INTRODUCTION

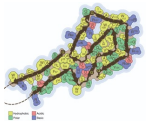
Neurodegenerative disorders such as Alzheimer's and Parkinson's Disease affect the lives of millions every year. Being a progressively degenerative condition caused by amyloidosis of proteins, early detection is imperative for the effectiveness of treatment down the road.[1] Similarly, mutations in human lysozyme can lead to systemic amyloidosis affecting essential organs, such as the liver and kidneys.[2] Thioflavin T (ThT) is a state-of-the-art fluorescent probe commonly used to label amyloidogenesis and fibril aggregation.[3] Though effective, its positive charge attracts the negative charges on DNA and RNA, potentially leading to false positives. As a result, identifying new fluorescent probes without a positive charge could lead to earlier diagnosis and improved patient care.

RESEARCH QUESTION

What components of organic molecules are important for their ability to fluoresce in the presence of amyloid fibrils?

HYPOTHESIS

Protein aggregation to form fibrils involves burying hydrophobic groups of the protein inside of the fibrils to form a hydrophobic core. I hypothesize that the more hydrophobic the components are within the probes, the better they will fluoresce with the amyloid fibrils.



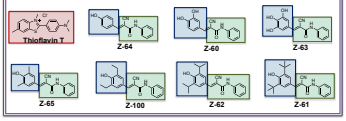
The amyloid fibril structure for lysozyme showing the location of the β-strands (black) with color-coded sidechains - hydrophobic residues are in yellow.[4] These hydrophobic residues become buried within the fibrils.

PROBE DESIGN

Thioflavin T (ThT - red below) is a fluorescence-based dye that is used widely to detect amyloid fibrils.[5] However, since ThT is positively charged, it can also bind to RNA and other elements in the cell, leading to false positives in cellular imaging. It is important, therefore, to identify new probes for amyloidogenesis.

The reagent **rottlerin** is known to dissolve amyloid fibrils of HEWL.[6] For my experiments, I had initially set out to use Z compounds (**Z-60 to 100**) that looked somewhat like rotlerin to reverse amyloid formation. While these compounds did not dissolve HEWL amyloid fibrils, I found that some of the compounds became highly fluorescent upon treatment with HEWL fibrils. This interesting observation changed the course of my project to developing new probes for amyloid fibrils.

The Z compounds all contain a similar amide region (**green** box), but have differing levels of hydrophobicity on the benzene ring (**blue** box). **Z-64**, **Z-60** and **Z-63** have increased numbers of the hydrophilic hydroxy (OH) groups, whereas **Z-65**, **Z-100**, **Z-62** and **Z-61** have increasingly hydrophobic alkyl groups.



MATERIALS

Proteins: hen egg white lysozyme (HEWL), α-synuclein fibrils (gift from Prof. Rochet, Purdue)

Reagents: phosphate buffer (pH 12.2, 7.4), thioflavin T (ThT), Z compounds (prepared by Zach St. John/Chmielewski group), rottlerin, DMSO

Lab Supplies: micropipettes, pipette tips, spatula, weighing paper, Eppendorf tubes, centrifuge tube,

Instruments: analytical balance, centrifuge, transmission electron microscope (TEM), fluorescence plate reader, fluorescence microscope.

METHODS – 1

HEWL was incubated at pH-12.2 at a concentration of 70 μM at room temperature for 120 h to induce formation of fibrils, as described before.[5,6] TEM was used to make sure that fibrils had formed. For subsequent experiments, a 10 μM HEWL fibril sample was diluted in 200 mM phosphate buffer pH 7.4. To screen for Z compounds that fluorescently label HEWL, 2.5 mM stock solutions of test compounds were prepared in DMSO. Samples for fluorimetry were prepared by adding 1 μL of test compound solution to 99 μL of the diluted HEWL fibril solution in a 96-well plate (final 25 μM test compound). Fluorescence was read on a plate reader, with an excitation wavelength at 485 nm and an emission wavelength at 540 nm. DMSO was the negative control, and a 2.5 mM ThT solution prepared in DMSO was the positive control. All experiments were performed at room temperature and in duplicate. Fluorescence microscopy was used to visually image if fibrils were fluorescent and TEM was used to make sure that fibrils were still intact.

