BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 8127–8133 8127
DOI: 10.1021/bi100939a

The Flavanol (−)-Epigallocatechin 3-Gallate Inhibits Amyloid Formation by Islet Amyloid Polypeptide, Disaggregates Amyloid Fibrils, and Protects Cultured Cells against IAPP-Induced Toxicity†

Fanling Meng,‡ Andisheh Abedini,§ Annette Plesner,∥ C. Bruce Verchere,∥,⊥ and Daniel P. Raleigh*,‡,#

‡*Department of Chemistry,* #*Graduate Program in Biochemistry and Structural Biology and Graduate Program in Biophysics, State University of New York at Stony Brook, Stony Brook, New York 11794-3400,* §*Department of Medicine, New York University Medical Center, 550 First Avenue, New York, NY 10016,* ∥*Department of Pathology and Laboratory Medicine, and* ⊥*Department of Surgery, Child and Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada V5Z 4H4*

Received June 10, 2010; Revised Manuscript Received August 12, 2010

ABSTRACT: Islet amyloid polypeptide (IAPP, amylin) is the major protein component of the islet amyloid deposits associated with type 2 diabetes. The polypeptide lacks a well-defined structure in its monomeric state but readily assembles to form amyloid. Amyloid fibrils formed from IAPP, intermediates generated in the assembly of IAPP amyloid, or both are toxic to β-cells, suggesting that islet amyloid formation may contribute to the pathology of type 2 diabetes. There are relatively few reported inhibitors of amyloid formation by IAPP. Here we show that the tea-derived flavanol, (−)-epigallocatechin 3-gallate [(2*R*,3*R*)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3-yl 3,4,5-trihydroxybenzoate] (EGCG), is an effective inhibitor of *in vitro* IAPP amyloid formation and disaggregates preformed amyloid fibrils derived from IAPP. The compound is thus one of a very small set of molecules which have been shown to disaggregate IAPP amyloid fibrils. Fluorescence-detected thioflavin-T binding assays and transmission electron microscopy confirm that the compound inhibits unseeded amyloid fibril formation as well as disaggregates IAPP amyloid. Seeding studies show that the complex formed by IAPP and EGCG does not seed amyloid formation by IAPP. In this regard, the behavior of IAPP is similar to the reported interactions of Aβ and α-synuclein with EGCG. Alamar blue assays and light microscopy indicate that the compound protects cultured rat INS-1 cells against IAPP-induced toxicity. Thus, EGCG offers an interesting lead structure for further development of inhibitors of IAPP amyloid formation and compounds that disaggregate IAPP amyloid.

Amyloid formation plays a key role in a wide range of diseases including Alzheimer's disease, Parkinson's disease, and Huntington's disease (*1*, *2*). Human islet amyloid polypeptide (amylin, IAPP)[1] is a 37-residue polypeptide which is the major component of the pancreatic islet amyloid associated with type 2 diabetes and is one of the most amyloidogenic polypeptides known (Figure 1) (*3*−*11*). IAPP has been identified in all mammalian species examined and is a member of the calcitonin-like family of peptides which includes calcitonin, adrenomedullin, and calcitonin gene-related peptide (*9*). IAPP normally functions as an endocrine partner to insulin, is processed in parallel with insulin in the pancreatic β-cells, and is secreted in response to the same stimuli that lead to insulin secretion (*8*, *12*, *13*). Synthetic aggregates of human IAPP are toxic to pancreatic β-cells, arguing that the process of IAPP amyloid fibril formation contributes to islet cell death in type 2 diabetes (*6*, *7*, *14*−*16*). Longitudinal studies using animal models suggest a role for islet amyloid in type 2 diabetes, while autopsies indicate varying amounts of amyloid deposits in individuals diagnosed with type 2 diabetes (*17*, *18*). Recent work has highlighted a potentially deleterious role for IAPP amyloid formation in islet transplantation (*19*−*22*). Thus, there is considerable interest in the development of inhibitors of IAPP amyloid formation. There is a very large body of work on inhibitors of the Alzheimer β amyloid peptide (Aβ), but much less attention has been paid to the development of IAPP amyloid inhibitors (*23*−*34*).

The ester of epigallocatechin and gallic acid, (−)-epigallocatechin 3-gallate [EGCG; (2*R*,3*R*)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3-yl 3,4,5-trihydroxybenzoate], is the most abundant biologically active compound in tea (*35*, *36*). This tea-derived flavanol has been reported to attenuate Aβ-induced neurotoxicity in cultured human neuronal cell lines and to modulate both tau pathology and Aβ-mediated cognitive impairment in transgenic mouse models of Alzheimer disease (*37*−*39*). The molecular basis of these effects is not well understood but has been postulated to be related to EGCG's radical scavenging properties, its potential to affect the processing of amyloid precursor protein, its ability to interfere

†Grant Sponsors NIH GM078114 to D.P.R. and Canadian Institute of Health Research MOP-14682 to C.B.V.

*To whom correspondence should be addressed. Phone: 631-632-9547. Fax: 631-632-7960. E-mail: draleigh@notes.cc.sunysb.edu.

[1]Abbreviations: Aβ, the proteolytic fragment of amyloid precursor protein which is responsible for amyloid formation in Alzheimer's disease; EGCG, (−)-epigallocatechin 3-gallate [(2*R*,3*R*)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3-yl 3,4,5-trihydroxybenzoate]; Fmoc, 9-fluorenylmethoxycarbonyl; HFIP, hexafluoroisopropyl alcohol; HPLC, high-performance liquid chromatography; IAPP, human islet amyloid polypeptide; MALDI-TOF MS, matrix-assisted laser desorption ionization−time-of-flight mass spectrometry; PAL-PEG, 5-(4′-Fmoc-aminomethyl-3′,5-dimethoxyphenyl)-valeric acid; TEM, transmission electron microscopy; TFA, trifluoroacetic acid.

