

# Inhibition of cytotoxicity and amyloid fibril formation by a D-amino acid peptide that specifically binds to Alzheimer's disease amyloid peptide

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**Alzheimer's disease (AD) is a progressive neurodegenerative disorder. The 'amyloid cascade hypothesis' assigns the amyloid-beta-peptide (A $\beta$ ) a central role in the pathogenesis of AD. Although it is not yet established, whether the resulting A $\beta$  aggregates are the causative agent or just a result of the disease progression, polymerization of A $\beta$  has been identified as a major feature during AD pathogenesis. Inhibition of the A $\beta$  polymer formation, thus, has emerged as a potential therapeutic approach. In this context, we identified peptides consisting of D-enantiomeric amino acid peptides (D-peptides) that bind to A $\beta$ . D-peptides are known to be more protease resistant and less immunogenic than the respective L-enantiomers. Previously, we have shown that a 12mer D-peptide specifically binds to A $\beta$  amyloid plaques in brain tissue sections from former AD patients. *In vitro* obtained binding affinities to synthetic A $\beta$  revealed a  $K_d$  value in the submicromolar range. The aim of the present study was to investigate the influence of this D-peptide to A $\beta$  polymerization and toxicity. Using cell toxicity assays, thioflavin fluorescence, fluorescence correlation spectroscopy and electron microscopy, we found a significant effect of the D-peptide on both. Presence of D-peptides (Dpep) reduces the average size of A $\beta$  aggregates, but increases their number. In addition, A $\beta$  cytotoxicity on PC12 cells is reduced in the presence of Dpep.**

**Keywords:** A $\beta$ /aggregation/Alzheimer's disease/cytotoxicity/D-amino acid peptide

## Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which is characterized by memory loss, confusion and a variety of cognitive disabilities (Selkoe, 1999). AD, the most common form of dementia, is a devastating disease and affects five million people in Europe alone (Ferri *et al.*, 2005). Today, the only definite way to diagnose AD is the *post mortem* identification of amyloid plaques and neurofibrillary tangles in the brain tissue of the patient. Recent studies indicate that imaging methods like computed tomography, positron emission tomography (PET) and magnetic resonance tomography (MRT) could be useful in distinguishing AD from other forms of dementia disorders, such as vascular dementia, Parkinson's disease and

Huntington's disease. Especially, new radiotracers like fluorine-18-labelled-FDDNP (2-(1-{6-[(2-[F-18]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene) malononitrile) and carbon-11-labelled-PIB (Pittsburgh Compound B) in combination with improved PET scanning methods are promising tools for the improvement of the early diagnosis of AD and the monitoring of disease progression. The major component of the amyloid plaques is the amyloid  $\beta$ -peptide (A $\beta$ ) consisting of 39 to 43 amino acid residues. It is still not established, whether these plaques are a result of AD or, vice versa, AD is the result of A $\beta$  plaques. Although final proof or disproof has not yet been provided, there is an increasing amount of evidence in favor of the latter hypothesis, and the corresponding 'amyloid model' has become more and more consistent during the last years (Selkoe, 1999; Kimura *et al.*, 2003). A $\beta$  is produced from the amyloid precursor protein (APP) by two distinct proteolytic activities, called  $\beta$ - and  $\gamma$ -secretases (Kang *et al.*, 1987; Weidemann *et al.*, 1989; Haass and Selkoe, 1993). These formerly proposed proteolytic activities have been assigned to certain proteins, called beta-site APP-cleaving enzyme (BACE) (Sinha *et al.*, 1999; Vassar *et al.*, 1999) and Presenilin-1 (Haass, 1996), the latter in combination with other factors (Edbauer *et al.*, 2003).

Therapeutical approaches currently available for AD are restricted to rather unspecific interventions in processes being located far downstream in the causal chain leading ultimately to AD clinical symptoms. Substances used for these interventions include inflammation inhibitors, acetylcholinesterase inhibitors, glutamic acid receptor activators, glutamate antagonists, secretase-inhibitors, NGF-agonists and antioxidants. Acetylcholine inhibitors like donepezil, galantamine and the NMDA receptor antagonist memantine are approved for clinical use for treatment of cognitive symptoms (Blennow *et al.*, 2006; Roberson and Mucke, 2006). Atypical antipsychotic drugs like risperidone are reported to show an effect in reducing disease-related behavioral symptoms like aggression, agitation and psychosis (Brodaty *et al.*, 2003).

