Appendix D - **UNIVERSITY HONORS PROGRAM**
SENIOR PROJECT - APPROVAL

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PROJECT TITLE: Studies on Protein Inhibitors of the Alzheimer's Fibrils (Aβ1-40) using a Sensitive Streptavidin-Europium Assay

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Ronald Wetzel, Faculty Mentor

Date: 5-12-2000

Comments (Optional):

Studies on Protein Inhibitors of the Extension of Alzhiemer's Fibrils ($A\beta_{1-40}$) using a Sensitive Streptavidin-Europium Assay

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Abstract:

Amyloid plaques, comprised mainly of a fibrillar form of amyloid β protein (Aβ), are a defining pathological feature of Alzheimer's disease (AD). Therefore, studies on Alzheimer's disease have concentrated on obtaining potent protein and peptide inhibitors of Aβ fibrillogeneses. We have explored the inhibitory activities of BSA, HSA, and ovarian ovalbumin on the rate of fibril extension *in-vitro* using a very sensitive microplate assay. In contrast to other extension assays that use high concentrations of Aβ (μM range) we can measure the extension of fibrils down to the sub-fmol range using physiological concentrations of Aβ (nM range). Sonicating Aβ fibrils greatly increases the fibril's ability to seed the extension reaction. This enhancement of seeding ability is most likely due to an increase in the number of fibril growing ends that are accessible to Aβ monomer binding.

All the proteins in our inhibition studies, even the ovarian ovalbumin, have similar inhibitory potencies. For example, similar concentrations of the inhibitors (2μM) inhibit the rate of fibril extension by 25-30%. This suggests that many polypeptides are be able to inhibit fibril extension. To test whether the proteins require a native folded structure to inhibit fibril extension, the inhibitors were denatured by breaking their disulfide bonds using reduction and alkylation. Surprisingly, we found that these modifications enhanced their inhibitory potencies by 2 fold in most cases. An IC_{50} value of 0.18μM was determined for modified HSA. These results suggest that the protein's native structure plays little to no role in the inhibition of fibril extension.

Introduction:

Alzheimer's disease (AD) is a progressive senile dementia that effects a significant amount of the elderly [*1*]. Amyloid plaques, hard waxy deposits consisting of proteins and polysaccharide, are the defining pathological feature of the AD brain [*2*]. The major constituent of amyloid is a hydrophobic 30-43 amino acid peptide, β-amyloid (Aβ) [*3*]. Aβ is a cleavage product of a large trans-membrane protein, amyloid precursor protein, that is encoded by the APP gene [*4*].

Similar concentrations of Aβ (nM) have been observed in the plasma and cerebral spinal fluid (CSF) of both normal and AD patients [*5*]. Also, Aβ is constitutively produced by cells in culture [*6*]. However, several studies show that the accumulation of insoluble rather than soluble Aβ is linked to AD. For example, the transition of Aβ from soluble to insoluble peptide is associated with the onset of cellular toxicity [*4*]. In addition, overproduction of the peptide in transgenic mice leads to both significant Aβ deposition and neuronal toxicity [*7*]. Therefore, the suppression or prevention of the transition of Aβ from monomeric to a toxic insoluble aggregate has emerged as a goal in the development of therapy for AD [*3*].

The majority of assays that are currently used to measure the inhibition of fibril growth by inhibitors suffers a major drawback. These assays require high concentrations of Aβ (in the μM range) that are significantly greater than the physiological concentration of Aβ (in the nM range) [*5*]. One assay uses physiological concentrations of Aβ, but it also uses radio-labeled Aβ [*8*]. In

contrast, we have studied the inhibitory potencies of native and denatured BSA, HSA, and ovalbumin using a sensitive microplate assay that uses physiological concentrations of biotinylated Aβ. The rate of fibril extension is monitored by measuring time-resolved fluorescence of europium. The europium probe is attached to biotinylated Aβ using streptavidin. This method eliminates the damaging ionizing radiation associated with radio-labeled Aβ.

Using the microplate assay we screened several proteins for any inhibitory activity on Aβ fibril extension. All of the proteins tested have similar inhibitory potencies. To determine if the proteins require a native folded structure to inhibit fibril extension the inhibitory proteins were modified by reduction and alkylation. We found that these modifications enhances the inhibitory potency of the proteins. The results suggest that the native structure of the protein plays little to no role in the protein's inhibitory activity of fibril extension.