© 2010 American Chemical Society
Published on Web 08/13/2010

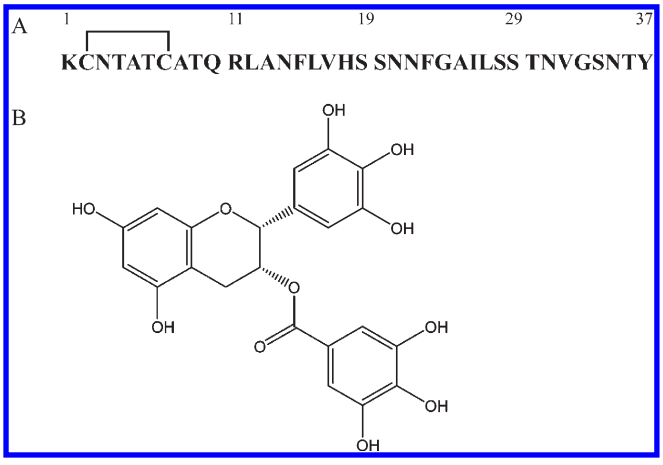

FIGURE 1: (A) The primary sequence of human IAPP. The peptide contains a disulfide bridge between Cys-2 and Cys-7 and has an amidated C-terminus. (B) The structure of EGCG.

with amyloid formation, or its inhibition of c-Abl/FE65 nuclear translocation and GSK3 β activation (*39*−*41*).

Early studies using model homopolymers of amino acids showed that catechins, a core component of the EGCG structure, interfered with and inhibited the coil to β-sheet transition (*42*). Recent work has shown that EGCG inhibits the *in vitro* amyloid formation of several natively unfolded polypeptides including Aβ, α-synuclein, polyglutamine peptides, and the model polypeptide κ-casein (*41*, *43*, *44*). The compound has also been shown to induce a transition of the cellular form of the prion protein into a detergent-insoluble form, which differs from the pathological scrapie protein conformation, and to eradicate formation of a variety of prion structures (*45*, *46*). It also inhibits *in vitro* amyloid formation by a malaria antigenic protein (*47*). However, its ability to inhibit amyloid formation by IAPP has not been tested, nor has its ability to protect cells against the toxic effects of IAPP amyloid formation been examined. These observations prompted us to examine the ability of EGCG to inhibit amyloid formation by IAPP and disaggregate amyloid fibrils and to test its ability to protect cells against IAPP toxicity.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification. Human IAPP was synthesized on a 0.25 mmol scale using an Applied Biosystems 433A peptide synthesizer by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry as described (*48*). Pseudoprolines were incorporated to facilitate the synthesis. 5-(4′-Fmoc-aminomethyl-3′,5-dimethoxyphenol)valeric acid (PAL-PEG) resin was used to afford an amidated C-terminal. The first residue attached to the resin, β-branched residues, residues directly following β-branched residues, and pseudoprolines were double coupled. The peptide was cleaved from the resin using standard TFA protocols. Crude peptides were oxidized by dimethyl sulfoxide (DMSO) for 24 h at room temperature (*49*). The peptides were purified by reverse-phase HPLC using a Vydac C18 preparative column. HCl was used as the counterion since the presence of TFA has been shown to affect amyloid formation by some IAPP-derived peptides (*50*). After the initial purification, the peptide was washed with ether, centrifuged, dried, and then redissolved in HFIP and subjected to a second round of HPLC purification. This procedure was necessary to remove residual scavengers that can interfere with toxicity assays. Analytical HPLC was used to check the purity of the peptide. The identity of the pure peptide was confirmed by mass spectrometry using a Bruker MALDI-TOF MS: IAPP observed 3904.6; expected 3904.8. An additional sample of human IAPP was purchased from Bachem.

Sample Preparation for in Vitro Biophysical Assays of Amyloid Formation. Stock solutions (1.58 mM) of IAPP were prepared in 100% hexafluoroisopropyl alcohol (HFIP) and stored at 4 °C. Aliquots of IAPP peptide in HFIP were filtered through a 0.45 μm filter and dried under vacuum. A Tris-HCl buffered (20 mM, pH 7.4) thioflavin-T solution was added to these samples to initiate amyloid formation. These conditions were chosen to match the method of sample preparation used for toxicity studies.

Thioflavin-T Fluorescence. Fluorescence measurements were performed using a Beckman model D880 plate reader. The samples were incubated at 25 °C in 96-well plates. An excitation filter of 430 nm and an emission filter of 485 nm were used. All solutions for these studies were prepared by adding a Tris-HCl buffered (20 mM, pH 7.4) thioflavin-T solution to IAPP peptide (in dry form) immediately before the measurement. The final concentration was 32 μM peptide and 25 μM thioflavin-T with or without 32 μM EGCG in 20 mM Tris-HCl. Seeding experiments were performed by adding IAPP to either preformed amyloid or to the final products of an IAPP plus EGCG kinetic experiment. The final concentration of seeds for the IAPP and IAPP−EGCG complex seeding experiments were 3.2 μM IAPP and 3.2 μM IAPP−3.2 μM EGCG, respectively, in monomer units. EGCG was purchased from Sigma-Aldrich.

Transmission Electron Microscopy (*TEM*). Peptide solution (5 μL) was blotted onto a carbon-coated Formvar 300 mesh copper grid for 1 min and then negatively stained with saturated uranyl acetate for 1 min. The same solutions that were employed for thioflavin-T fluorescence measurements were used for TEM studies so that samples could be compared under as similar conditions as possible.

Analysis of the Effect of EGCG on IAPP-Induced Toxicity. Rat insulinoma (INS-1) β-cells were used to assess the ability of EGCG to protect against the toxic effects of human IAPP. INS-1 cells were grown in RPMI 1640 (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), 11 mM glucose, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 units/mL penicillin (Gibco-BRL), and 100 units/mL streptomycin (Gibco-BRL). Cells were maintained at 37 °C in a humidified environment supplemented with 5% CO_2. Cells were grown for two passages prior to use and used in assays between passages 59 and 65. For toxicity experiments, cells were seeded at a density of 30000 cells per well in 96-well plates and cultured for 24 h prior to addition of solutions. Solutions of EGCG−IAPP at 1:1 molar ratio (30 μM IAPP and 30 μM EGCG) were prepared by adding aliquots of a 1.09 mM EGCG stock solution to dry IAPP (prepared as described in the sample preparation subsection) and diluting with Tris-HCl buffer (pH 7.4). Peptide samples and samples of peptide plus EGCG in Tris-HCl buffer (pH 7.4) were added directly to cells (30% final media concentration) after 14 h of incubation at room temperature. The redox-sensitive dye alamar blue (resazorin) (Biosource International, CA) was used to assess INS-1 cell viability (*51*). Alamar blue was diluted 10-fold in 30% culture media, and cells were incubated for 5 h at 37 °C. Fluorescence (excitation 530; emission 590 nm) was measured with a Fluoroskan Ascent plate reader (Thermo Labsystems). Data are representative of a minimum of three independent experiments performed in triplicate. The experiments were repeated using human IAPP

synthesized by two independent sources, and similar results were obtained.

