics, transgenic animal studies and aggregation/toxicity studies with recombinant α-syn (and various synthetic peptide fragments derived from it) to suggest that the conversion of this protein from soluble monomers to aggregated, insoluble forms in the brain is a key event in the pathogenesis of PD and related diseases (reviewed in references (8, 11, 12)). It seems increasingly likely that early “soluble oligomers” are actually the toxic species responsible for neurodegeneration and neuronal cell death (13–18). This suggests that inhibition of α-syn oligomerization may be a viable strategy for therapeutic intervention in PD and related disorders. α-Syn is normally a soluble, unfolded protein, with little or no ordered structure under normal physiological conditions (18, 19, 23), but it has an inbuilt tendency to self-oligomerize and to assemble into fibrillar aggregates (18–23). The assembly of α-syn into amyloid-like fibrils is accompanied by a transition from random-coil to β-sheet conformation (18, 19, 22, 23). During aggregation, α-syn can also generate hydrogen peroxide, which could be responsible for its toxic effects (50, 51). In this study, we attempted to inhibit this aggregation process by designing small peptides that can specifically interact with the region of α-syn responsible for its self-aggregation. To identify this critical region, we synthesized an extensive library of overlapping α-syn peptides, each of which was seven amino acids long. We considered this to be an appropriate length to test for binding to full-length α-syn. The binding sequence identified using the peptide library was located in the central hydrophobic region of α-syn covering amino acid residues 64–100, and the highest levels of binding to α-syn were observed with peptides covering amino acid residues 64–86 of α-syn, which corresponds to the N-terminal region of NAC.

Interestingly, recent studies have shown that substitution of the hydrophobic residues in the NAC sequence of full-length α-syn for charged ones impairs fibril formation (52). Furthermore, we have previously shown that the N-terminal half of NAC α-syn (61–78) is the amyloidogenic region, which is responsible for the aggregation and toxicity of the NAC peptide (22). During the preparation of our manuscript, Du et al. also reported that the hydrophobic region α-syn (66–74) is critical for its aggregation and toxicity (53). Taken together, all of these results suggest that α-syn self-aggregation is mainly driven by hydrophobic-hydrophobic interactions. Therefore, our attempt to design compounds as potential inhibitors to arrest the aggregation of α-syn was based
on peptides taken from the hydrophobic binding sequence α-syn (68−72) linked to a solublizing component. Using this new strategy, we designed several peptides as α-syn inhibitors (ASI). Our results show that some of these ASI peptides could inhibit the formation of α-syn β-sheet amyloid fibrils, as detected by EM and Th-T binding. It is likely that the peptides inhibit fibril formation by binding to monomeric and/or dimeric α-syn, thereby blocking the formation of early soluble aggregates, as shown by immunoblot analysis and the oligomer-specific ELISA. Furthermore, any ASI peptides that could inhibit α-syn aggregation were also capable of inhibiting the aggregation of NAC. In a test for specificity, we found that the ASI peptides were only able to delay the aggregation of Aβ (1−40) peptide. This effect is probably due to nonspecific hydrophobic interactions between the ASI peptides and Aβ. Taken together, our data support the idea that the ASI peptides do bind specifically to their homologous native sequences in α-syn and NAC, and so inhibit their self-oligomerization. We have determined that the minimum peptide domain for inhibiting α-syn aggregation corresponds to AVVT, and reducing the AVVT domain length causes the loss of ability to inhibit α-syn aggregation.

Recently, several strategies have been employed to design peptide inhibitors of Aβ aggregation and toxicity. All of the peptides designed in these studies are based on Aβ (16−22) fragment, which is responsible for the self-aggregation of Aβ. Soto and co-workers have designed peptides, termed β-sheet breaker peptides, by incorporating proline residues into the peptide sequence (24). The β-sheet breaker peptides can bind to soluble Aβ and prevent its conversion into toxic aggregates. The second strategy reported recently uses N-Methyl amino acids in alternating positions of the Aβ (16−22) sequence (54). The methylated peptides appear to act by binding to Aβ through one hydrogen-bonding face and simultaneously blocking the propagation of the hydrogen-bond array of the β-sheet with a nonhydrogen bonding face. Recently, Murphy and co-workers have reported another strategy for designing peptides to inhibit Aβ toxicity (55). These peptide inhibitors are composed of residues 15−25 of Aβ, designed to function as the recognition element, linked to an oligolysine-disrupting element. Interestingly, these inhibitors neither alter the apparent secondary structure of Aβ, nor prevent its aggregation; rather, they cause a change in aggregation kinetics and the higher-order structural characteristics of the aggregate. It is possible that our own strategy, as reported here for α-syn and the ASI peptides, could also be used successfully to design peptides to inhibit other protein aggregation systems. Using this strategy, we have designed peptides composed of residues 16–20 of Aβ. These peptides did inhibit the formation of early aggregates and the toxicity of Aβ (El-Agnaf, unpublished results).