Methods and Materials:

Materials: Aβ fibrils and biotinylated Aβ were prepared by others using unconjuqated and cysteine (N-terminal) conjugated Aβ peptides obtained from Quality Controlled Biochemicals (Hopkinton, MA). Essentially fat free bovine serum albumin (BSA), human serum albumin (HSA), and ovarian ovalbumin were obtained from sigma. All chemicals were of analytical grade.

Preparation of sonicated fibrils and coating of the microtiter plate: To Aβ fibrils (a gift from B. O'Nuallian; UT Medical Center, Knoxville TN) in phosphate buffered saline (PBS) and 0.05% sodium azide, at pH 7.4 (standard buffer), a solution of 1mM dithiothreirol and 0.5mM EDTA was added. The fibril solution was sonicated on ice for 0-250 seconds (30s burst) using a Tekmar sonicator. Aliquots (100μL) of 0.25μg/mL Aβ fibrils in standard buffer were pipetted into the wells of a high binding microtiter plate (Corning EIA/RIA plate). Control wells were not coated with fibrils. The plate was then sealed, and allowed to incubate at room temperature for 2.5 days. After incubation the plate was washed twice with a wash buffer (PBS and 0.01% tween). Wells of the microtiter plate were blocked with 200μL of PBS containing 0.3% gelatin, sealed and incubated for 2 hours at 37^0C. The plate was then washed with washing buffer, and 100μL of standard solution was placed in each well. The plate was sealed, and stored for up to two weeks at 4^0C (Figure 1).

Extension Assay: Aliquots (100μL) of 5nM biotinylated Aβ monomer (a gift from R. Wetzel UT Medical Center, Knoxville, TN) in standard buffer with or without inhibitor present (0.2-30μM) was added to specific control and sample

wells of the microplate over a period of 6h at 37^{0}C (Figure 1). Experimental data was measured in triplicate. After the final time point, the plate was washed twice and 100μL of 0.1μg/ml strepavidin-europium complex (Wallac corp.) was added to each well. The plate was sealed and incubated for 1h at room temperature. After 1 hour the plate was washed three times and 100μL of enhancement solution (Wallac corp.) was added to each well for 5-7min, at room temperature (Figure 1). Time resolved Europium fluorescence was measured using a Wallac 1420 Victor^2 Multilabel Counter. The rates of extension were then calculated by plotting the amount of Aβ that deposited onto the fibril seed (corrected using control readings) and obtaining the initial rate of the extension reaction directly from the slope of the straight line that best fits the data.

Fluorimetry: Protein and ANS fluorescence were monitored at room temperature using a Perkins Elmer Luminescence Spectrometer LS50B. Readings were carried out using 1μM protein in PBS, with or without 2μM ANS, pH 7.4. The protein and ANS fluorescence were measured with excitation at 280nm and 350nm respectively. The emission and excitation slits were both set at 5nm for protein fluorescence measurements. Slits of 2.5nm and 5nm were used when measuring ANS fluorescence.