Light Microscopy. Changes in cell morphology were examined by light microscopy in order to provide a second method of evaluating cell viability. Transformed rat INS-1 β-cells were photographed immediately prior to assessment of toxicity by alamar blue cell viability assays. Images were captured using a Nikon Eclipse TS100 light microscope.

RESULTS AND DISCUSSION

EGCG Inhibits Amyloid Formation by IAPP in Vitro. The primary structure of IAPP is shown in Figure 1, which also displays the structure of EGCG. The kinetics of *in vitro* amyloid formation are generally complex, and IAPP is no exception. Like other amyloidogenic polypeptides, it displays a lag phase during which no detectable amyloid fibrils are formed followed by a more rapid growth phase, also called the elongation phase, which leads to a final state in which amyloid fibrils are in equilibrium with soluble peptide. The rate of amyloid formation by IAPP was measured in the presence and in the absence of EGCG using thioflavin-T binding assays. Thioflavin-T is a small molecule whose fluorescent quantum yield increases significantly when it binds to amyloid fibrils (*52*). The dye is thought to bind in grooves formed on the surface of the amyloid fibril which are generated by the in-register alignment of side chains in the regular cross-β-sheet structure. Binding of the dye in a planar conformation eliminates rotation of the benzothiozole and benzaminic rings and reduces self-quenching, resulting in an increase in fluorescence quantum yield.

Thioflavin-T-monitored kinetic progress curves for IAPP in the presence and absence of EGCG are displayed in Figure 2. The sigmoidal curve observed in the absence of EGCG is typical of that observed for IAPP *in vitro*. The lag time is on the order of 20 h for the IAPP sample in the absence of EGCG. The time course of amyloid formation observed here is comparable to other studies that have used similar sample preparation protocols, but is slower than has been observed for some biophysical studies that were conducted with constant stirring and which initiate amyloid formation by dilution of stock solutions of IAPP in fluorinated alcohols, typically HFIP, into buffer. Stirring is well-known to increase the rate of amyloid formation, most likely by inducing fiber fragmentation, and small amounts of residual HFIP have been shown to drastically accelerate amyloid formation by IAPP (*53*−*57*).

Experiments conducted in the presence of EGCG give strikingly different results than observed in the absence of the compound. No increase in thioflavin-T fluorescence is observed for the 1:1 mixture of IAPP with EGCG. The standard interpretation of curves such as those shown in Figure 2 is that the lack of thioflavin-T fluorescence indicates that no amyloid is formed. However, it is important to independently confirm the results of the thioflavin-T binding assays (*58*). Consequently, TEM images were recorded of aliquots removed at the end of the reaction. The TEM images of the sample without inhibitor revealed extensive amyloid fibrils with a morphology typical of that found for *in vitro* IAPP amyloid deposits (Figure 2B). In contrast, very few aggregates were observed on the grid when EGCG was present at the 1:1 ratio, and the few fibrils detected had a different morphology (Figure 2C, Supporting Information). The TEM and thioflavin-T studies indicate that EGCG inhibits amyloid formation by IAPP *in vitro*.

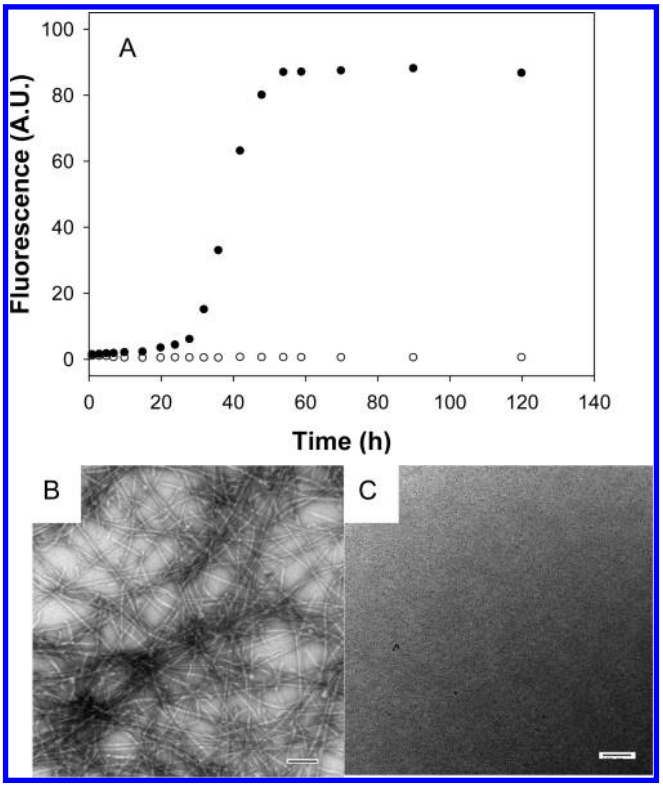

FIGURE 2: EGCG inhibits amyloid formation by IAPP *in vitro*. (A) Fluorescence-detected thioflavin-T binding assays in the presence and absence of EGCG: IAPP alone (●); a 1:1 molar ratio mixture of EGCG and IAPP (○). (B) TEM image of IAPP alone. (C) TEM image of a 1:1 mixture of IAPP and EGCG. Aliquots were removed from the kinetic experiments depicted in panel A after 200 h and blotted for TEM. Scale bars represent 100 nm. Samples contained 32 μM IAPP. and experiments were performed in 20 mM Tris-HCl, pH 7.4, 25 °C.

