



Attenuation of β -Amyloid Toxicity *In Vitro* and *In Vivo* by Accelerated Aggregation

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Abstract Accumulation and aggregation of β -amyloid (A β) peptides result in neuronal death, leading to cognitive dysfunction in Alzheimer's disease. The self-assembled A β molecules form various intermediate aggregates including oligomers that are more toxic to neurons than the mature aggregates, including fibrils. Thus, one strategy to alleviate A β toxicity is to facilitate the conversion of A β intermediates to larger aggregates such as fibrils. In this study, we designed a peptide named A3 that significantly enhanced the formation of amorphous aggregates of A β by accelerating the aggregation kinetics. Thioflavin T fluorescence experiments revealed an accelerated aggregation of A β monomers, accompanying reduced A β cytotoxicity. Transgenic *Caenorhabditis elegans* over-expressing amyloid precursor protein exhibited paralysis due to the accumulation of A β oligomers, and this phenotype was attenuated by feeding the animals with A3 peptide. These findings suggest that the A β aggregation-promotion effect can potentially be useful for developing strategies to reduce A β toxicity.

Keywords A β · Alzheimer's disease · Aggregation · A β oligomer · Amorphous aggregate · Cell death · *C. elegans* · β -sheet

Introduction

Aggregation of amyloid peptides is known to play a key role in Alzheimer's disease (AD). These amyloid aggregates are composed mainly of β -amyloid (A β) peptide. *In vivo*, A β is produced by endoproteolytic cleavage of amyloid precursor protein (APP) [1–5]. A β monomers self-assemble into variable intermediate aggregates, such as oligomers, protofibrils, annular aggregates, and the insoluble mature fibrils [6–8]. Recent studies suggest that the soluble A β oligomers, rather than A β monomers or the insoluble large A β fibrils, are the most toxic, leading to neuronal pathology and the clinical manifestations of AD [9–12]. Extensive efforts have been directed towards A β reduction, either by inhibiting the enzymes that generate A β peptide or neutralizing A β antibodies. These strategies have not been successful so far [13]. An alternative strategy to alleviate A β toxicity is to promote A β oligomers to further aggregate into A β fibers or other insoluble aggregates. There have been reports that [G3]-Mor (a gallic acidtriethylene glycol dendrimer, functionalized with 27 terminal morpholinoethyl groups) and PEI-P (polyethylenimineperphenazine-P) accelerate aggregation, which would significantly reduce the toxicity of A β aggregates [14, 15]. Aggregation of proteins can be affected by various environmental conditions, such as temperature, salt concentration, or the presence of other catalysts [16]. In addition, surface chemistry has also been shown to appreciably affect the aggregation kinetics of proteins and peptides [17, 18].

Attempts have been made to find molecular species that can interfere with the aggregation kinetics of β amyloid. These molecules can be categorized as polypeptides, dyes, and organic or inorganic compounds [19–26]. Amyloid-binding dyes such as thioflavin T (ThT) have been reported

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to reduce the aggregation of A β (3–42) and significantly extend the lifespan of adult *Caenorhabditis elegans* [22]. Phage display experiments have shown that some peptides have a high affinity for either monomeric or aggregated A β [27]. These findings have improved our understanding of the molecular pathways of the protein aggregation process. However, most of these studies were conducted *in vitro*. Transgenic mouse models of AD exhibit some of the pathological and behavioral changes seen in humans, but such models are expensive, time-consuming, and not amenable to drug screening [28, 29]. Simple invertebrate models of A β aggregation may offer advantages, not only in drug screening but also in studying the basic cellular processes conserved among animals. Transgenic *C. elegans* can express human A β and exhibit pathological and behavioral abnormalities relevant to A β [30, 31].

In the present study, we set out to identify peptide modulators that significantly affect the aggregation kinetics of A β , leading to the attenuation of β -amyloid toxicity.

Materials and Methods

Synthetic Peptides and A β

Four peptide modulators were designed and custom-synthesized by GL Biochem Ltd (Shanghai, China). The peptide purity (>98%) was verified by high-performance liquid chromatography and mass spectrum analyses. A β 42 was from GL Biochem. The peptide modulators were dissolved in triple-distilled H₂O and A β was dissolved in 1% dimethyl sulfoxide (DMSO) for *in vivo* experiments.