Potential problems associated with peptide therapy include transport through the blood−brain barrier, the generation of an immune response, and high sensitivity of peptides to proteolytic degradation. These difficulties can be minimized by reducing the peptide length and by using D-amino acids or N-methylated amino acid derivatives.

Recently, Soto and co-workers have shown that short peptides (β-sheet breaker peptides) designed to inhibit amyloid β-protein aggregation, have the ability to cross the blood−brain barrier and reduce amyloid deposition and cerebral damage in vivo using transgenic mouse models of AD (30). The potential use of our ASI peptides as drugs for PD, DLB and MSA would depend, of course, on their ability to cross the blood−brain barrier and enter brain cells to block and/or reverse the formation of α-syn inclusions. The ASI peptides without any delivery system
could be problematic in this respect, because of expected limited transport across the blood–brain-barrier and potential problems with degradation. Methods have been developed for the delivery of exogenous proteins into living cells and across the blood–brain barrier with the help of membrane-permeable carrier peptides such as HIV-1 Tat (48–60), flock house virus coat (35–49), Drosophila antennapedia (43–58) and basic peptides such as octa and hexa arginine peptides (44–46). The efficacy of such approaches is illustrated by the example of the Tat-β-galactosidase fusion protein (56), which has a molecular mass as high as 120 kDa. Intraperitoneal injection of mice with Tat-β-galactosidase results in delivery of the protein to the brain, with retained biological activity. Recently, it has been reported that polyglutamine-binding peptides targeted for intracellular delivery by fusion to TAT retain their ability to inhibit polyglutamine aggregation and cell death in transfected cells (27). More recently, it has been shown that cell-permeable inhibitors of calcineurin using the polyarginine peptide delivery system were active in vitro in cell culture models and in vivo in animal models (57). Interestingly, in this paper, we have also shown that the ASI1 peptide fused to an arginine-rich carrier (ASI1D) can enter cells and inhibit DNA damage induced by Fe(II) in M17-A53T cells. Therefore, it would be of great interest to test the effect of ASI1D on PD animal models. Because the peptides we identified here are relatively small, these could also represent the starting point for designing peptidomimetic molecules more suitable for chronic therapy, which may be used as new drugs for the treatment of PD and related disorders in the future.

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REFERENCES


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Table 1

Primary structure of the native sequence α-syn(68–75) and the ASI and NTC peptides that were synthesised and studied