We also examined the ability of EGCG to inhibit amyloid formation by IAPP when added at substoichiometric levels. Significant effects were observed at a 2:1 ratio of IAPP to EGCG and at a 5:1 ratio of IAPP to EGCG. The final thioflavin-T fluorescence intensity was reduced by 94% for the 2:1 experiment (IAPP in 2-fold excess) and was reduced by 80% even when IAPP was in 5-fold excess (Supporting Information). The lag phase was also much longer in the presence of substoichiometric amounts of EGCG and was increased approximately 2-fold for the 5:1 (IAPP to EGCG) sample. The observation that EGCG is a significant inhibitor at substoichiometric levels suggests that it binds to oligomeric species; of course, it may also bind to monomers, or it may bind to monomers and form a structure which can associate with additional monomers to generate a complex that does not lead to amyloid.

The Complex Formed by IAPP and EGCG Does Not Seed Amyloid Formation. Studies with Aβ and α-synuclein have shown that the EGCG peptide complexes formed are unable to seed amyloid formation by the parent protein (*41*). Seeding refers to the process of adding preaggregated species to a sample of unaggregated polypeptide. Seeding normally significantly accelerates amyloid formation by eliminating the lag phase. The inability of the EGCG−Aβ and the EGCG−α-synuclein complexes to seed aggregation of the parent proteins was taken as evidence that the species are off-pathway (*41*). It is extremely difficult to determine if an intermediate is on- or off-pathway (*59*). Strictly speaking, the results with Aβ and α-synuclein indicate that the species formed are not capable of supporting growth of

amyloid fibrils but do not prove that they are off-pathway. Instead, EGCG may have trapped the respective polypeptides in an early intermediate state which is on-pathway but which has not yet reached the state where it is capable of promoting growth of cross-β-structure. Nonetheless, seeding experiments can provide important mechanistic insight. In particular, the observation of the ability to seed amyloid formation is consistent with the species being on-pathway. Thus, a positive result in a seeding study is easily interpreted, while a negative result, although informative, is more ambiguous. Consequently, we investigated the ability of the material present at the end of the kinetic experiments to seed amyloid formation by IAPP. Figure 3 displays the results of IAPP seeding studies. Adding pure IAPP seeds eliminates the lag phase and leads to a thioflavin-T curve which is very similar to those reported in other seeding studies (*55*). In contrast, seeding by aliquots of the 1:1 EGCG−IAPP mixture collected at the end of the kinetic experiment displayed in Figure 2 had no detectable effect. This shows that the EGCG−IAPP complex does not seed amyloid formation by IAPP under these conditions (Figure 3).

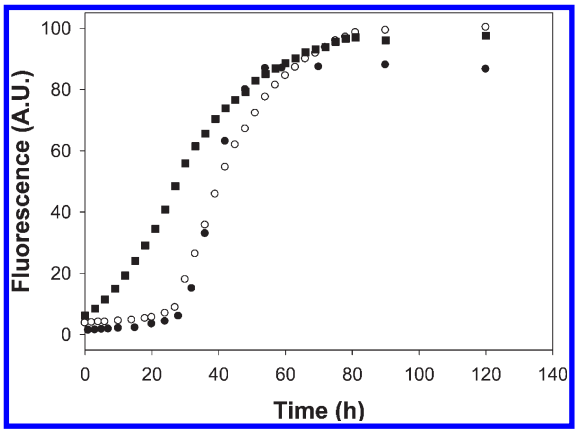

FIGURE 3: IAPP−EGCG complexes do not seed amyloid formation by IAPP. The results of thioflavin-T-monitored kinetic experiments are shown: unseeded IAPP (●); IAPP seeded with IAPP amyloid fibrils (■); IAPP seeded with the IAPP−EGCG complex (○). Experiments were conducted in 20 mM Tris-HCl, pH 7.4, 25 °C. The IAPP concentration was 32 μM. Seeds, when present, were added at a concentration of 3.2 μM (monomer units).

EGCG Disaggregates IAPP Amyloid Fibrils. We also tested the ability of EGCG to disaggregate IAPP amyloid fibrils. To the best of our knowledge, there are no small molecules which have been reported to disaggregate IAPP amyloid, although one large peptide-based inhibitor has been shown to do so (*27*). Figure 4 displays the results of a kinetic experiment for an IAPP control without EGCG; the second curve is from an experiment in which EGCG was added after the plateau region was reached. An initial rapid decrease in thioflavin-T fluorescence is observed after EGCG is added, followed by a slower decay of fluorescence. Samples of the solution were removed at various time points after addition of EGCG and used for TEM analysis. Images recorded from samples removed after the end of the initial, rapid decay of thioflavin-T fluorescence revealed that the amyloid fibrils had converted to much shorter aggregates which qualitatively appeared to have less tendency to clump together. Images were also recorded after the end of the second, slower decay phase. Most of the TEM grids were blank, while a few regions contained amorphous aggregates or very thin aggregates (Figure 4, Supporting Information).

EGCG Protects INS-1 β-Cells against the Toxic Effects of Human IAPP. We compared the effects of 30 μM human IAPP and a 1:1 mixture of 30 μM human IAPP and 30 μM EGCG on rat INS-1 cells in order to determine whether EGCG was able to protect β-cells from the toxic effects of human IAPP. Rat INS-1 cell area transformed β-cell line which is widely used in