ThT Fluorescence

A β was incubated in sterile phosphate-buffered saline (PBS) with or without the peptide modulators for up to 4.5 days at 37 °C. The final concentration of A β was 40 μ mol/L. Aggregation was recorded by fluorescence after addition of ThT. A fluorescent dye, ThT identifies structures containing β -sheets. We chose a series of time points for quantitative analysis, and A β aggregation was accompanied by a characteristic increase in fluorescence intensity at 480 nm in Corning 96-well black plates (New York, NY) using a PerkinElmer Victor microplate reader (Waltham, MA). To exclude background fluorescence, the fluorescence intensity of a blank solution (ThT alone in PBS) was subtracted from all readings.

Transmission Electron Microscopy (TEM)

We incubated A β (40 μ mol/L) with the peptide modulators (10 μ mol/L) at 37 °C for 3 days in PBS. A β or peptide

modulators alone were set up as control groups. At 6 and 72 h, a 50 μ L sample was dropped onto carbon-coated copper grids (200 mesh) for 20 min and dried by air flow after removal of the excess sample drop. Afterwards, the samples were negatively stained with 20 μ L phosphotungstic acid (1 mg/mL) for 10 min and the excess solution was removed. Then the grids were washed three times with triple-distilled water and dried under vacuum overnight. All experiments were performed using a FEI Tecnai G2 20 S-TWIN TEM (Hillsboro, TX) with an accelerating voltage of 200 kV.

Atomic Force Microscopy (AFM)

Samples were diluted in the same way as for TEM. At 72 h, $\sim$ 10 μ L of the solution was applied to a freshly-cleaved mica surface and dried with air flow. AFM measurements were performed on a Bruker Dimension 3100 scanning probe microscope (Karlsruhe, Germany) in tapping mode at room temperature. A silicon probe was oscillated at $\sim$ 330 kHz and images were collected at a scan rate of 1 Hz.

Cell Line and Culture

The human neuroma cell line SH-SY5Y was purchased from the Cell Culture Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Cells were cultured in Hyclone RPMI-1640 medium (Logan, UT) supplemented with 15% Gibco newborn bovine serum (Grand Island, NY), penicillin G (100 kU/L), and kanamycin (0.1 g/L). The cells were routinely subcultured once every 3 days and maintained at 37 °C in 5% CO₂ and 95% air in 75-cm² tissue culture flasks.

MTT Assay

SH-SY5Y cells were plated in full medium on 96-well plates in a 100 μ L total volume at 1×10^4 cells/mL and cultured at 37 °C in 5% CO₂ for 24 h. Then 10 μ L A β (final concentration, 40 μ mol/L) and 10 μ L peptide modulator (final concentration as indicated) were added and incubated for another 30 h. Then the medium was carefully removed from each well and 150 μ L DMSO was added. Cell viability was determined based on the mitochondrial conversion of MTT dye to formazan crystals. Absorbance was read at 540 nm on a Thermo microplate reader (Waltham, MA). Data are expressed as percentage cell viability compared to a 100% signal from untreated cells.

C. elegans Strains

The wild-type strain N2, and the transgenic nematode strains CL4176 and CL802 were kindly provided by

professor Guangjun Nie (National Center for Nanoscience and Technology, China). The CL4176 strain constitutively produces body-wall muscle-specific A β 42, the expression of which depends on upshifting the temperature from 16 to 23 °C. The CL802 strain does not express body-wall muscle-specific A β 42 and was used as a control. The wild-type N2 and the transgenic CL4176 and CL802 were propagated at 16 °C on solid nematode growth medium seeded with 200 μ L spots of *Escherichia coli* (OP50) for food. After obtaining age-synchronized *C. elegans*, the worms were fed with the designed peptides either from stage L1 (1 day old) or starting from the egg stage.