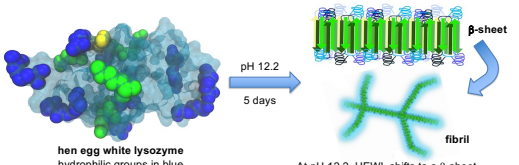
METHODS - 2

The rottlerin assay was performed to determine if test compounds can be used to monitor fibril disassembly. A 10 mM rottlerin solution in DMSO was added to a 10 μM HEWL fibril sample diluted in 200 mM phosphate buffer pH 7.4, such that the final concentration ratio is 100 μM rottlerin: 10 μM HEWL.[5] At different time points, aliquots of 99 uL were removed from this sample, and 1 uL of the 2.5 mM stock solutions of the Z compound will be added. The fluorescence values were read on a plate reader, with an excitation wavelength at 485 nm and an emission wavelength at 540 nm. Values were taken at 0, 2 and 8 minutes at room temperature.

To determine if the Z compounds fluorescently labeled other types of fibrils, alpha-synuclein pre-formed fibrils were examined. The ability for the test compounds to label a 10 μM solution of alpha-synuclein pre-formed fibrils in 200 mM phosphate buffer pH-7.4 was screened. Samples for fluorimetry were prepared by adding 1 μL of the Z compound solution (2.5 mM) to 99 μL of the alpha-synuclein fibril solution in a 96-well plate (final 25 μM test compound). Fluorescence was read on a plate reader, with an excitation wavelength at 485 nm and an emission wavelength at 540 nm. DMSO was the negative control, and a 2.5 mM ThT solution prepared in DMSO was the positive control. All experiments were performed at room temperature and in duplicate.

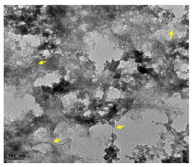
RESULTS

Formation of Hen Egg White Lysozyme (HEWL) Amyloid Fibrils



hen egg white lysozyme
hydrophilic groups in blue
hydrophobic groups in green

At pH 12.2, HEWL shifts to a β-sheet conformation and aggregates, burying hydrophobic groups within the fibril



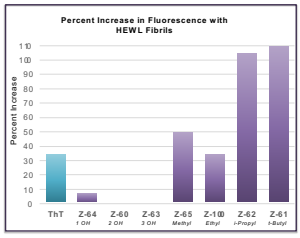
Transmission electron microscope (TEM) image of HEWL amyloid fibrils I formed. Yellow arrows show distinct fibrils.

Detecting HEWL Amyloid Fibrils with Fluorescent Probes

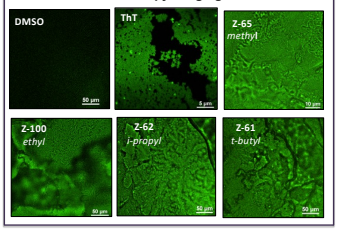
Table 1. Fluorescence Values of Z compounds and ThT with HEWL Fibrils

	ThT	Z-64 1 OH	Z-60 2 OH	Z-63 3 OH	Z-65 Methyl	Z-100 Ethyl	Z-62 i-Propyl	Z-61 t-Butyl
Buffer -HEWL	1653 (± 117)	1904	1619 (± 80)	3140 (± 520)	6654 (± 769)	3289 (± 157)	1792 (± 65)	1816 (± 240)
Buffer +HEWL	2240 (± 47)	2056 (± 145)	1561 (± 37)	2228 (± 88)	9994 (± 999)	4409 (± 1438)	3665 (± 43)	3802 (± 623)
Percentage Increase	35%	8%	-	-	50%	35%	105%	110%

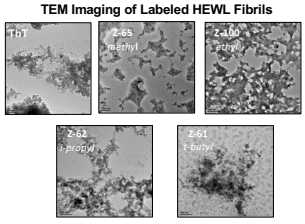
Table 1 has the fluorescence data obtained for the Z compounds with and without the HEWL amyloid fibrils. The percent increase data is also graphed on the right for comparison.