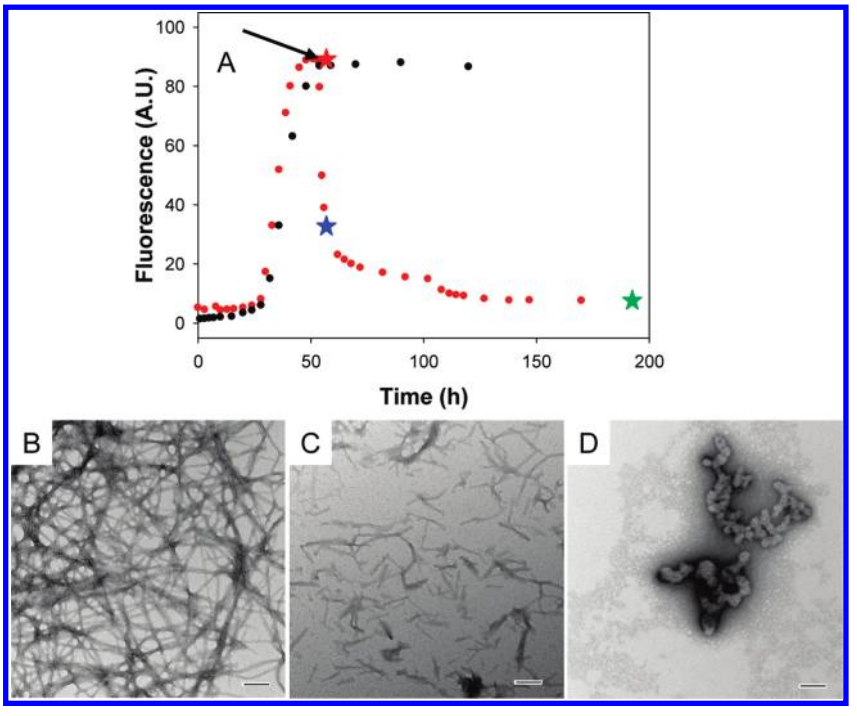

FIGURE 4: EGCG disaggregates IAPP amyloid fibrils. (A) thioflavin-T-monitored kinetic experiments: black, IAPP alone; red, EGCG added at the point indicated by the arrow. (B) TEM image of IAPP before the addition of EGCG. The sample was removed at the time point indicated by the red star. (C) TEM images of IAPP after the addition of EGCG. The sample was removed at the time point indicated by the blue star. (D) TEM image of IAPP after the addition of EGCG. The sample was removed at the time point indicated by the green star. Scale bars represent 100 nm. Kinetic runs were conducted at pH 7.4, 25 °C, 20 mM Tris-HCl with 32 μM IAPP.

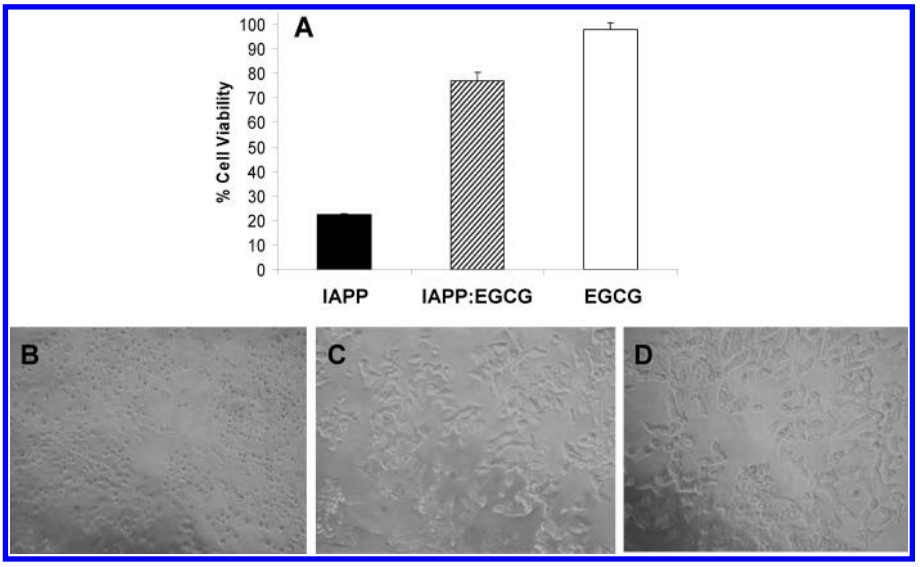

FIGURE 5: EGCG protects rat INS-1 cells against the toxic effects of human IAPP. (A) Cell viability as determined via alamar blue assays, plotted as percent viability: black, the effect of the addition of a 30 μM solution of human IAPP; hatched, the effect of a 1:1 mixture of human IAPP (30 μM) and EGCG (30 μM); white, the effect of a 30 μM solution of EGCG. All solutions were incubated at room temperature for 14 h and then applied to rat INS-1 cells in 96-well plates. Values are relative to those of control cells treated with buffer only. All values represent means ± SEM (n = 3). (B) Evaluation of apoptotic cell morphology by light microscopy. Transformed rat INS-1 β-cells were photographed immediately prior to assessment of cell viability by alamar blue. (B) The effect of the addition of 30 μM human IAPP. (C) The effect of a 1:1 mixture of human IAPP (30 μM) and EGCG (30 μM). (D) The effect of a 30 μM solution of EGCG.

studies of β-cell toxicity. Incubation of INS-1 cells with 30 μM human IAPP for 5 h resulted in significant toxicity; cell viability was only 22 ± 0.3% relative to untreated control determined by alamar blue assays. The 1:1 mixture of EGCG and IAPP was significantly less toxic, increasing the percentage of viable cells to 77 ± 4% (Figure 5A). Changes in cell morphology were examined by light microscopy in order to provide a second method of evaluating cell viability. Cells were photographed immediately prior to assessment of toxicity by alamar blue assay. Analysis of 30 μM IAPP-treated INS-1 cells demonstrated induction of cell shrinkage and extensive detachment of cells from the cell culture substratum, indicative of cell death. In contrast, INS-1 cells treated with a 1:1 molar ratio of EGCG−IAPP or with just EGCG demonstrated no observable signs of cell death (Figure 5B−D).

The experiments demonstrate a significant level of EGCG-induced protection and indicate that EGCG is an effective inhibitor of human IAPP-induced *in vitro* toxicity. Similar results were obtained using human IAPP from two independent sources, in-house prepared human IAPP and human IAPP purchased from Bachem. This is an important control since there have been reports of significant lot-to-lot variability in the toxicity of human IAPP from different sources (*60*).