Paralysis Assays

The CL4176 strain maintained at 16 °C was egg-synchronized onto 50 $\times$ 10 mm² culture plates containing either vehicle or drug. Transgene expression was induced by upshifting the temperature from 16 to 23 °C, starting on the 24th hour after egg-laying and lasting until the end of the paralysis assay. Paralysis was scored at 2-h intervals until the last worm became paralyzed.

Results

Effect of A3 Peptide on A β Aggregation Kinetics

A β peptide has several core amyloidogenic sequences, regarded as self-recognition regions, by which A β peptides interact with each other and can further assemble into β -sheet-rich fibrils [32, 33]. Several studies have shown that the onset of aggregation (nucleation) is accelerated by the addition of peptides with pro-aggregative activity [34–36]. In addition, some small molecular inhibitors have been shown to reduce A β aggregation by targeting the hydrophobic residues of A β sequences [15, 37].

In the present study, we set out to develop peptide-based A β modulators, focusing on the core amyloidogenic sequences of the A β 42 molecule. The main principle of our design was based on the amino-amino electrostatic forces, but we also considered the hydrophilic and hydrophobic interactions, as well as steric hindrance. We designed four peptides with the following sequences (Fig. 1A):

- A1: VDDQEL
- A2: KSGYKV
- A3: DDKSGYKVDDQD
- A4: RKGVS

It is believed that A β fibrils contain many more β -sheet motifs than A β oligomers [38]. Thus, an increase in β -sheets can be viewed as an indicator of oligomer-to-fibril conversion. The ThT fluorescence assay has been widely

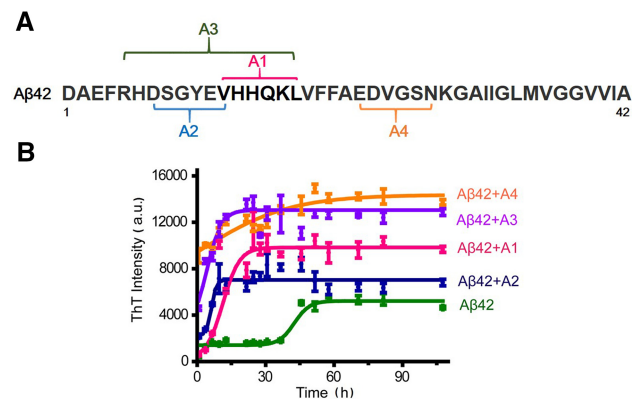


Fig. 1 Schematic of the design of peptide modulators. **A** Specific A β sequences with specific targets of the peptide modulators indicated. **B** The peptide modulators altered A β 42 aggregation. ThT fluorescence was monitored during incubation of 40 μ mol/L A β 42 in the absence or presence of 10 μ mol/L peptide modulator. Error bars are standard deviations of the mean of three replicates.

used to detect the β -sheet conformation in A β 42 aggregates [39, 40]. The fluorescence intensity of ThT depends strictly on the presence of β -sheet structures in the system—the more β -sheets are formed, the higher the ThT fluorescence intensity. Newly-synthesized A β 42 was incubated for 72 h in the absence or presence of each of the peptide modulators, and the aggregation process was monitored over time by ThT fluorescence.

In the absence of any peptide, the ThT fluorescence was 0 a.u. or slightly lower (data not shown). A β 42 alone exhibited detectable fluorescence at time point 0, valued at $\sim$ 1000 a.u. (Fig. 1B), and then increased to reach a maximum at 50 h (Fig. 1B). In the presence of any of the four peptide modulators, the ThT fluorescence not only reached much higher levels but also increased at a faster rate. Among the four modulators, A3 appeared to have the greatest effect on ThT fluorescence (Fig. 1B). At 0 h, the ThT intensity was already high ($\sim$ 4000 a.u.) and it only took 16 h to reach the peak. At the plateau, the intensity was almost three times that with A β alone (A β alone, 4500 a.u.; A β + A3, 13000 a.u.). Thus, the A β aggregation process was significantly accelerated by the A3 peptide. Interestingly, the ThT fluorescence of the A β 42 + A3 solution had no lag time (Fig. 1B). It should be pointed out that the starting point was not really 0; the time interval from mixing the A β 42 and A3 peptides to finishing the measurement was $\sim$ 5 min. Moreover, the A3 peptide might act as a nucleating agent, leading to much faster nucleation of the A3–A β complexes.