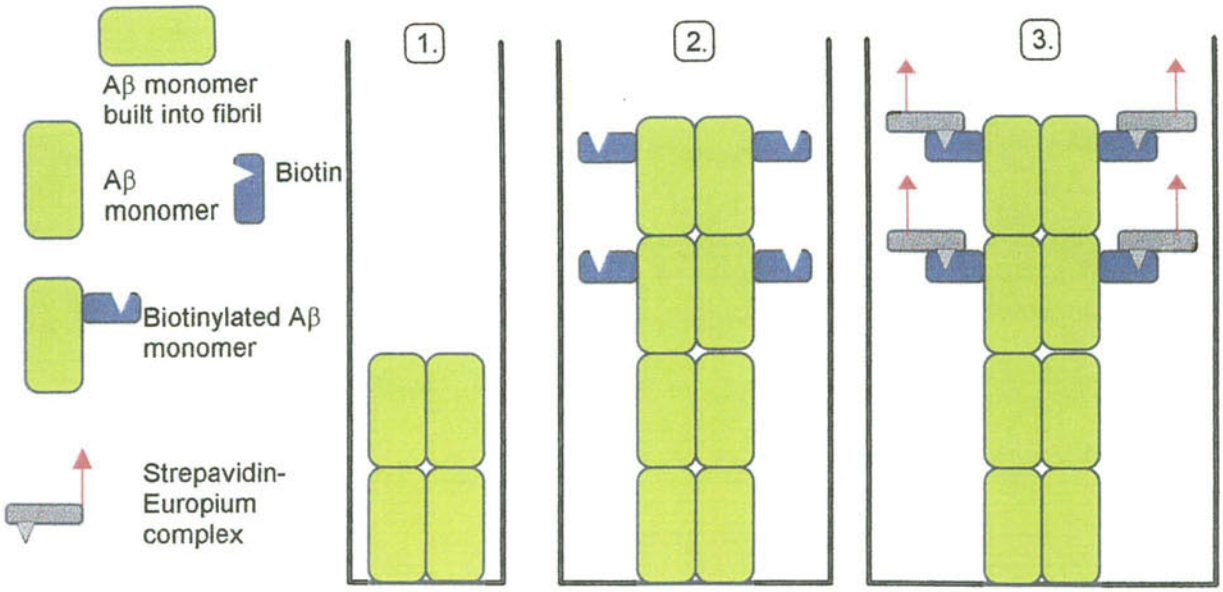

Figure 1. Schematic Representation of the Aβ Fibril Extension Microplate Assay. Step 1: The microplate is coated with sonicated synthetic fibrils then blocked with gelatin. Step 2: A biotinylated Aβ monome-r solution is added and allowed in incubate with or without inhibitor at intervals for up to 6 h. Step 3: The Strepavidin-Europium complex is added to the plate and allowed to incubate at room temperature for 1 hour. Between each step the wells were washed. For a more detailed explanation of the experiment see Methods and Materials.

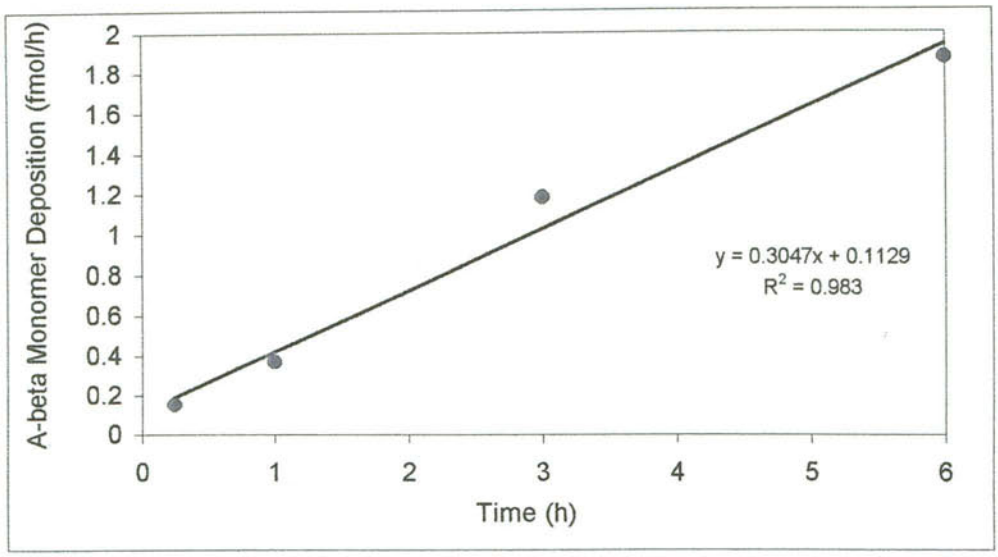

Figure 2. Determination of the Initial Rate of Aβ Fibril Extension. The reaction was monitored using the microplate assay with each well containing 25ng/100μL of sonicated fibrils and 5nM biotinylated Aβ monomer with no inhibitor present at 37°C for 0-6 hours as is described in the Methods and Materials.