CONCLUSIONS

The data reported here demonstrate that EGCG inhibits *in vitro* amyloid formation by IAPP and disaggregates IAPP amyloid. EGCG is one of the first small molecule that has been shown to disaggregate IAPP-derived amyloid fibrils. Studies of the interaction of EGCG with α-synuclein and Aβ lead to the proposal, based in part on seeding studies, that EGCG functions by a universal mechanism which involves diverting polypeptides from their normal amyloid formation pathway into nonproductive off-pathway states (*41*). It is worth noting, however, that it is extraordinarily difficult to prove if a species is on or off the pathway of amyloid formation (*59*, *61*). Along these lines, studies with another polyphenol, exifone [3,4,5,2′,3′,4′-hexahydroxybenzophenone [(2,3,4-trihydroxyphenyl)-(3,4,5-trihydroxyphenyl)methanone]], has provided evidence that such compounds can function by an alternative mechanism in which they trap amyloidogenic proteins in an on-pathway intermediate state (*62*). Recent work with reduced carboxymethylated κ-casein showed that EGCG maintained the protein in a preamyloid state but did not redirect the normal aggregation pathway (*43*). Thus EGCG can inhibit amyloid by a variety of ways. Under the conditions used here, EGCG appears to interact with IAPP in a fashion more similar to its interaction with Aβ and α-synuclein. Irrespective of the details, it is clear that EGCG has the ability to interact with a broad range of natively unfolded proteins and inhibit their *in vitro* aggregation while at the same time protecting cultured cells against toxicity.

ACKNOWLEDGMENT

We thank Ms. Ping Cao for assistance with the synthesis and purification of human IAPP and for helpful discussions. We also thank Prof. Martin T. Zanni and Dr. Chris T. Middleton for helpful discussions and for continued interest in this work. We thank Dr. Mahiuddin Ahmed for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Additional TEM images of IAPP inhibited with EGCG and of the products of the disaggregation study and thioflavin-T kinetic studies for 2:1 and 5:1 mixtures of IAPP and EGCG. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Sipe, J. D. (1994) Amyloidosis. *Crit. Rev. Clin. Lab. Sci. 31*, 325–354.
2. Selkoe, D. J. (2004) Cell biology of protein misfolding: The examples of Alzheimer's and Parkinson's diseases. *Nat. Cell Biol. 6*, 1054–1061.
3. Westermark, P., Wernstedt, C., Wilander, E., Hayden, D. W., Obrien, T. D., and Johnson, K. H. (1987) Amyloid fibrils in human insulinoma and islets of langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. *Proc. Natl. Acad. Sci. U.S.A. 84*, 3881–3885.