Morphological Change of A β Aggregates in the Presence of A3 Peptide

TEM and AFM experiments were carried out to observe the morphological changes of A β intermediates incubated in

the presence or absence of the A3 peptide (Fig. 2). TEM revealed that while A β 42 peptide appeared as small granular particles without a clear form or shape at 6 h, it clearly formed a typical fibril structure after 3 days (72 h) *in vitro*, as reported previously [41] (Fig. 2A, D). The A3 peptide alone did not form any discernible structure under TEM (Fig. 2B, E). We expected that A β oligomers would be converted to long fibrils after co-incubation with the A3 peptide for 6 h and more so for 72 h. To our surprise, we found a time-dependent accumulation of smaller amorphous aggregates (Fig. 2C, F). Compared with A β alone, the amount of oligomer-like structures was significantly reduced and small amorphous aggregates were observed

instead. AFM experiments provided a different way of looking at protein aggregates. Consistent with the TEM results, A β 42 alone exhibited clustering structures indicative of A β oligomers. Co-incubation of A β with the A3 peptide revealed almost no A β oligomer-like structures, but a marked increase in small amorphous aggregates at 72 h (Fig. 2G–I). The EM and ThT experiments together support the notion that the A3 peptide interacts with A β at the early stage of aggregation and promotes A β aggregation into amorphous rather than fibrillary structures. The accelerated aggregation with faster nucleation and cross-linking effects gave rise to a less-ordered morphology, but their secondary structures were still mainly β -sheets.