Results from A $\beta$  vaccination studies by active or passive immunization of humans (Hock *et al.*, 2003) and transgenic mice (Schenk *et al.*, 1999; Bard *et al.*, 2000; Dodart *et al.*, 2002; Seabrook *et al.*, 2007) are promising. However, data on potential long-term side effects of vaccination against endogenous host proteins are not available yet. In one study, phase 2 clinical trials were halted when 6% of the patients treated with an A $\beta$ -antibody (AN1792; Elan Pharmaceuticals Inc.) developed meningoencephalitis (Orgogozo *et al.*, 2003). Other approaches targeting A $\beta$  are the reduction of A $\beta$  production by  $\gamma$ - or  $\beta$ -secretase-modulators (Citron, 2004) or the disruption of A $\beta$  aggregation by small molecules or peptides. Thus, substances being able to inhibit A $\beta$  aggregation and reduce its toxic effects are still highly

desirable. A variety of such substances were described, e.g. Congo red, haloperidol, nicotine, hexadecyl-*N*-methylpiperidiniumbromid, laminin and rifampicin (Soto, 1999). Oligomeric acylated aminopyrazoles, which prevent A $\beta$  aggregation, were constructed by rational design (Rzepecki *et al.*, 2004). Small peptides that inhibit A $\beta$  aggregation and reduce its toxic effects are also described. Soto *et al.* designed a pentapeptid based on the central hydrophobic region in the N-terminal domain of A $\beta$  that acts as a  $\beta$ -sheet breaker (Soto *et al.*, 1998; Soto, 1999). Tjernberg *et al.* identified a short A $\beta$  fragment that binds to full-length A $\beta$ , thus preventing its assembly into amyloid fibrils (Tjernberg *et al.*, 1996). The corresponding all-D-amino acyl analogue peptide of the A $\beta$  fragment LVFFA was proven to be a A $\beta$  fibrillogenesis inhibitor (Findeis *et al.*, 1999). All these aforementioned peptides were derived by variations of the A $\beta$  peptide itself.

Earlier, we described a mirror image phage display approach with the D-amino acid enantiomer of A $\beta$ (1–42) as target. We identified an A $\beta$  binding D-peptide (Dpep). We were able to show that Dpep specifically binds only to A $\beta$  deposits in human brain tissue sections (Wiesehan *et al.*, 2003). In the present study, we explore the influence of Dpep on polymerization and cytotoxicity of A $\beta$ .

## Materials and methods

### Peptides

L-A $\beta$ (1–42), DAEFRHDSGYEVHHQKLVFFAEDVGSNKG AIIGLMVGGVVIA, Dpep, QSHYRHISPAQV, all amino acids such as L-enantiomers and D-enantiomers were purchased as reversed phase high performance liquid chromatography purified products (Jerini Biotech, Berlin, Germany). Identity was confirmed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) (Karas and Hillenkamp, 1988).

### Preparation of A $\beta$ samples

L-A $\beta$ (1–42) was dissolved in sterile filtered H<sub>2</sub>O at a concentration of 10  $\mu$ M. The solution was aliquoted and lyophilized. These aliquots were dissolved in an adequate volume of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), which did contain zero, 100  $\mu$ M or 1 mM Dpep, to obtain 5-fold end concentrations of L-A $\beta$ (1–42) in the respective assay.

### Cell toxicity assays

For the investigation of Dpep influence on the cellular toxicity of L-A $\beta$ (1–42), the MTT assay (Shearman *et al.*, 1994) was accomplished in this way: 20  $\mu$ l of each A $\beta$ -solution were directly (0 h) or after one day (24 h, 37°C), respectively, added to a collagen-IV-coated microtiter plate (BD Biosciences) well containing 80  $\mu$ l DMEM ('Dulbecco's modified Eagle medium' with acetyl-alanine-glutamine, 10% fetal bovine serum, 5% horse serum and 10 mg/ml gentamycin) and  $2 \times 10^4$  PC12 cells grown for 24 h at 37°C in a 7.5% (v/v) CO<sub>2</sub> atmosphere.