4. Cooper, G. J. S., Willis, A. C., Clark, A., Turner, R. C., Sim, R. B., and Reid, K. B. M. (1987) Purification and characterization of a peptide from amyloid-rich pancreases of type-2 diabetic-patients. *Proc. Natl. Acad. Sci. U.S.A. 84*, 8628–8632.
5. Kahn, S. E., Andrikopoulos, S., and Verchere, C. B. (1999) Islet amyloid: A long-recognized but underappreciated pathological feature of type 2 diabetes. *Diabetes 48*, 241–253.
6. Clark, A., Lewis, C. E., Willis, A. C., Cooper, G. J. S., Morris, J. F., Reid, K. B. M., and Turner, R. C. (1987) Islet amyloid formed from diabetes-associated peptide may be pathogenic in type-2 diabetes. *Lancet 2*, 231–234.
7. Hull, R. L., Westermark, G. T., Westermark, P., and Kahn, S. E. (2004) Islet amyloid: A critical entity in the pathogenesis of type 2 diabetes. *J. Clin. Endocrinol. Metab. 89*, 3629–3643.
8. Marzban, L., Park, K., and Verchere, C. B. (2003) Islet amyloid polypeptide and type 2 diabetes. *Exp. Gerontol. 38*, 347–351.
9. Westermark, P., Wernstedt, C., Wilander, E., and Sletten, K. (1986) A novel peptide in the calcitonin gene related peptide family as an amyloid fibril protein in the endocrine pancreas. *Biochem. Biophys. Res. Commun. 140*, 827–831.
10. Jaikaran, E. T. A. S., Higham, C. E., Serpell, L. C., Zurdo, J., Gross, M., Clark, A., and Fraser, P. E. (2001) Identification of a novel human islet amyloid polypeptide beta-sheet domain and factors influencing fibrillogenesis. *J. Mol. Biol. 308*, 515–525.
11. Cooper, G. J. S. (1994) Amylin compared with calcitonin-gene-related peptide—Structure, biology, and relevance to metabolic disease. *Endocr. Rev. 15*, 163–201.
12. Hutton, J. C. (1989) The insulin secretory granule. *Diabetologia 32*, 271–281.
13. Nishi, M., Sanke, T., Nagamatsu, S., Bell, G. I., and Steiner, D. F. (1990) Islet amyloid polypeptide—A new beta-cell secretory product related to islet amyloid deposits. *J. Biol. Chem. 265*, 4173–4176.
14. Lorenzo, A., Razzaboni, B., Weir, G. C., and Yankner, B. A. (1994) Pancreatic-islet cell toxicity of amylin associated with type-2 diabetes-mellitus. *Nature 368*, 756–760.
15. Clark, A., Wells, C. A., Buly, I. D., Cruickshank, J. K., Vanhegan, R. I., Matthews, D. R., Cooper, G. J. S., Holman, R. R., and Turner, R. C. (1988) Islet amyloid, increased alpha cells, reduced beta cells and exocrine fibrosis: Quantitative changes in the pancreas in type 2 diabetes. *Diabetes Res. Clin. Pract. 9*, 151–159.
16. Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., and Butler, P. C. (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes 52*, 102–110.
17. Hayden, M. R., Karuparthi, P. R., Manrique, C. M., Lastra, G., Habibi, J., and Sowers, J. R. (2007) Longitudinal ultrastructure study of islet amyloid in the HIP rat model of type 2 diabetes mellitus. *Exp. Biol. Med. 232*, 772–779.
18. Rocken, C., Linke, R. P., and Saeger, W. (1992) Immunohistology of islet amyloid polypeptide in diabetes-mellitus—Semiquantitative studies in a postmortem series. *Virchows Arch. A 421*, 339–344.
19. Westermark, G. T., Westermark, P., Berne, C., Korsgren, O., and Transpla, N. N. C. I. (2008) Widespread amyloid deposition in transplanted human pancreatic islets. *N. Engl. J. Med. 359*, 977–979.
20. Westermark, G. T., Westermark, P., Nordin, A., Tornelius, E., and Andersson, A. (2003) Formation of amyloid in human pancreatic islets transplanted to the liver and spleen of nude mice. *Upsala J. Med. Sci. 108*, 193–203.
21. Udayasankar, J., Kodama, K., Hull, R. L., Zraika, S., Aston-Mourney, K., Subramanian, S. L., Tong, J., Faulenbach, M. V., Vidal, J., and Kahn, S. E. (2009) Amyloid formation results in recurrence of hyperglycaemia following transplantation of human IAPP transgenic mouse islets. *Diabetologia 52*, 145–153.
22. Potter, K. J., Abedini, A., Marek, P., Klimek, A. M., Butterworth, S., Driscoll, M., Baker, R., Nilsson, M. R., Warnock, G. L., Oberholzer, J., Bertera, S., Trucco, M., Korbutt, G. S., Fraser, P. E., Raleigh, D. P., and Verchere, C. B. (2010) Islet amyloid deposition limits the viability of human islet grafts but not porcine islet grafts. *Proc. Natl. Acad. Sci. U.S.A. 107*, 4305–4310.
23. Feng, B. Y., Toyama, B. H., Wille, H., Colby, D. W., Collins, S. R., May, B. C. H., Prusiner, S. B., Weissman, J., and Shoichet, B. K. (2008) Small-molecule aggregates inhibit amyloid polymerization. *Nat. Chem. Biol. 4*, 197–199.
24. Blazer, L. L., and Neubig, R. R. (2009) Small molecule protein-protein interaction inhibitors as cns therapeutic agents: Current progress and future hurdles. *Neuropsychopharmacology 34*, 126–141.
25. Takahashi, T., and Mihara, H. (2008) Peptide and protein mimetics inhibiting amyloid beta-peptide aggregation. *Acc. Chem. Res. 41*, 1309–1318.
26. Abedini, A., Meng, F. L., and Raleigh, D. P. (2007) A single-point mutation converts the highly amyloidogenic human islet amyloid polypeptide into a potent fibrillization inhibitor. *J. Am. Chem. Soc. 129*, 11300.
27. Yan, L. M., Tatarek-Nossol, M., Velkova, A., Kazantzis, A., and Kapurniotu, A. (2006) Design of a mimic of nonamyloidogenic and bioactive human islet amyloid polypeptide (IAPP) as nanomolar affinity inhibitor of IAPP cytotoxic fibrillogenesis. *Proc. Natl. Acad. Sci. U.S.A. 103*, 2046–2051.
28. Scrocchi, L. A., Chen, Y., Wang, F., Han, K., Ha, K., Wu, L., and Fraser, P. E. (2003) Inhibitors of islet amyloid polypeptide fibrillogenesis, and the treatment of type-2 diabetes. *Lett. Pept. Sci. 10*, 545–551.
29. Porat, Y., Mazor, Y., Efrat, S., and Gazit, E. (2004) Inhibition of islet amyloid polypeptide fibril formation: A potential role for heteroaromatic interactions. *Biochemistry 43*, 14454–14462.
30. Mishra, R., Bulic, B., Sellin, D., Jha, S., Waldmann, H., and Winter, R. (2008) Small-molecule inhibitors of islet amyloid polypeptide fibril formation. *Angew. Chem., Int. Ed. 47*, 4679–4682.
31. Porat, Y., Abramowitz, A., and Gazit, E. (2006) Inhibition of amyloid fibril formation by polyphenols: Structural similarity and aromatic interactions as a common inhibition mechanism. *Chem. Biol. Drug Des. 67*, 27–37.
32. Aisen, P. S., Gauthier, S., Vellas, B., Briand, R., Saurnier, D., Laurin, J., and Garceau, D. (2007) Alzhemed: A potential treatment for Alzheimer's disease. *Curr. Alzheimer Res. 4*, 473–478.
33. Kisilevsky, R., Lemieux, L. J., Fraser, P. E., Kong, X. Q., Hultin, P. G., and Szarek, W. A. (1995) Arresting amyloidosis in-vivo using small-molecule anionic sulfonates or sulfates—Implications for Alzheimers-disease. *Nat. Med. 1*, 143–148.
34. Mishra, R., Sellin, D., Radovan, D., Gohlke, A., and Winter, R. (2009) Inhibiting islet amyloid polypeptide fibril formation by the red wine compound resveratrol. *ChemBioChem 10*, 445.
35. Higdon, J. V., and Frei, B. (2003) Tea catechins and polyphenols: Health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. 43*, 89–143.
36. Jeong, W. S., and Kong, A. N. T. (2004) Biological properties of monomeric and polymeric catechins: Green tea catechins and procyanidins. *Pharm. Biol. 42*, 84–93.
37. Rezai-Zadeh, K., Arendash, G. W., Hou, H. Y., Fernandez, F., Jensen, M., Runfeldt, M., Shytle, R. D., and Tan, J. (2008) Green tea epigallocatechin-3-gallate (EGCG) reduces beta-amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer transgenic mice. *Brain Res. 1214*, 177–187.
38. Li, Q., Gordon, M., Tan, J., and Morgan, D. (2006) Oral administration of green tea epigallocatechin-3-gallate (EGCG) reduces amyloid beta deposition in transgenic mouse model of Alzheimer's disease. *Exp. Neurol. 198*, 576–576.
39. Rezai-Zadeh, K., Shytle, D., Sun, N., Mori, T., Hou, H. Y., Jeanniton, D., Ehrhart, J., Townsend, K., Zeng, J., Morgan, D., Hardy, J., Town, T., and Tan, J. (2005) Green tea epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice. *J. Neurol. 25*, 8807–8814.
40. Lin, C. L., Chen, T. F., Chiu, M. J., Way, T. D., and Lin, J. K. (2009) Epigallocatechin gallate (EGCG) suppresses beta-amyloid-induced neurotoxicity through inhibiting c-Abl/FE65 nuclear translocation and GSK3 beta activation. *Neurobiol. Aging 30*, 81–92.
41. Ehrnhoefer, D. E., Bieschke, J., Boeddrich, A., Herbst, M., Masino, L., Lurz, R., Engemann, S., Pastore, A., and Wanker, E. E. (2008) EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nat. Struct. Mol. Biol. 15*, 558–566.
42. Tilstra, L. F., Maeda, H., and Mattice, W. L. (1988) Interaction of (+)-catechin with the edge of the beta-sheet formed by poly(S-carboxymethyl-L-cysteine). *J. Chem. Soc., Perkin Trans. 2*, 1613–1616.
43. Hudson, S. A., Ecroyd, H., Dehle, F. C., Musgrave, I. F., and Carver, J. A. (2009) (−)-Epigallocatechin-3-Gallate (EGCG) Maintains kappa-casein in its pre-fibrillar state without redirecting its aggregation pathway. *J. Mol. Biol. 392*, 689–700.
44. Bieschke, J., Russ, J., Friedrich, R. P., Ehrnhoefer, D. E., Wobst, H., Neugebauer, K., and Wanker, E. E. (2010) EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity. *Proc. Natl. Acad. Sci. U.S.A. 107*, 7710–7715.
45. Rambold, A. S., Miesbauer, M., Olschewski, D., Seidel, R., Riemer, C., Smale, L., Brumm, L., Levy, M., Gazit, E., Oesterhelt, D., Baier, M., Becker, C. F. W., Engelhard, M., Winklhofer, K. F., and Tatzelt, J. (2008) Green tea extracts interfere with the stress-protective activity of PrPC and the formation of PrPSc. *J. Neurochem. 107*, 218–229.
46. Roberts, B. E., Duennwald, M. L., Wang, H., Chung, C., Lopreiato, N. P., Sweeny, E. A., Knight, M. N., and Shorter, J. (2009)

