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Testing synthetic amyloid-β aggregation inhibitor using single molecule atomic force spectroscopy

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ABSTRACT

Alzheimer's disease is a neurodegenerative disease with no known cure and few effective treatment options. The principal neurotoxic agent is an oligomeric form of the amyloid-β peptide and one of the treatment options currently being studied is the inhibition of amyloid aggregation. In this work, we test a novel pseudopeptidic aggregation inhibitor designated as SG1. SG1 has been designed to bind at the amyloid-β self-recognition site and prevent amyloid-β from misfolding into β sheet. We used atomic force spectroscopy, a nanoscale measurement technique, to quantify the binding forces between two single amyloid peptide molecules. For the first time, we demonstrate that single molecule atomic force spectroscopy can be used to assess the effectiveness of amyloid aggregation inhibitors by measuring the experimental yield of binding and can potentially be used as a screening technique for quick testing of efficacy of inhibitor drugs for amyloid aggregation.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease associated with progressive memory loss and dementia. Current research supports the hypothesis that Alzheimer's is caused by the misfolding of the amyloid-β (Aβ) peptide into β sheets which then aggregate into toxic oligomers (Chiti and Dobson, 2009) and fibrils. AD is a complex disease associated with a number of potential factors including aging (Bertram and Tanzi, 2005), genetics (Bertram and Tanzi, 2005), metals dyshomeostasis (Bush and Tanzi, 2008), protein–lipid interactions (DiPaolo and Kim, 2011), vascular disorders (Roy and Rauk, 2005), and loss of acetylcholine receptors (Whitehouse et al., 1986). Pharmaceutical therapies have focused on attacking underlying factors associated with Alzheimer's (Robertson and Mucke, 2006). For example, γ-secretase inhibitors have been developed in an attempt to stop the cleaving of amyloid-β from its precursor protein, the amyloid precursor protein (APP) (Citron, 2004). Despite extensive research and funding, no therapy has yet been shown to reverse the symptoms associated with Alzheimer's in human trials, and even the most efficacious therapies are not curative, but only delay the onset of symptoms (Neugroschl and Sano, 2010).

Aβ begins its pathological aggregation by misfolding into an internal β sheet between the amino acids 17–23 and 28–35. The amino acids (24–27) form a β-hairpin to allow the peptide to fold back on itself (Petkova et al., 2002; Sciarretta et al., 2005). After misfolding into a hairpin, the peptide may bind to another Aβ peptide, acting as a nucleus for further peptide aggregation. The self-recognition site (17–23) (Tjernberg et al., 1996, 1997) is responsible for the aggregation of Aβ into dimers and higher oligomers. Blocking this site effectively prevents the misfolding and subsequent aggregation. In the absence of sufficient peptide clearance, this aggregation leads to the formation of toxic amyloid aggregates (Harper and Lansbury, 1997).

The Aβ sequence (13–23), which incorporates the self-recognition site, has been a target for amyloid aggregation inhibitors (Mothana et al., 2009) which are designed to have a high affinity for the self-recognition site and "block" the peptide from self-dimerization. Rauk and co-workers developed a series of pseudopeptidic novel amyloid inhibitors that bind to and act on this self-recognition site (Mothana et al., 2009; Roy, 2010). These inhibitors consist of a series of modified amino acids including methylated amino acids. A structure of SG1 is show in Fig. 2b. These inhibitors were synthesized and tested using a combination of circular dichroism and thioflavin T fluorescence assay to look for evidence of peptide aggregation. These methods have a

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characteristically low throughput because a period of 24 h or more is needed for the peptide to aggregate into a recognizable form to determine the effect of the inhibitor on the aggregation properties. Atomic force spectroscopy (AFS) is a nanoscale technique based on atomic force microscopy (AFM) and is often used to probe interactions between two single molecules. We used this technique to test the efficacy synthetic amyloid inhibitor SG1 and demonstrated that SG1 reduces the number of binding events between two Aβ peptides by as much as 81%. In addition, we used atomic force microscopy and molecular dynamics simulations for comparison with AFS data and demonstrated with all these methods that the aggregation of Aβ is greatly reduced in the presence of SG1.

2. Material and methods

To follow the effect of SG1 on Aβ aggregation and fibril formation, AFM was used in both imaging mode and force spectroscopy mode. In imaging mode a sharp scanning probe (AFM tip) scans the sample surface and the forces of interaction at each point are measured to produce an image of surface morphology (Binnig et al., 1986), which allows for high resolution imaging and visualization of the formation of Aβ oligomers and fibrils. To measure single molecule binding forces between peptides, AFM was used in force spectroscopy mode, in which the AFM probe, with an Aβ peptide attached to it was moved repeatedly towards and away from the mica surface, also bound with peptides, and the interaction force was measured as a function of probe-sample separation at pN resolution. For these experiments attachment of the Aβ peptide to both AFM probe and the surface is required. Except as noted, we used chemical modification protocols as previously described (Hane et al., 2013). The details of experimental procedures are outlined below.