Determination of cellular 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) reduction was carried out after a 24-h incubation period of the cells together with the A $\beta$ -Dpep-DMEM mixtures described

above at 37°C in a 7.5% (v/v) CO<sub>2</sub> atmosphere. Then, 10  $\mu$ l sterile filtered solution of 5 mg/ml MTT in PBS was added and incubated for another 3 h. Medium was removed and 100  $\mu$ l cell lysis buffer (99.4 ml DMSO, 0.6 ml 100% acetic acid, 10 g SDS) were added and incubated for 30 min while gently shaking. MTT reduction was determined by measuring the difference between absorbencies at 570 and 630 nm. A cell viability value of 100% was defined corresponding to MTT reduction of cells treated neither with A $\beta$  nor with Dpep. A cell viability value of 0% was defined by treatment of the PC12 cells with 0.2% Triton-X. The percentage of MTT reduction for each measurement was calculated as the fraction of the value relative to the 100 and 0% values.

### Thioflavin T assay

Thioflavin T (ThT) assays (LeVine, 1993) were carried out with minor modifications. Five microliter of each A $\beta$ -Dpep mixture were added to 195  $\mu$ l 5  $\mu$ M ThT (Sigma) in 50 mM Glycin-NaOH, pH 8.5. Fluorescence was monitored immediately ( $t = 0$  h) and after 24 h with a microplate reader at excitation and emission wavelengths of 440 and 490 nm, respectively (Polarstar Optima, BMG). Fluorescence of the ThT solution without addition of A $\beta$  was subtracted from each value to correct for the fluorescence background.

### Electron microscopy

Aliquots (10  $\mu$ l) of the respective L-A $\beta$ -Dpep mixtures as well as control samples of the Dpep and PBS-buffer (data not shown) were placed on 200 mesh carbon-coated formvar copper grids. After 5–10 min, excess fluid was discarded and the samples and simple PBS-buffer, as negative controls, were negatively stained with 2% (w/v) ammonium molybdate for several seconds. Finally, the specimens were viewed in a Zeiss EM-910 transmission electron microscope (TEM).

### FCS measurements

Fluorescence correlation spectroscopy (FCS) measurements were performed with a Confocor I instrument (Zeiss-Evotec), equipped with an argon ion laser and filter systems for  $\lambda_{\text{EX}} = 488$  nm. For fluorescence detection, A $\beta$ (1–42)-peptide (P. Henklein, Charité Berlin) was labeled at the N-terminus with the dye OregonGreen (Molecular Probes). A 24 well micro carrier with 20  $\mu$ l sample volume (MC 384/15, Evotec Technologies) was used. Adjustment of the instrument was performed before each measurement with rhodamine 6G. Pinhole was 45  $\mu$ m in diameter. Focus was set 200  $\mu$ m above bottom of the well. The reaction mixture contained 22  $\mu$ M A $\beta$ (1–40) unlabeled (prepared according to Fezoui *et al.*, 2000), 10 nM A $\beta$ (1–42), labeled with OregonGreen in 10 mM sodiumphosphate-buffer, pH 7.2, and different concentrations of the D-peptide. A $\beta$  (1–42)-OG was prepared as 500 nM stock solution in 100% DMSO, stored in aliquots at –20°C and filtered through 0.45  $\mu$ m nylon filters directly prior to use. Reaction mixtures contained only one OregonGreen labeled peptide per 2200 unlabeled molecules. Fluorescence fluctuations in each well were recorded forty times for 30 s per sample with 500 datapoint resolution. Corresponding autocorrelation functions were calculated by a hardware correlator card. Data evaluation was carried out with the Evotec software Multi-FCSaccess.