A synergistic small-molecule combination directly eradicates diverse prion strain structures. *Nat. Chem. Biol. 5*, 936–946.
47. Chandrashekaran, I. R., Adda, C. G., MacRaild, C. A., Anders, R. F., and Norton, R. S. (2010) Inhibition by flavonoids of amyloid-like fibril formation by *Plasmodium falciparum* merozoite surface protein 2. *Biochemistry 49*, 5899–5908.
48. Abedini, A., and Raleigh, D. P. (2005) Incorporation of pseudoproline derivatives allows the facile synthesis of human IAPP, a highly amyloidogenic and aggregation-prone polypeptide. *Org. Lett. 7*, 693–696.
49. Abedini, A., Singh, G., and Raleigh, D. P. (2006) Recovery and purification of highly aggregation-prone disulfide-containing peptides: Application to islet amyloid polypeptide. *Anal. Biochem. 351*, 181–186.
50. Nilsson, M. R., and Raleigh, D. P. (1999) Analysis of amylin cleavage products provides new insights into the amyloidogenic region of human amylin. *J. Mol. Biol. 294*, 1375–1385.
51. Hamid, R., Rotshteyn, Y., Rabadi, L., Parikh, R., and Bullock, P. (2004) Comparison of alamar blue and MTT assays for high throughput screening. *Toxicol. In Vitro 18*, 703–710.
52. Levine, H. (1995) Thioflavine-T interaction with amyloid beta-sheet structures. *Amyloid 2*, 1–6.
53. Collins, S. R., Douglass, A., Vale, R. D., and Weissman, J. S. (2004) Mechanism of prion propagation: Amyloid growth occurs by monomer addition. *PLoS Biol. 2*, 1582–1590.
54. Knowles, T. P. J., Waudby, C. A., Devlin, G. L., Cohen, S. I. A., Aguzzi, A., Vendruscolo, M., Terentjev, E. M., Welland, M. E., and Dobson, C. M. (2009) An analytical solution to the kinetics of breakable filament assembly. *Science 326*, 1533–1537.
55. Larson, J. L., and Miranker, A. D. (2004) The mechanism of insulin action on islet amyloid polypeptide fiber formation. *J. Mol. Biol. 335*, 221–231.
56. Abedini, A., and Raleigh, D. P. (2005) The role of His-18 in amyloid formation by human islet amyloid polypeptide. *Biochemistry 44*, 16284–16291.
57. Xue, W. F., Homans, S. W., and Radford, S. E. (2008) Systematic analysis of nucleation-dependent polymerization reveals new insights into the mechanism of amyloid self-assembly. *Proc. Natl. Acad. Sci. U.S.A. 105*, 8926–8931.
58. Meng, F. L., Marek, P., Potter, K. J., Verchere, C. B., and Raleigh, D. P. (2008) Rifampicin does not prevent amyloid fibril formation by human islet amyloid polypeptide but does inhibit fibril thioflavin-T interactions: Implications for mechanistic studies beta-cell death. *Biochemistry 47*, 6016–6024.
59. Ling, Y. L., Strasfeld, D. B., Shim, S. H., Raleigh, D. P., and Zanni, M. T. (2009) Two-dimensional infrared spectroscopy provides evidence of an intermediate in the membrane-catalyzed assembly of diabetic amyloid. *J. Phys. Chem. B 113*, 2498–2505.
60. Konarkowska, B., Aitken, J. F., Kistler, J., Zhang, S. P., and Cooper, G. J. S. (2006) The aggregation potential of human amylin determines its cytotoxicity towards islet beta-cells. *FEBS J. 273*, 3614–3624.
61. Abedini, A., and Raleigh, D. P. (2009) A critical assessment of the role of helical intermediates in amyloid formation by natively unfolded proteins and polypeptides. *Protein Eng., Des. Sel. 22*, 453–459.
62. Masuda, M., Hasegawa, M., Nonaka, T., Oikawa, T., Yonetani, M., Yamaguchi, Y., Kato, K., Hisanaga, S., and Goedert, M. (2009) Inhibition of alpha-synuclein fibril assembly by small molecules: Analysis using epitope-specific antibodies. *FEBS Lett. 583*, 787–791.