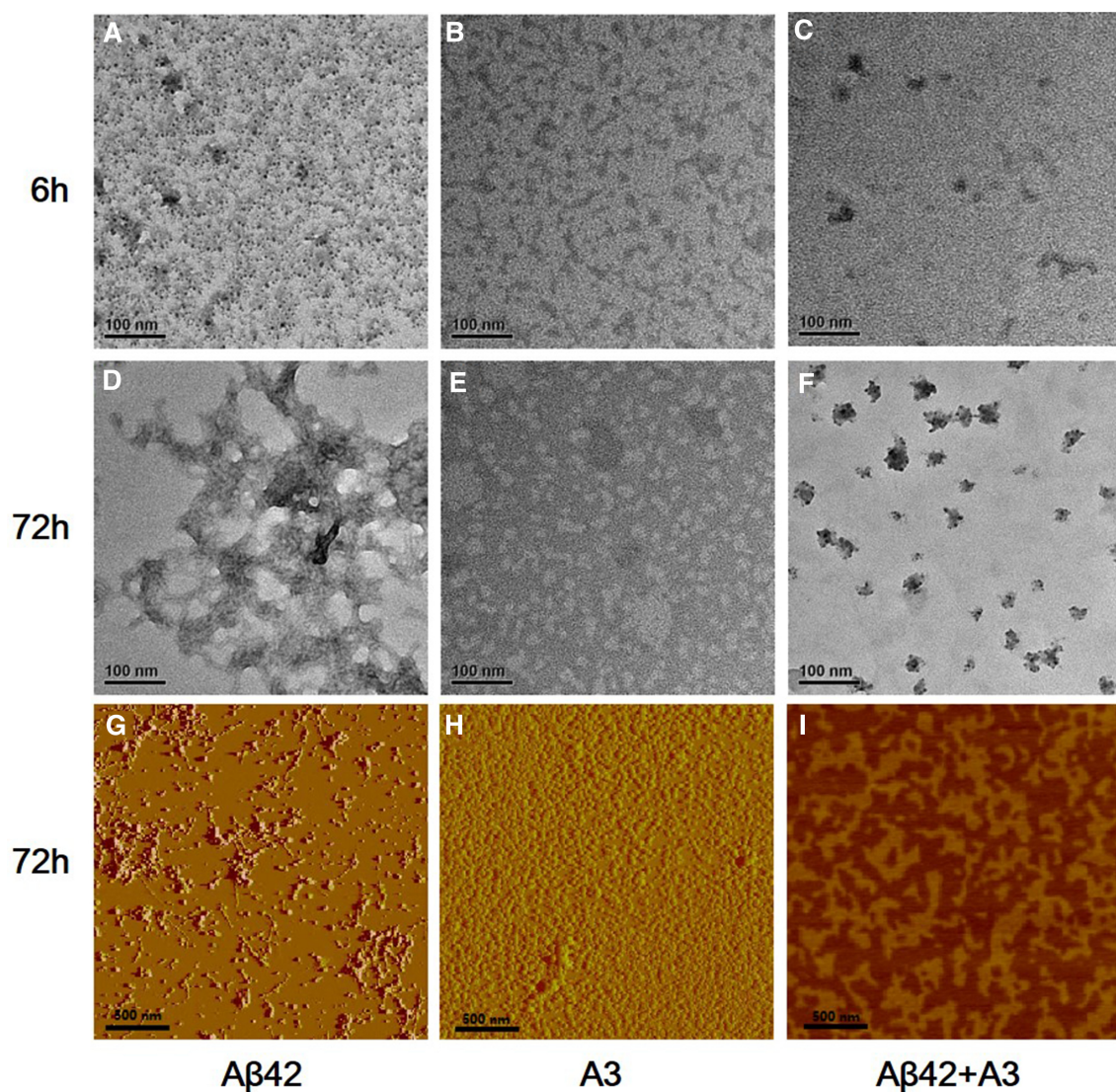


Fig. 2 A3 peptide facilitated A β aggregation into amorphous aggregates, as revealed by TEM and AFM. **A–F** Negatively-stained TEM images of A β , A3 peptide, and A β + A3 after 6 h and 3 days

incubation *in vitro*. Scale bars, 100 nm. **G–I** AFM images of A β , A3 peptide, and A β + A3 after 3 days of incubation. Scale bars, 500 nm.

Reduced Cytotoxicity of A β Aggregates in Association with A3 Peptide

Next, we determined whether the A3 peptide counters A β toxicity by using SH-SY5Y cells. Application of A β aggregates to the cultured cells induced a dose-dependent decrease in viability (Fig. 3A). At 40 μ mol/L, A β reduced the viability to nearly 60%. In contrast, exposure of SYHY-5Y cells to the A1, A2, or A3 peptide alone induced no change in viability (Fig. 3B–D). The A4 peptide appeared to have a small effect on cell survival even at 2.5 μ mol/L, so it was not useful in cytotoxicity experiments (Fig. 3E).

In addition, we found that the A3 peptide inhibited A β -induced cell death over a wide range of concentrations, from 2.5 to 60 μ mol/L (Fig. 3F). At the highest concentration (180 μ mol/L), cell viability dropped to the control level (60%). This “biphasic” viability curve might be due to a pH decrease in the medium, when too much A3 peptide (isoelectric point 3.85) was added. In contrast, none of the other peptides (A1, A2, and A4) countered the effect of A β on viability (Fig. 3F). Taken together, our

experiments suggest that the A3 peptide attenuates A β -induced cytotoxicity, possibly by facilitating A β aggregation.

A3 Peptide Alleviates A β -Induced Paralysis in Transgenic *C. elegans*

We further investigated whether the A3 peptide specifically protects against A β -induced toxicity *in vivo*, in a transgenic *C. elegans* model of human AD, the CL4176 strain (smg-1^{ts} [myo-3/A β _{1–42}long 3'-untranslated region]). The CL4176 strain over-expresses human A β in muscle cells, leading to an A β -dependent paralysis phenotype (Fig. 4A). As a control, we used the CL802 strain that does not express A β . A3 peptide solution (50 mg/mL) was fed to CL4176 and CL802 animals. A β expression was induced by a temperature upshift from 16 to 23 °C at 24 h [22]. Paralysis did not occur in the CL802 control (Fig. 4B). The CL4176 line fed with the A3 peptide showed a notable delay of paralysis compared with those without A3 (Fig. 4B). For quantitative analysis, we defined PT50 as the time interval at which 50%