## Results

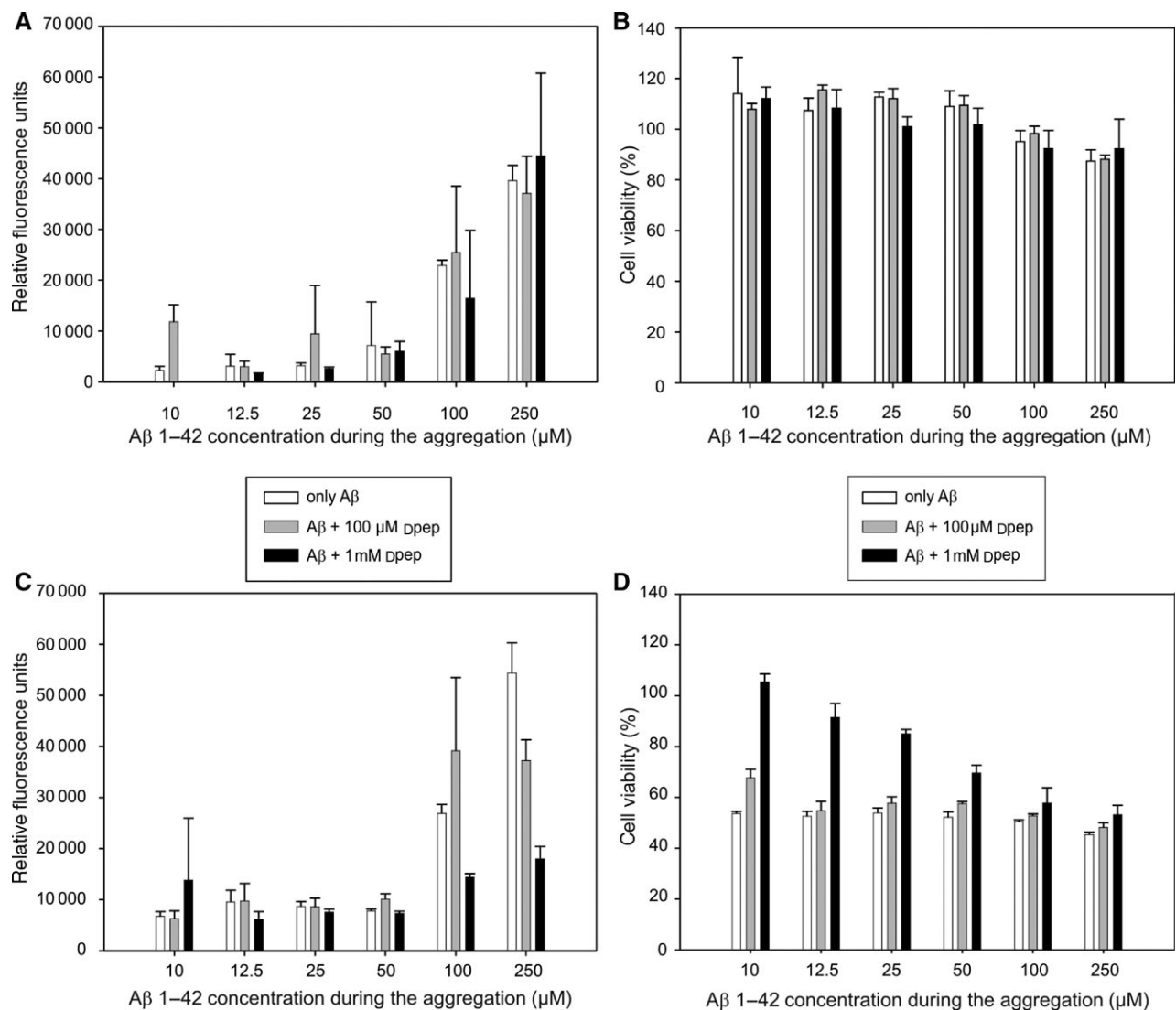
### Influence of dpep on A $\beta$ (1–42) toxicity

The influence of dpep on the viability of PC12 cells was investigated by MTT reduction (Shearman *et al.*, 1994) in the presence of A $\beta$  concentrations between 10 and 250  $\mu$ M. To achieve these A $\beta$  concentrations in the cell assay, A $\beta$  was pre-dissolved at a concentration of 10  $\mu$ M in H<sub>2</sub>O, lyophilized and re-dissolved in an adequate volume of PBS, with or without dpep to obtain 5-fold concentrations of A $\beta$  (10, 12.5, 25, 50, 100 and 250  $\mu$ M) when compared with its end concentrations in the cell assays (2, 2.5, 5, 10, 20 and 50  $\mu$ M). The toxicity of the A $\beta$  solutions, in the presence of three different dpep concentrations (0, 100 and 1000  $\mu$ M), was determined immediately and 24 h after preparation of the A $\beta$  solutions with or without dpep (Fig. 1B and D).

Cell viability measured immediately after preparation of the A $\beta$ –dpep mixtures yielded only a small dependence on the A $\beta$  concentrations applied in the assays (Fig. 1B). Only high A $\beta$  concentrations (100 and 250  $\mu$ M) lead to about 10% reduction of cell viability. At this time point, cell viability was not dependent on dpep either.