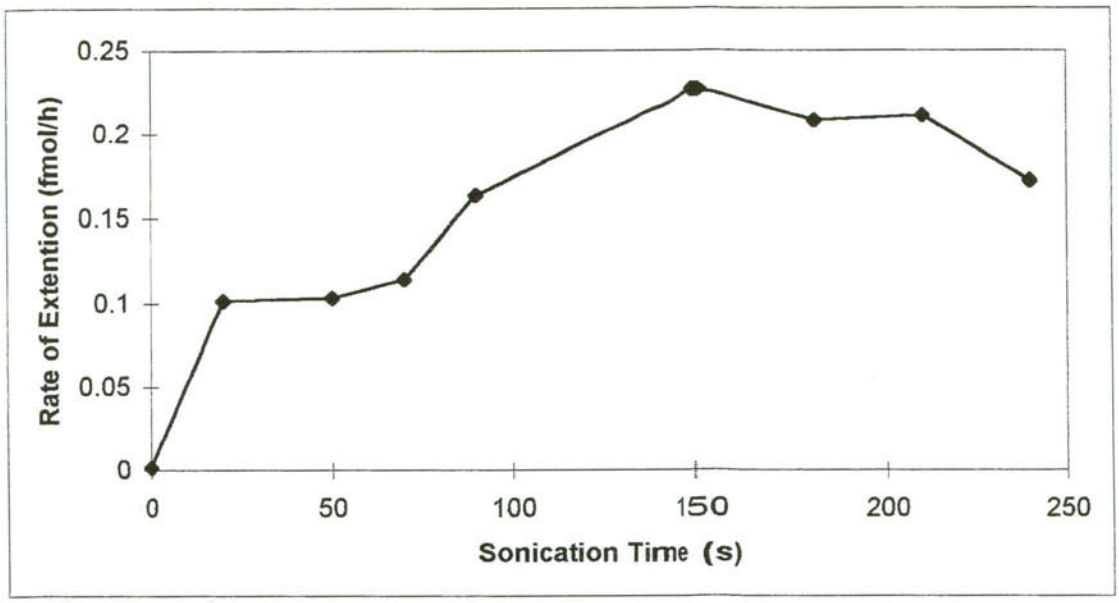

Figure 3. Effect of Sonication on Initial Fibril Extension. The reaction was monitored using the microplate assay with each well 25ng/100μL of fibrils and 5nM biotinylated Aβ monomer at 37C^0 for 0-6 hours.

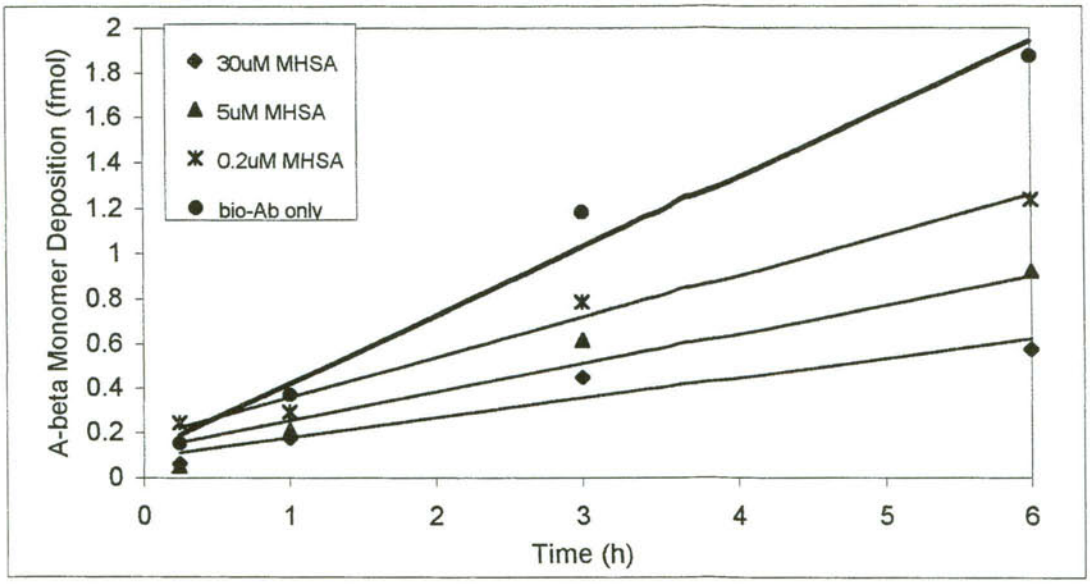

Figure 4. The Effects of Modified HSA on the Rate of Aβ Fibril Extension. The reaction was monitored at 37C^0 as is described in Figure 3 and as described in the Methods and Materials.