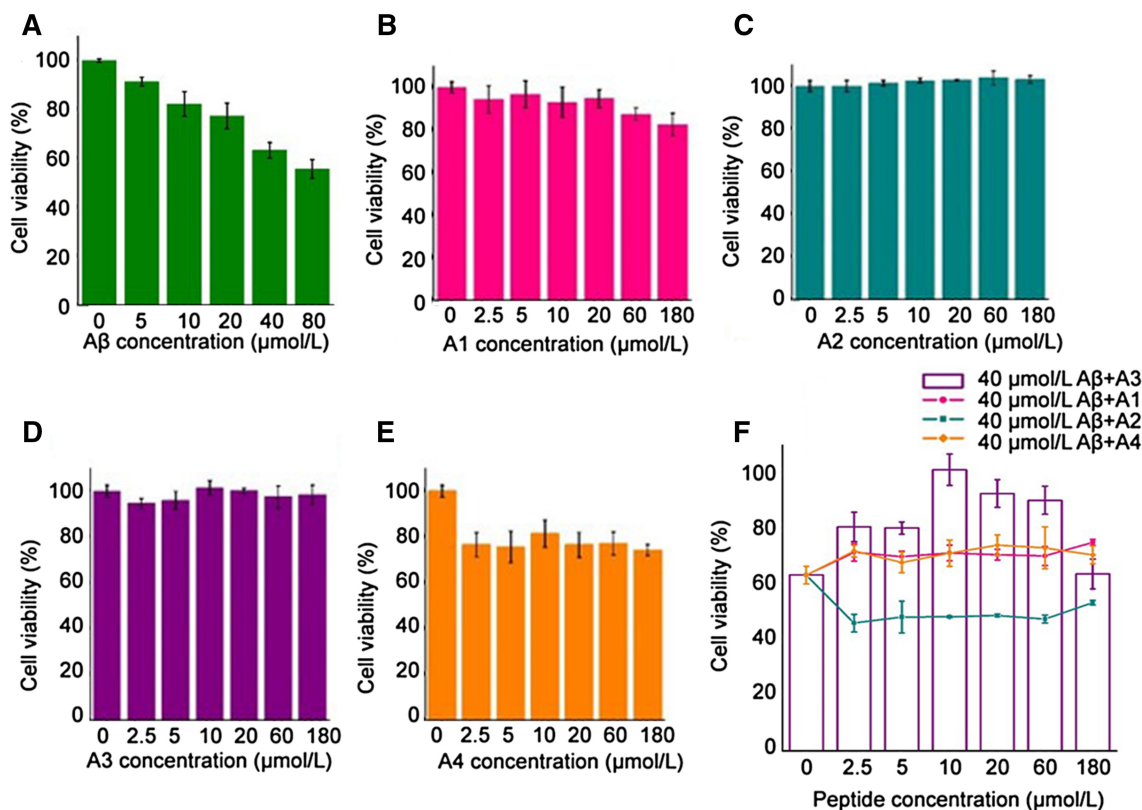


Fig. 3 The peptides affected A β aggregation-induced cytotoxicity in SH-SY5Y cells at different concentrations. SH-SY5Y cell viability was measured by MTT assay after incubation. **A** A β 42 reduced the viability of SH-SY5Y cells in a concentration-dependent manner. **B–E** Cytotoxicity of designed peptides in SH-SY5Y cells. **F** Viability

changed in the presence of designed peptides over a wide range of concentrations (2.5–180 μ mol/L). The cell survival in PBS controls was taken to be 100%. Error bars correspond to standard deviations of six sets of experiments.