### dpep is able to inhibit cell toxicity of A $\beta$

When the A $\beta$ –dpep mixtures were applied to the cell cultures for MTT reduction assay after a 24 h incubation period at 37°C, the situation had completely changed (Fig. 1D). In the absence of dpep, a clear reduction of cell viability by about 50% was observed for all A $\beta$  concentrations under investigation (10–250  $\mu$ M). Presence of 100  $\mu$ M dpep yielded an increase of viability to almost 70% at low A $\beta$



**Fig. 1.** Influence of dpep on cytotoxicity and aggregate formation of A $\beta$ . (A and C) Binding of ThT to amyloid A $\beta$ -fibrils was determined by ThT fluorescence at Ex 440/Em 490 and shown as relative fluorescence units. All values are given as means from three measurements with the respective standard deviation. One single measurement (10  $\mu$ M A $\beta$  and 100  $\mu$ M dpep) was completely out of range for unknown reasons and was not used for further analysis. All measurements were carried out on freshly prepared A $\beta$ (1–42) (A) and A $\beta$ (1–42) incubated for 24 h at 37°C (C). (B and D) Cell viability for A $\beta$ (1–42)-treated cells was measured using the MTT assay with PC12-cells. Percentages of cell viability were derived as follows: the 100% value was obtained from cells treated neither with dpep nor with A $\beta$ (1–42); a value of 0% was obtained by treatment of the PC12 cells with 0.2% Triton-X. All values are given as means from three measurements with the respective standard deviation. All measurements were carried out on freshly prepared A $\beta$ (1–42) (B) and L–A $\beta$ (1–42) incubated for 24 h at 37°C (D).

concentration (10  $\mu\text{M}$ ). Presence of 1 mM dpep yielded an increase of viability up to 100% depending on the A $\beta$  concentration. dpep itself did not show any toxic effects, as demonstrated with cell viability values between 98 and 111% for dpep concentrations of up to 1 mM dpep in the absence of A $\beta$  (data not shown).

Taken together, the data clearly demonstrate inhibitory effects of dpep for A $\beta$  toxicity; although, concentrations of dpep necessary to obtain this effect are rather high and may withstand its therapeutical application.

#### *A $\beta$ amyloid fibril formation in the presence of dpep as measured by ThT assay*

Simultaneous to each cell viability assay, the effect of dpep on amyloid fibril formation was determined by thioflavinT (ThT) assays (Levine *et al.*, 1993). All A $\beta$ -dpep mixtures prepared for the cell toxicity assays were diluted 1:40 with ThT solution for determination of amyloid fibril formation (Fig. 1A and C). dpep solutions in the absence of A $\beta$  did not yield any detectable fluorescence signals for dpep concentrations of up to 1 mM dpep (data not shown).

ThT assays carried out with A $\beta$  solutions immediately after they were prepared yielded values that roughly correlate with the concentrations of A $\beta$  applied to the assay. ThT assay results of A $\beta$  solutions with and without dpep did not show significant differences from each other (Fig. 1A).

#### *dpep is able to reduce A $\beta$ $\beta$ -sheet formation*

When the ThT assays were carried out after a 24 h incubation period of the A $\beta$ -dpep mixtures, a remarkably different result was obtained (Fig. 1C). Mixtures with A $\beta$  concentrations up to 50  $\mu\text{M}$ , with or without dpep, yielded very similar fluorescence values among each other. The mixtures without dpep containing 100 and 250  $\mu\text{M}$  A $\beta$  show a small but significant increase of  $\beta$ -sheet formation after 24 h. This increase is dramatically inverted for the 250  $\mu\text{M}$  A $\beta$  mixture containing 1000  $\mu\text{M}$  dpep. Thus, high concentrations of dpep are able to reduce the amyloid fibril content of mixtures with elevated A $\beta$  concentrations.

#### *Cell toxicity and $\beta$ -sheet formation do not correlate*

Cell viability assays of A $\beta$  samples of various concentrations with or without dpep yielded values between 90 and 110%, regardless of the  $\beta$ -sheet contents as obtained from the ThT assays. After 24 h incubation, the dpep containing A $\beta$  samples exert highly diverse values in the cell viability assays. In contrast, the ThT assays of the same samples yielded values rather similar to each other. The most intriguing example is the 250  $\mu\text{M}$  A $\beta$  sample with three different dpep concentrations (0, 100 and 1000  $\mu\text{M}$ ) after 24 h incubation. Although the total amount of A $\beta$  in amyloid fibrils, as measured by the ThT assay, is highly dependent on the dpep concentration, the presence of dpep obviously did not have a significant effect on A $\beta$  toxicity. This indicates that the ThT signal does not correlate with cytotoxicity in general. This is a very clear and interesting conclusion from the data.