Fluorescence Microscopy Imaging of HEWL Fibrils

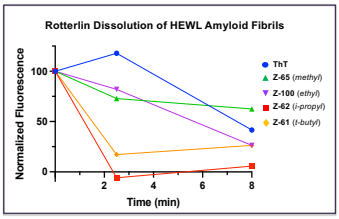


TEM Imaging of Labeled HEWL Fibrils



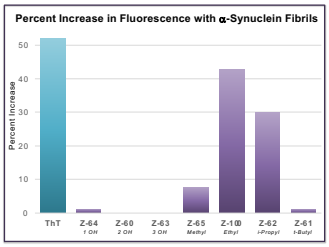
The data **above left** shows the fluorescence microscopy images obtained for the Z compounds and ThT with the HEWL amyloid fibrils. The data **above right** shows the same samples imaged by TEM to see if fibrils are still in the samples.

Monitoring Dissolution of HEWL Amyloid Fibrils



The graph above shows the fluorescence of the HEWL samples after being treated with the fibril dissolving compound rottlerin, followed by the quick addition of the Z compounds and ThT. The normalized value was obtained by subtracting the fluorescence without fibrils.

Probing α-Synuclein Amyloid Fibrils



The above graph shows the percent increase in fluorescence of the Z compounds and ThT with and without the alpha-synuclein amyloid fibrils.

DATA ANALYSIS

Formation of HEWL Amyloid Fibrils

- After 5 days at pH 12.2, the HEWL solution was cloudy and I suspended the precipitate using vortexing to image the aggregates by TEM. In the TEM, I observed varying spread-out groupings of fibrils, with some forming condensed clumps and some strings (yellow arrows).

Probing HEWL Amyloid Fibrils

- I confirmed that my fluorescence assay was working by using the known ThT. I observed a 35% increase in the ThT fluorescence in the presence of amyloid fibrils of HEWL.
- All of the Z compounds were fluorescent in buffer, but only the Z compounds with hydrophobic groups (methyl, ethyl, *i*-propyl and *t*-butyl) showed a notable increase in fluorescence with the HEWL amyloid fibrils.
- The two best Z compounds with HEWL amyloid fibrils were Z-61 and Z-62, with about 3-times better fluorescence increase than ThT. Z-65 was 1.4-times better than ThT and Z-100 was equal to ThT.
- The Z compounds with 1, 2 and 3 OH groups showed little or no fluorescence increase with HEWL amyloid fibrils, most likely because they do not have the hydrophobic groups.
- The larger alkyl groups (*i*-propyl and *t*-butyl) were more effective than the smaller alkyl groups (methyl and ethyl) at binding to HEWL amyloid fibrils, because their hydrophobic groups should interact more strongly with the buried hydrophobic groups within the fibrils.
- Fluorescent microscopy overall confirmed the fluorescence data; the four most effective Z compounds and ThT all showed strongly fluorescent fibrils, with ThT showing distinct clumps and the Z compounds showing larger green fibrils.
- TEM images also confirmed that fibrils were present with some differences in the shape of the fibrils.

Monitoring Dissolution of HEWL Amyloid Fibrils

- Rottlerin is known to rapidly dissolve HEWL amyloid fibrils.[5] I wanted to use my probes to see if I could monitor this dissolution. I found that the four hydrophobic Z compounds and ThT were all able to show a drop in fluorescence at 2 and 8 minutes after adding rottlerin to HEWL amyloid fibrils. The two best performing probes in the above fluorescence assay were found to most rapidly decrease in fluorescence, with Z-62 decreasing the fastest and the most completely. The small variations below zero and above 100, could be due to a lack of replicates for this experiment.