2.1. Tip and mica modification for force spectroscopy.

A Bruker MSNL Silicon Nitride AFM cantilever was cleaned in ethanol, washed with MilliQ water and dried in a gentle stream of nitrogen. The cantilever was then placed under UV light for 30 min. Freshly cleaved mica slides and cantilevers were modified with 1-(3-aminopropyl) silatrane (APS), incubated in a 167 μM solution of 35 nm *N*-hydroxysuccinimide-polyethylene glycol-maleimide (NHS-PEG-mal) (Laysan Bio (Arab, AL)) for 3 h, rinsed with DMSO, and then dried with a gentle stream of nitrogen.

2.2. Amyloid-β (1–42) preparation and surface binding force spectroscopy measurements

Cys-Amyloid-β (1–42) was purchased from Anaspec (Fremont, CA) and dissolved in DMSO at a concentration of 1 mg/mL. The Aβ stock solution was diluted in HEPES buffer (50 mM, pH 7.4, NaCl ionic strength 150 mM) to a final concentration of 20 nM. *Tris*(2-carboxyethyl)phosphine (TCEP, 200 nM) was added to the dilute Aβ solution in equal volume. The Aβ was stored for 15 min, and then centrifuged at 14,000 RPM for 15 min (Kim et al., 2011). Mica and cantilevers were immersed in the Aβ solution for 30 min, rinsed with HEPES buffer, treated for 10 min with β-mercapto-ethanol. Both cantilever and mica were rinsed with HEPES buffer, and stored in HEPES buffer until use.

2.3. Preparation of SG1 inhibitor solution

SG1 was synthesized using fmoc-chemistry method (Roy, 2010). SG1 was lyophilized and stored in powdered form in −20 °C. When ready for experimental use, SG1 was dissolved in HEPES buffer (50 mM, 150 mM NaCl, pH 7.4). A 2 μM stock solution was prepared using serial dilutions. From the stock solution, final aliquots of 20 nM, 40 nM and 200 nM were prepared for experimental force spectroscopy experiments.

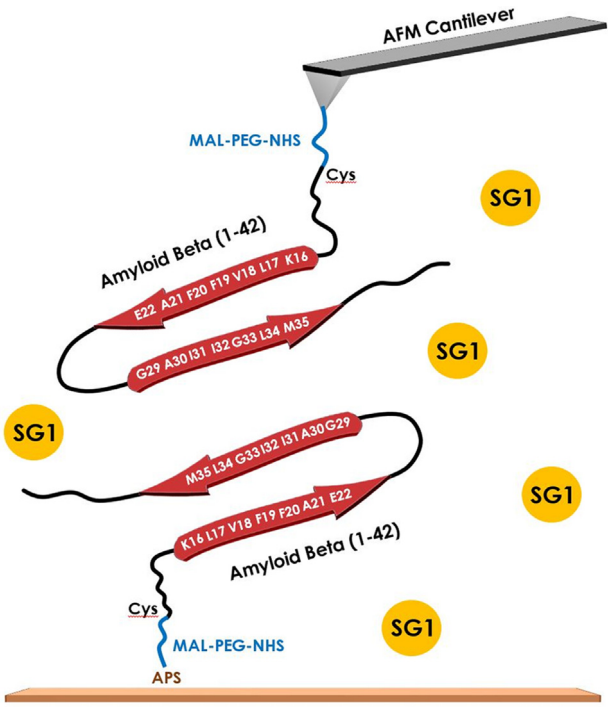

Fig. 1. Schematic of force spectroscopy experiment. A schematic of the experimental setup of force spectroscopy experiments, showing Aβ bound to the substrate and AFM tip via the PEG linker with aggregation inhibitor SG1 in the aqueous solution. A silanated surface is modified with NHS-PEG-maleimide. A cysteine residue is bound to the N-terminus of Aβ peptide to act as a binding site for the maleimide group of the linker molecule.

2.4. Atomic force spectroscopy

A JPK Nanowizard II atomic force microscope (AFM) was used for force measurements in contact mode, where deflection of cantilever versus distance was recorded. The mica substrate with attached Aβ peptide was placed on the stage and the liquid cell was filled with HEPES buffer. An AFM probe with Aβ attached was brought into contact with the substrate (approach) to allow the peptides attached to the probe to bind to peptides attached to substrate (Fig. 1) and then the probe was retracted from the substrate to force the unbinding of the two peptides. A series of force curves were taken with an approach and retract velocity of 400 nm/s. The ratio of observed events to the total number of approaches is referred to as the experimental yield. The yields from separate experiments varied from 3% to 30%. At least 900 force plots were captured in each experiment. More than 10,000 approaches were made and force plots collected to achieve a statistically valid number of rupture events. Nine hundred approaches were done for each experiment (i.e. 900 approaches for Aβ only, 900 approaches with 20 nM SG1, 900 approaches with 40 nM SG1, 900 approaches 200 nM SG1). Each experiment was repeated 3 times. We used Bruker MLCT cantilevers with spring constants between 100 and 700 mN/m, calibrated using JPK software via the thermal noise method (Hutter and Bechhoefer, 1993). In our experiments, we prepared a set of chemically modified cantilevers and substrates in advance, and once the inhibitor solution is ready, the cantilever can be loaded into the AFM and a control experiment can be completed consisting of ~1000 approaches. The inhibitor solution can then be added to the liquid