Consequently, dpep's effect on the amyloid fibril content of A $\beta$  samples is not mirrored by a similar effect on cell viability values of the respective samples. Vice versa, at A $\beta$  concentrations lower than 100  $\mu\text{M}$ , dpep did not have a

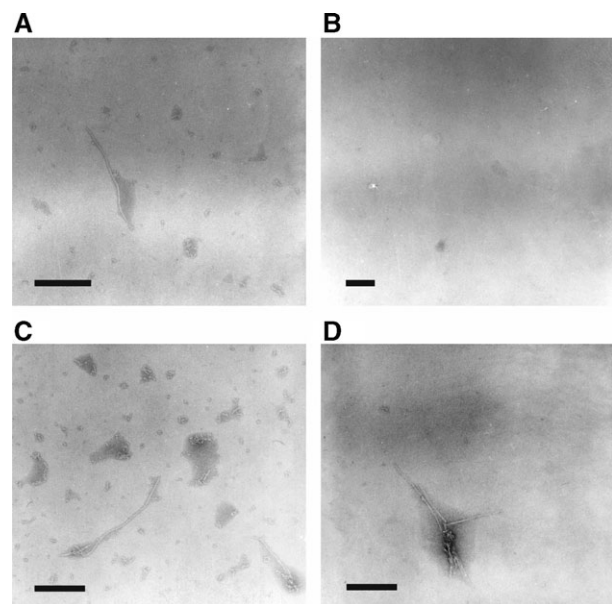
significant effect on amyloid fibril content, but increased cell viability values dramatically.

That strongly suggests that dpep is able to modify the toxicity of A $\beta$  amyloid fibrils either by simply covering them or by influencing their composition without changing the ThT signal.

#### *Aggregate formation of A $\beta$ in the presence of dpep as detected by electron microscopy*

To examine the amyloid fibril formation in the A $\beta$  dpep mixtures, we prepared electron micrographs (EM) from A $\beta$  dpep mixtures with A $\beta$  concentrations of 10 and 250  $\mu\text{M}$  A $\beta$  in absence or presence of 1 mM dpep with an incubation time of 24 h (Fig. 2).

The micrographs of 10  $\mu\text{M}$  A $\beta$  without dpep yielded mainly short amyloid fibrils with small amounts of amorphous material and a small amount of longer amyloid fibrils (Fig. 2A). Ten micromolar samples in the presence of dpep showed after 24 h of co-incubation almost no EM-detectable amyloid fibrils (Fig. 2B). This is not in perfect accordance with the respective ThT assays (Fig. 1C). Possibly, the ThT assay is not sensitive enough to discriminate between different samples with A $\beta$  aggregates below a certain threshold. Another explanation could be that ThT values do not exactly correlate with amyloid fibril content, but rather with  $\beta$ -sheet content of amyloid fibrils and amorphous material in sum. The EM pictures of 250  $\mu\text{M}$  A $\beta$  in absence of dpep (Fig. 2C) show more amyloid fibrils and aggregates than those of 250  $\mu\text{M}$  A $\beta$  in presence (Fig. 2D) of 1 mM dpep. This is much more in accordance with the respective ThT values (Fig. 1C).



**Fig. 2.** Electron microscopic analysis of negatively stained samples showing the effect of dpep on A $\beta$  amyloid fibril formation. (A and B) Ten micromolar A $\beta$ (1-42) with (B) and without (A) 1 mM dpep were incubated for 24 h. (C and D) Two hundred and fifty micromolar A $\beta$ (1-42) with (D) and without (C) 1 mM dpep were incubated for 24 h. The samples were subsequently placed on 200 mesh carbon-coated formvar copper grids and negatively stained with 2% (w/v) ammonium molybdate. The black bars indicate 200 nm.

Control samples with PBS and Dpep solved in PBS did not yield any detectable structures. The EM results suggest that Dpep strongly influences fibrillogenesis of A $\beta$ .

#### Aggregate formation of A $\beta$ in the presence of Dpep as measured by FCS

Fluorescence correlation spectroscopy (FCS) allows the observation of single fluorescently labeled molecules diffusing free in solution. By measuring the fluorescence fluctuations caused by single molecules excited within the confocal volume element, the diffusion coefficient can be determined, which together with an assumption regarding the shape of the molecule allows for an estimation of the corresponding molecular weight of the diffusing species. In the case of aggregating A $\beta$ , the system is expected to be highly polydisperse. Aggregates of many different sizes and different shapes will be present. In addition, some of the material might already be insoluble and therefore inaccessible to the method. The obtained autocorrelation curves were fitted with a two component (fluorescent probe free and bound to aggregated species) system. The diffusion time of the first component was determined separately by measuring the fluorescent probe alone. This was used to calculate the diffusion time of the second component as well as the relative amounts of both from the measurements of the fluorescent probe in A $\beta$  Dpep mixtures. The diffusion time of the second component represents a weight averaged diffusion time for all aggregated species in solution.