Probing α-Synuclein Amyloid Fibrils

- When probing α-synuclein amyloid fibrils, only two of the seven Z compounds showed fluorescence. Compared to ThT, Z-62 performed worse, and Z-100 performed most closely to ThT. The lack of fluorescence from Z-61 and Z-65 indicated that they were specific for HEWL fibrils, whereas Z-62 and Z-100 could label both.
- The best performing Z compound in this assay (Z-100) was not the most hydrophobic. α-Synuclein amyloid fibrils are known to be less hydrophobic than lysozyme amyloid fibrils,[7] so it is likely that less hydrophobic compounds can interact with α-synuclein fibrils better.

CONCLUSIONS

- My hypothesis was that the more hydrophobic the components are within the probes, the better they will fluoresce with the amyloid fibrils. Overall, my hypothesis was confirmed with HEWL amyloid fibrils as the four best compounds (Z-61, Z-62, Z-65 and Z-100) had the hydrophobic groups, and the more hydrophilic compounds Z-60, Z-63, and Z-64 showed limited or no fluorescence with fibrils.
- My hypothesis was partially confirmed with α-synuclein fibrils. Two of the lesser hydrophobic compounds (Z-62 and Z-100) showed an increase in fluorescence with these fibrils, whereas the compounds with the more hydrophobic groups (Z-61 and Z-65) were not very effective. α-Synuclein amyloid fibrils are known to be less hydrophobic than fibrils of HEWL,[7] so this may explain the observed differences.
- My findings also showed that compounds Z-61 and Z-65 were specific only for HEWL fibrils, which could be very useful to probe specific diseases caused by lysozyme amyloid fibrils, such as systemic amyloidosis.
- In the future, I would like to conduct more experimental runs monitoring the dissolution of HEWL amyloid fibrils, so that I can acquire more accurate results. I would also like to try other amyloid fibrils with my probes, such as Aβ1-42 for Alzheimer's disease.
- In the future, I would also be very interested to see if this research can be mobilized for cell-based imaging experiments and leveraged into a small-molecule drug for amyloid diseases.

BIBLIOGRAPHY

1. Aguzzi A, O'Connor, T. Protein aggregation diseases: pathogenicity and therapeutic perspectives. Nat Rev Drug Discov. 2010, 9, 237–248.
2. Sattianayagam PT, Gibbs SD, Rowczenio D, Pinney JH, Wechalekar AD, Gilbertson JA, Hawkins PN, Lachmann HJ, Gillmore JD. Hereditary lysozyme amyloidosis -- phenotypic heterogeneity and the role of solid organ transplantation. J Intern Med. 2012, 272, 36-44.
3. Biancalana M, Koide S. Molecular mechanism of Thioflavin-T binding to amyloid fibrils. Biochim Biophys Acta. 2010, 1804, 1405-12.
4. Frey L, Zhou J, Cereghetti G, *et al.* A structural rationale for reversible *vs* irreversible amyloid fibril formation from a single protein. Nat Commun. 2024, 15, 8448.
5. Sarkar N, Kumar M, Dube VK. Rottlerin dissolves pre-formed protein amyloid: A study on hen egg white lysozyme, Biochimica et Biophysica Acta. 2011, 1810, 809–814.
6. Kumar S, Ravi VK, Swaminathan R. Suppression of lysozyme aggregation at alkaline pH by tri-N-acetylchitotriose, Biochimica et Biophysica Acta. 2009, 1794, 913–920.
7. Waxman EA, Mazzulli JR, Giasson BI. Characterization of hydrophobic residue requirements for alpha-synuclein fibrillization. Biochemistry. 2009, 48, 9427-36.

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