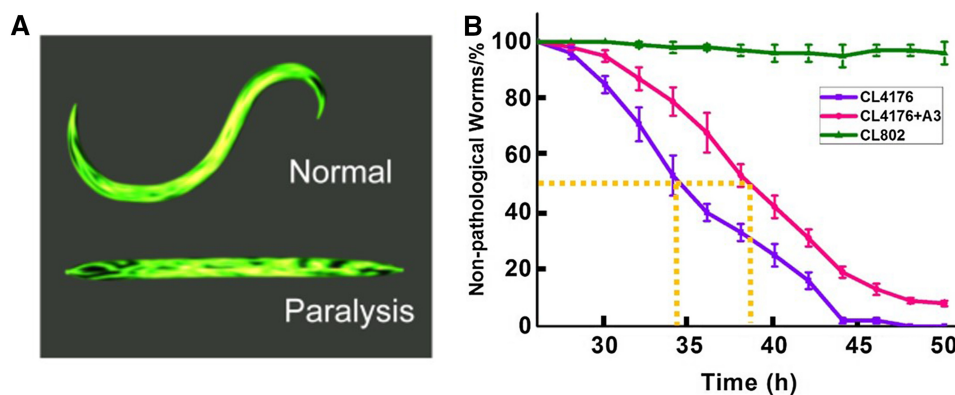


Fig. 4 The A3 peptide delayed the rate of paralysis in the muscle A β strain CL4176. **A** Normal *C. elegans* can move freely (*above*). *C. elegans* with transgene expression paralysis cannot move or only move the head (*below*). **B** Time course of paralysis in CL4176 worms

of the worms were paralyzed. Treatment with the A3 peptide caused an approximately 5 h delay of PT50, compared with the untreated CL4176 as the control group ($P < 0.03$; $n = 9$ assays; 100 worms in each assay group). Moreover, the peptide feeding was found to delay paralysis at different concentrations (data not shown). These results suggest that the A3 peptide is capable of attenuating the behavioral phenotype induced by A β .

Discussion

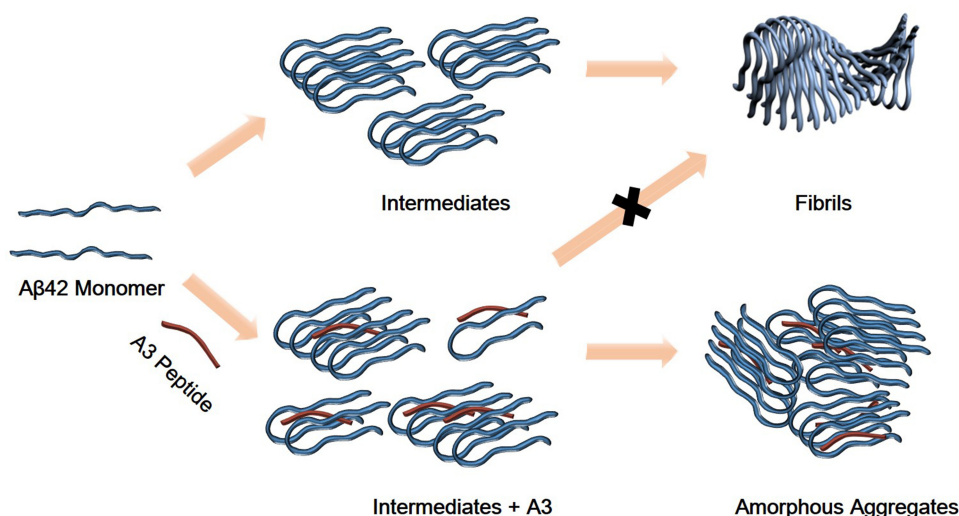
The present study aimed at identifying molecular species that interfere with the A β aggregation process. Our results showed that the A3 peptide accelerated the conversion of A β oligomers into β -sheet-rich amorphous aggregates, rather than A β fibrils. A cartoon illustrating how the A3 peptide modulates A β aggregation is shown in Fig. 5. In

parallel, the A3 peptide also led to a reduction in the A β -induced toxicity in cultured SH-SY5Y cells and muscle paralysis in *C. elegans in vivo*. Taken together, our results reveal a new approach to attenuating A β -induced cell death, and raise the possibility of a new strategy for A β -related disease, including AD therapy.