Presence of Dpep reduces the diffusion time of the A $\beta$  aggregates (Fig. 3A) indicating that on average, the size of A $\beta$  aggregates is significantly smaller than in absence of Dpep. Interestingly, the number of A $\beta$  aggregates is increased in the presence of Dpep when compared with samples without Dpep (Fig. 3B).

The mean diffusion time of 1 ms matches to a mean diffusion coefficient of  $1.8 \times 10^{-7} \text{ cm}^2/\text{s}$  and corresponds to aggregates of five million Da assuming a spherical shape (Equation:  $D = \omega_1^2/4\pi\text{diff}$ ;  $\omega_1$  is the radius of the confocal volume element). In the presence of Dpep, the mean diffusion coefficient of measured aggregates is  $5.5 \times 10^{-7} \text{ cm}^2/\text{s}$  and corresponds to a molecular weight of only 200 000 Da. Molecular weights will appear smaller the more the shape deviates from a sphere. The reduction in aggregate size caused by Dpep seems to be at least in parts be balanced by an increase of the relative amount of aggregates as shown in Fig. 3B.

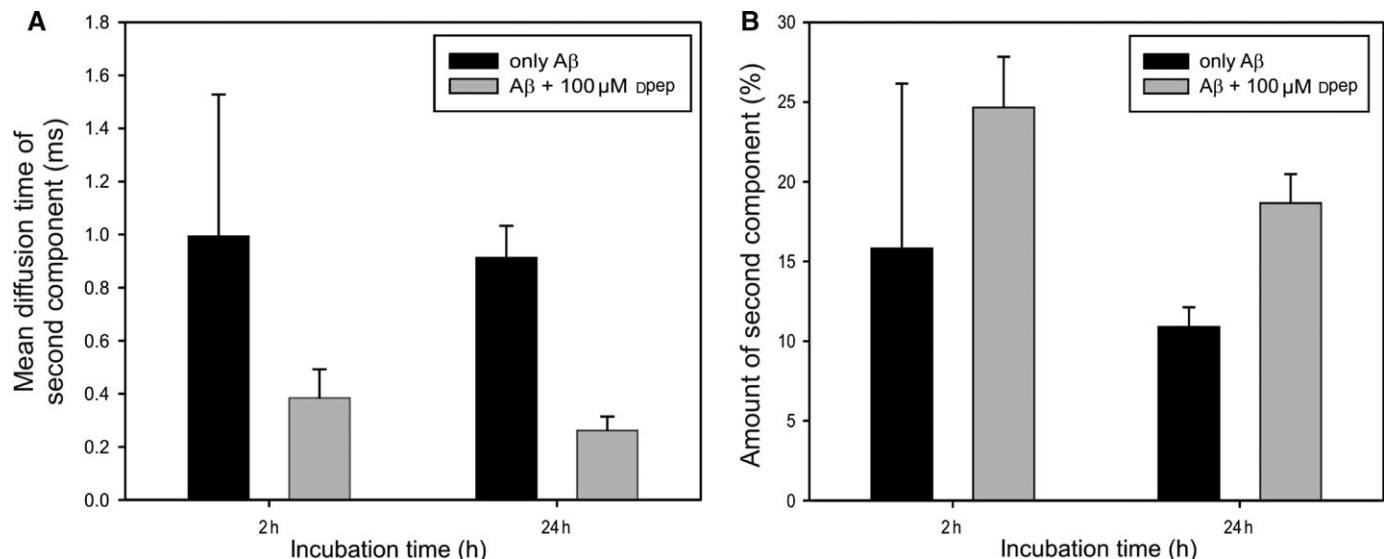
Thus, Dpep leads to a decrease of the average A $\beta$  particle size, but increases the number of A $\beta$  particles.

Therefore, the results from the FCS measurements are well suited to bring the ThT assay results (Fig. 1A and C) in accordance with the EM pictures (Fig. 2). In the presence of Dpep, the average particle size of A $\beta$  aggregates drops to only 200 000 Da. This suggests that most of the aggregates were not EM-visible but contributed to the ThT signal.