Table I Comparison of the Intrinsic and Extrinsic Fluorescence Properties of Native and Modified Protein Inhibitors of Aβ Fibril Extension. Protein and ANS fluorescence wavelength scans were carried out with excitation at 280nm and 396nm respectively. The excitation and emission slits used were 5nm and 10nm respectively. Each fluorescence reading was carried out with 1μM of protein at room temperature as is described in Methods and Materials.

Protein	Protein Fluorescence		ANS Fluorescence	
	λ max (nm)	Yield (arbitrary units)	λ max (nm)	Yield (arbitrary units)
BSA	347	715	475	475
Modified BSA	334	704	475	280
Ovalbumin	337	377	482	74
Modified Ovalbumin	337	628	474	278
HSA	341	153	476	270
Modified HSA	324	155	476	193

Table II Comparison of the Abilities of Native and Modified Proteins to Inhibit the Rate of Aβ Fibril Extension. The percentages of inhibition were determined by comparing the initial rate of fibril extension with and without an inhibitor present for example Figures 1 and 4. The initial rate of fibril extension was monitored using the microplate assay as is described in Methods and Materials.

Inhibitor Protein	Inhibition (%)
BSA	26.8
Modified BSA	51.3
Ovalbumin	30.1
Modified Ovalbumin	38.8
HSA	25.9
Modified HSA	40.7

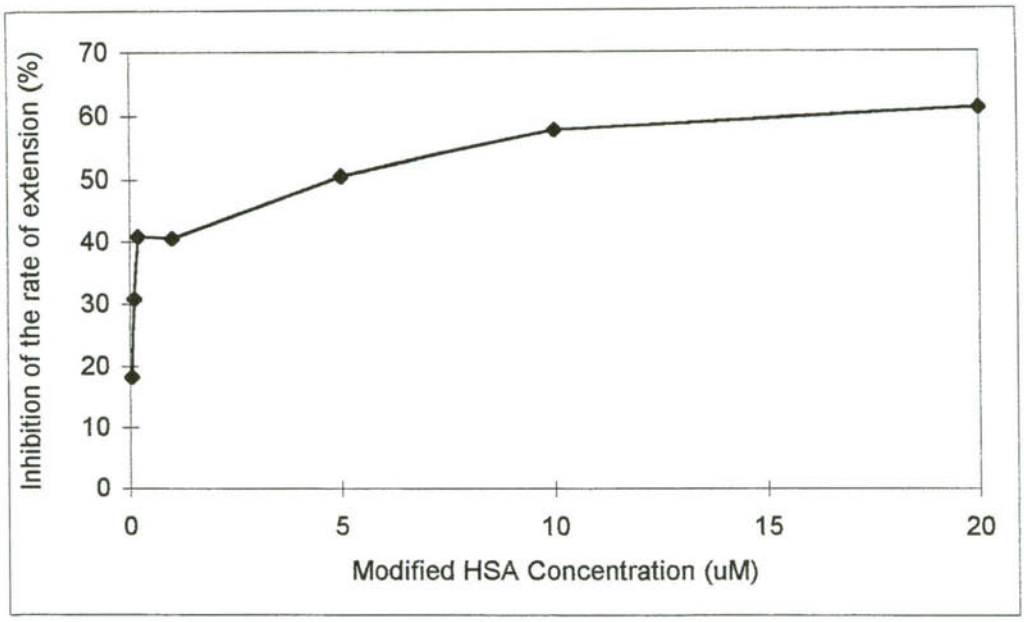

Figure 5. Determination of the IC_{50} Value for the Inhibition of the Rate of Aβ Fibril Extension by Modified HSA. The percentage inhibition of the reaction by modified HSA was calculated using values for the initial rate of the reaction as is described in Figures 1,4 and Methods and Materials. An IC_{50} of 0.18μM was determined for Modified HSA.

Results:

The time resolved fluorescence microplate assay that was used to monitor the extension of Aβ fibrils is very sensitive. This sensitivity is because of the high fluorescence of the probe (Europium) and because the fluorescence of the probe is measured after a brief waiting period that allows the background fluorescence to decay and therefore be reduced. In contrast, the use of real time fluorescence to monitor the reaction can often be limited by background noise. Using the microplate assay, we can measure the deposition of Aβ onto Aβ fibrils down to the sub fmol range (Figure 2). The initial rate of fibril extension (0.3047 fmol h^{-1}) with physiolgical concentrations (nM) of Aβ [5] is reproducibly linear up to six hours (Figure 2). The signal for the deposition of Aβ monomer at onto fibrils at a sub fmol range is consistently at least 3 fold greater than the signal obtained for control wells that contain no fibril seed (data not shown).