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<td>RGKKEGGGR-NH$_2$ α-syn(21–25; 32–36; 43–47; 58–62; 80–84)</td>
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Figure 1. Screening of the peptide library peptides 1–80 (A) and peptides 83–134 (B) for binding to recombinant α-syn immobilized on 96-well microtiter plates. The biotinylated peptides were added to the immobilized α-syn and incubated for 2 h (see Materials and Methods for details). Binding of the peptides to the immobilized α-syn was measured by the subsequent binding of ExtrAvidin-alkaline phosphatase. The data shown are representative of four independent experiments, from two different preparations of α-syn. Measurements were taken in triplicate, and the results show the mean ± standard deviation for each point.
Figure 2. Effect of ASI and control peptides on α-syn fibrillogenesis. α-Syn samples were incubated for 4 days with continuous mixing at 37°C in the presence of various concentrations of ASI and NAC(8–15) peptides (A) and NTC peptides (B), and amyloid fibril formation was measured by the Th-T binding assay (see Materials and Methods section for details). Data were collected and presented as a percentage of the signal obtained from α-syn incubated in the absence of ASI peptides. The data shown are representative of at least three independent experiments. The assays were performed in triplicate, and mean ± standard deviations are shown.
Figure 3. Electron microscopic examination of the effect of ASI and control peptides on α-syn fibril formation. α-Syn samples at 50 µM were incubated alone or in the presence of peptides at 1:2 (molar ratio α-syn:ASI peptide) for 4 days with continuous shaking at 37°C (see Materials and Methods section for details). The figure shows negatively stained samples. A) α-Syn alone. B) α-Syn incubated with ASI1. C) α-Syn incubated with ASI2. D) α-Syn incubated with ASI3. E) α-Syn incubated with ASI4. F) α-Syn incubated with ASI5. G) α-Syn incubated with NAC(8–15). H) α-Syn incubated with NTC1. I) α-Syn incubated with NTC2. J) α-Syn incubated with NTC3. Scale bar = 100 nm.
Figure 4. Effect of ASI and control peptides on α-syn oligomerization. α-Syn samples were incubated for 4 days with continuous shaking at 37°C in the presence of various concentrations of peptides, at the indicated molar ratios of α-syn:ASI peptide, and α-syn oligomerization was measured by ELISA (see Materials and Methods section for details). The effect of ASI1 (A), ASI2 (B), and ASI3 (C) on α-syn oligomerization was monitored over 4 days by the ELISA. In (D), data were collected after 4 days from α-syn samples incubated with ASI and control peptides and presented as a percentage of the signal obtained from α-syn sample incubated in the absence of ASI peptides. The data shown are representative of at least three independent experiments. The assays were performed in triplicate, and mean ± standard deviations are shown.
Figure 5. Immunoblot analysis of the effect of ASI peptides on α-syn oligomerization. Fresh or aged α-syn samples alone or in the presence of ASI peptides at 1:1 molar ratio (α-syn:ASI peptide) incubated for 4 days with continuous shaking at 37°C were analyzed. Lane 1, fresh α-syn; lane 2, aged α-syn; lane 3; aged α-syn with ASI1; lane 4, aged α-syn with ASI2.
Figure 6. Effect of ASI and control peptides on NAC fibrillogenesis. NAC samples were incubated for 9 days at 37°C in the presence of various concentrations of peptides, and amyloid fibril formation was measured by the Th-T binding assay (see Materials and Methods section for details). The effect of ASI1 (A), ASI3 (B), and ASI4 (C) on NAC fibril formation was monitored over 9 days by the Th-T assay. D) Data were collected after 9 days from NAC samples incubated with ASI and control peptides and presented as a percentage of the signal obtained from NAC sample incubated in the absence of ASI peptides. The data shown are representative of at least three independent experiments. The assays were performed in triplicate, and mean ± standard deviations are shown.
Figure 7. Effect of ASI peptides on Aβ(1–40) oligomerization. Aβ samples were incubated for 12 days at 37°C in the presence of various concentrations of ASI peptides, and Aβ oligomerization was measured by ELISA (see Materials and Methods section for details). The effect of ASI1 (A), ASI2 (B), ASI3 (C) and ASI4 (D) on Aβ oligomerization was monitored over 12 days by the ELISA (see Materials and Methods section for details). The assays were performed in triplicate, and mean ± standard deviations are shown.
Figure 8. Frequency distributions of the levels of DNA single-strand breaks in M17-A53T cells following treatment with Fe(II) in the presence or absence of ASI peptides. Cells were grown to confluence before the following treatments: (A) M17-A53T cells treated with vehicle (1% DMSO), (B) 10 µM Fe(II)-treated M17-A53T cells; (C) M17-A53T cells treated with 1 µM ASI1 peptide; (D) M17-A53T cells treated with 10 µM Fe(II) in the presence of 1 µM ASI1 peptide; (E) M17-A53T cells treated with 1 µM ASI2 peptide; (F) M17-A53T cells treated with 10 µM Fe(II) in the presence of 1 µM ASI2 peptide; (G) M17-A53T cells treated with 1 µM ASI1D peptide; and (H) M17-A53T cells treated with 10 µM Fe(II) in the presence of 1 µM ASI1D peptide. Following a 2 h of treatment, cells were disaggregated with trypsin/EDTA before incorporation into the Comet assay, as described in Materials and Methods. Comet tail lengths (CTLs) (µm) were used as a measure of DNA damage. CTLs were compared using the Mann-Whitney test. P, as compared with the corresponding control; P*, as compared with vehicle control.
Figure 9. Effect of ASI1 and ASI1D peptides on α-syn aggregation. α-Syn samples were incubated for 4 days with continuous mixing at 37°C in the presence of various concentrations of peptides. The amyloid fibril formation was measured by the Th-T binding assay (A), and α-syn oligomerization was measured by ELISA (B) (see Materials and Methods section for details). Data were collected and presented as a percentage of the signal obtained from α-syn incubated in the absence of ASI peptides. The data shown are representative of at least three independent experiments. The assays were performed in triplicate, and mean ± standard deviations are shown.
Figure 10. Internalization of the ASI1D peptide through the cell membrane. Phase-contrast (left) and confocal microscopic observation (right) of the M17-A53T cells treated with ASI1D peptide (10 µM) for 30 min. Images captured at low magnification 10× (A) and at high magnification 40× (B).
Figure 11. Immunohistochemical analysis of M17-A53T cells treated with 10 μM Fe(II) in the presence or absence of ASI1D peptide. Cells were treated, as indicated, for 4 h on cover slips, after which they were analyzed for protein expression, as described in Materials and Methods. The antibodies employed were Bcl-2 mouse anti-human monoclonal (124, Isotype: IgG1) and BAX rabbit anti-human polyclonal. The percentage of cells staining positive was determined following five separate counts of 100 cells. Results are presented as mean ± SD. Slides were coded prior to counting to avoid bias. *P < 0.05, ***P < 0.0005 as determined by an Unpaired t-test with Welch’s correction. Cells treated with 10 μM Fe(II) alone are compared with vehicle control. Cells treated with 10 μM Fe(II) in the presence of 1 μM ASI1D are compared with cells treated with 10 μM Fe(II) alone.