The ThT fluorescence assay was used to quantify the amount of β -sheet structure, and therefore indirectly the aggregation kinetics of A β intermediates. In prior experiments, we had measured the ThT fluorescence of the A3 peptide alone, and none was detectable (data not shown). Thus, the ThT fluorescence in the mixture was derived from the β -sheet structures of A β alone, or the A3 peptide-A β complex. A faster increase and higher intensity of ThT fluorescence was measured in the A β + A3 peptide mixture than with A β alone. However, at the starting point of the A β + A3 peptide curve, the ThT fluorescence was already quite high (~ 4000 a.u.), and there was no lag time

parallel, the A3 peptide also led to a reduction in the A β -induced toxicity in cultured SH-SY5Y cells and muscle paralysis in *C. elegans in vivo*. Taken together, our results reveal a new approach to attenuating A β -induced cell death, and raise the possibility of a new strategy for A β -related disease, including AD therapy.

Fig. 5 Cartoon of A β 42 aggregation with or without A3 peptide. A β 42 monomers assemble into cross- β structure oligomeric intermediates and finally into amyloid fibrils (*above*). In the presence of A3 peptide, A β 42 aggregation is accelerated into amorphous aggregates (*below*).



(Fig. 1B). Two factors might contribute to the higher fluorescence at the start. First, the starting point was not really “0” time—the interval from mixing to finishing the measurement was ~ 5 min. Second, the A3 peptide might act as a nucleating agent, leading to much faster nucleation of A3 peptide-A β complexes.

It is generally believed that the A β 42 peptide does not exist as a monomer in the brain. Instead, it aggregates into more toxic A β oligomers, which in turn can be converted into larger and insoluble A β fibrils [41] (Fig. 5). TEM and AFM images at different time points revealed that, in the presence of the A3 peptide, A β oligomers form amorphous structures rather than fibril structures (Fig. 2). It is unclear how the A3 peptide directs A β to form amorphous aggregates rather than fibrils. We speculate that the nucleation and crosslinking capacity of the A3 peptide might accelerate A β aggregation, giving rise to a less-ordered amorphous morphology. Interestingly, the main secondary structures of amorphous aggregates remained largely β -sheets. It is possible that the A3 peptide interacts with the β -sheets of A β molecules by electrostatic attraction, Van der Waals attraction, and hydrophobic force, which would neutralize the electrostatic repulsion to prevent the self-assembly of A β oligomers.

The cytoprotective effect of the A3 peptide against A β is quite different from the previously reported inhibitors, which prevent neuronal death by inhibiting A β aggregation [42]. Our results support the notion that the A3 peptide reduced the toxicity of A β aggregation in SH-SY5Y cells and *C. elegans* by accelerating the conversion of A β oligomers to amorphous aggregates, which are less toxic than A β oligomers or even fibrils. However, the A3 peptide had its protective effects at low concentrations (up to 10 $\mu\text{mol/L}$), but not a high concentration (180 $\mu\text{mol/L}$). Since the A3 peptide alone had little cytotoxicity, the decrease in cell viability was most likely due to changes induced by the A3 peptide-A β complex. At a high concentration (180 $\mu\text{mol/L}$), the A3 peptide (with an isoelectric point of 3.85) may change the pH of the culture medium, affecting cell viability. Moreover, A β 42 (with an isoelectric point of 5.43) has polar chains with a potential for negative and positive charges at neutral pH. A large increase in the A3 peptide-to-A β ratio (180:40 $\mu\text{mol/L}$) may alter their charge interaction, making it difficult to form the less toxic amorphous structures.

Our studies indicate that the A3 peptide has the ability to significantly accelerate A β aggregation, leading to changes in the morphology of A β aggregates. Instead of fibrils, amorphous structures, which contained more β -sheets, were observed in the mature aggregates. Furthermore, the A3 peptide protected cells from A β -associated toxicity *in vitro* and alleviated A β -induced paralysis of transgenic *C. elegans in vivo*. The cytoprotective effect of the A3

peptide is quite different from that of inhibitors, which protect neurons by inhibiting A β aggregation. This aggregation-promotion effect may benefit the design of anti-amyloid therapy and ultimately benefit the treatment of amyloid diseases.

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