The sonication of Aβ fibrils is known to increase the potency as a seed in fibril extension reactions [9]. Therefore, as expected sonication of the Aβ fibrils up to the sonication time (150 s) that we use as our standard condition in our assay increased the rate of the extension reaction (Figure 3). In contrast, sonication for greater than 150 seconds causes the rate of the reaction to decrease by about 20%. Interestingly, no rate of fibril extension was monitored when the fibrils were unsonicated (Figure 3).

The extension assay is a potentially powerful tool to screen the abilities of protein to inhibit fibril extension at physiological concentrations of Aβ.

Therefore, we have screened the abilities of proteins that are known to inhibit fibril formation (BSA,HSA) and a protein, ovarian ovalbumin, that is not known to inhibit the reaction. All of the proteins even the ovarian ovalbumin similarly inhibited the rate of fibril extension (Figures 4&5; Table I). The effects of denaturing (modifying) the inhibitors ,by breaking their disulfide bridges, on their activities was tested using their reduced and alkylated forms (A gift from B. O'Nuallain; UT Medical Center, Knoxville TN). Surprisingly, modification of the proteins improves their inhibitory abilities (Figure 4; Table I). For example there are 2 fold and 3.5 fold decrease in the rate of fibril extension in the prescence of 0.2μM and 30μM modified HSA (Figure 4).

To confirm the modification of the protein inhibitors their intrinsic and extrinsic fluorescence properties were measured. Modification of the proteins significantly affects intrinsic and extrinsic fluorescent properties (Table II). For protein fluorescence, modification of the albumins results in a blue shift of their maximum wavelength emissions by 13-17nm respectively (Table I). ANS fluorescence is used for studying the hydrophobic binding sites on the proteins [*10,11*]. The ANS fluorescence yield for modified albumins is about half that of the native proteins (Table II). Modification of ovalbumin results in about a 2 fold and 4 fold increases in protein and ANS fluorescence respectively (Table II).

An IC_{50} value is defined as the concentration of inhibitor that gives 50% of its total inhibition potency. An IC_{50} value of 0.18μM was determined for the inhibitory activity of modified HSA (Figure 5). None of the proteins tested, either in the native or the modified forms were able to inhibit fibril extension 100%.

Discussion:

The microplate extension assay (developed by R. Wetzel; UT Medical Center, Knoxville, TN) that was used to screen protein inhibitors is a very sensitive assay that cam measure fibril extension down to the sub-fmol level with minimum background noise. This microplate assay is based on an fibril extension assay [*8*] that uses radio-labeled Aβ which requires considerable care, and is unstable, producing damaging ionizing radiation. In contrast, our assay uses biotinylated Aβ which is not toxic and relatively cheap. The results of our studies show that the extension assay is amenable to high-throughput screening for the identification of Aβ deposition inhibitors using physiological concentrations of Aβ.

The enhanced seed potencies of sonicated fibrils relative to unsonicated has previously been described by Jarrett and Landsbury [*9*]. Sonication of fibrils is likely to increase the available growing ends for the deposition of Aβ monomer. In contrast, the decline in the fibrils ability to undergo extension after 150s is interesting. This reduction in seed potency by repeated sonication is evidently because of effects on the higher order structure of the fibril. In this regard, studies in our lab (B. O'Nuallian) confirm that sonication of fibrils for long periods results in a reduction in their abilities to interact with Thioflavin-T, a fluorescent die that bind specifically to amyloid.

The inhibitory activities of all the proteins tested suggest that many polypeptides can inhibit fibril extension. Yet the mechanism of inhibition remains unknown. However, information can be drawn from the protein screenings.

Proteins exist in both native and non-native states. All of the native proteins screened have inhibitory activity. However, in all cases there was an increase in inhibitory potency with the modification of the proteins, where there are no native structures present. One hypothesis is that the three dimensional structure of the native protein plays little to no role in the inhibition of fibril extension. Future studies could concentrate on the inhibitory mechanism of the modified proteins. Conditions that favor the non-native states of the inhibitors such as denaturants and temperature could be used to investigate our hypothesis.

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