#### Discussion

Substances that preferentially bind A $\beta$  oligomers or amyloid fibrils can be expected to promote their formation, as was indeed observed (Kuner *et al.*, 2000; Lowe *et al.*, 2001). Other substances act like beta-sheet breakers and affect the amyloid fibril formation and cytotoxicity of A $\beta$  (Soto, 1999). Furthermore, an increasing number of investigations report that besides amyloid fibrils, protofilaments (Walsh *et al.*, 1999), oligomers or even smaller A $\beta$  units may be toxic to cells (Lambert *et al.*, 1998).

Thus, it was of great interest and of importance for potential diagnostic applications of Dpep, to investigate any effect of Dpep on the cytotoxicity of A $\beta$ . Although extrapolation of the results obtained from *in vitro* systems to *in vivo*



**Fig. 3.** FCS measurement of A $\beta$ -aggregation. Reaction mixtures containing 22  $\mu$ M A $\beta$ (1–40), 10 nM A $\beta$ (1–42)-OG in  $1 \times$  PBS were incubated with and without 100  $\mu$ M Dpep for 2 or 24 h at room temperature. From a two-component fit of the autocorrelation functions, a weight-averaged diffusion time (A) and the relative amount of aggregated species (B) were determined. Standard deviations result from averaging 40 measurements of 30 s each and from averaging two independent experiments. The obtained fluorescence correlation data were analyzed applying a model of two independent components as implemented in the Evotec Access 2.0 software. The diffusion time of the first component comprising the monomeric A $\beta$  was fixed to the values determined for a solution containing fluorescently labeled A $\beta$  alone measured within the same experiment. The diffusion time of the second component comprises a weight average for all associates of A $\beta$  present in the solution and is used as a measure of weight averaged size of aggregates.

mechanisms of the disease requires all necessary caution, we want to discuss potential mechanisms of dpep for either inhibition of toxicity or amyloid fibril formation.

Taken together all FCS, ThT and EM data, dpep dramatically reduces A $\beta$  particle size, but increases the overall number of aggregates. Further, the higher the dpep–A $\beta$  ratio is, the more significant is the positive effect of dpep on cell viability.

If we assume that dpep binds to A $\beta$  amyloid fibrils, protofilaments and other oligomers, it can be expected to reduce their toxic effects by simply covering them. Such an effect should depend roughly on the ratio of dpep and A $\beta$ . That is exactly what could be observed in the experiments. The highest cell viability was observed for the highest dpep–A $\beta$  ratio (Fig. 1C).

Formation and growth of amyloid fibrils requires existence of free binding sites on aggregates for further adsorption of A $\beta$  molecules. If dpep binds to all kinds of A $\beta$  aggregates, it should hinder formation of amyloid fibrils from smaller intermediates as well as growth of existing amyloid fibrils or protofibrils. Thus, in a simple competition reaction, dpep can be expected to suppress ongoing attachment of A $\beta$  molecules during amyloid fibril growth. The same competition hinders fusion of A $\beta$  aggregates to larger structures. The FCS data reported here show an increase in the number of particles and a decrease in average size (Fig. 2). This is in accordance with this model.

Most interestingly, after a 24 h incubation period, a lower amyloid fibril content is observed than before, indicating that dpep in fact can actively reduce  $\beta$ -sheet content or destroy amyloid fibrils. The concentrations of dpep necessary to observe these effects may be too high to be suited for preventive or therapeutic use in its present form. It should, however, be considered that A $\beta$  concentrations within the brain are by far lower than those used in the described *in vitro* cytotoxicity assays, and therefore, treatment of AD patients with dpep could possibly lead to a breakdown and clearance of existing amyloid deposits.

Taken all data together, dpep may not only serve as a molecular marker for A $\beta$  aggregates as shown previously (Wiesehan et al., 2003), but also is able to inhibit toxic effects of A $\beta$ . Although both effects of dpep on A $\beta$ , reduction of amyloid fibril formation and reduction of toxicity, do not perfectly correlate to each other, they may have a common basis, which could simply be binding to and covering A $\beta$  aggregates.

## Funding

This work has been supported by a grant of the Volkswagen-Stiftung to DW (I/82 649).

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Received April 22, 2007; revised September 14, 2007;  
 accepted September 14, 2007

Edited by Leo James