Detecting Amyloidogenesis: Developing Probes to Analyze Pre-Formed Amyloid Protein Fibrils

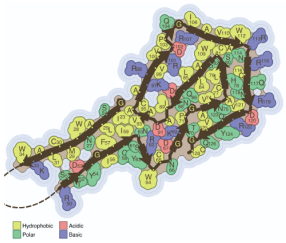
Alana Daniel

Jefferson High School

Lafayette, Indiana

RATIONALE: Each year, the accumulated progression of neurodegenerative disease kills millions, making prompt diagnosis vital in curbing its impact. Thioflavin T (ThT) is a state-of-the-art fluorescent dye; however, its positive charge attracts to cellular components, leading to potential false positives. This research aims to develop compounds more effective than ThT with no net positive charge. These compounds could then be applied to sample testing or mobilized into small-molecule drugs as a non-invasive alternative to biomarker screening, advancing both lab work and inpatient care.

HYPOTHESIS: Due to the amyloid fibrils' hydrophobic core, I hypothesize that the more hydrophobic the probe components are, the more they will fluoresce in the presence of amyloid fibrils.



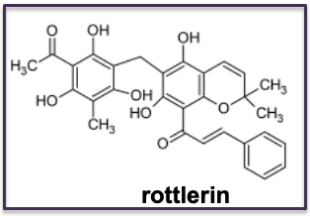
The amyloid fibril structure for lysozyme showing the location of the β-strands (black) with color-coded sidechains - hydrophobic residues are in yellow.[4] These hydrophobic residues become buried within the fibrils.

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

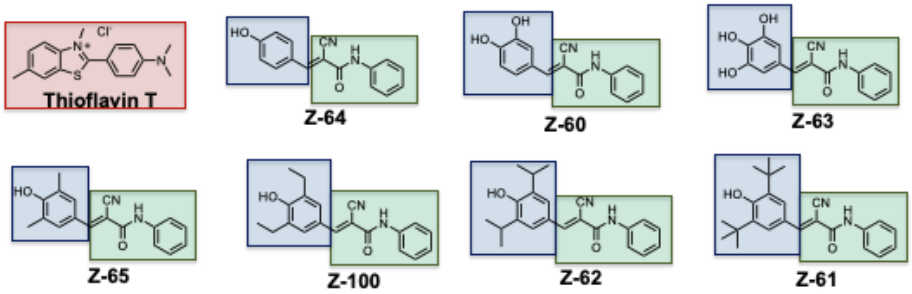
EXPERIMENTAL DESIGN

Thioflavin T (**ThT** - **red** below) is a fluorescence-based dye that is used widely to detect amyloid fibrils.[3] 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.

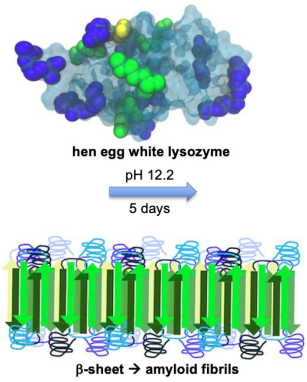
The reagent **rotterlin** is known to dissolve amyloid fibrils of hen egg white lysozyme (HEWL).[5] For my experiments, I had initially set out to use Z compounds (**Z-60** to **Z-100**) that looked somewhat like rotterlin to reverse amyloid formation. While these compounds did not dissolve HEWL amyloid 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.



EXPERIMENTAL METHODS



Amyloid Fibril Formation: Amyloid fibrils of hen egg white lysozyme (HEWL were incubated at 12.2 for 120 hours. A TEM microscope was used to confirm fibril formation. For experimentation, 10 μM HEWL fibrils were diluted in 200 mM phosphate buffer, pH 7.4. Fibril samples were treated with either ThT or a Z-compound (Z-60-65, Z-100). Sample fluorescence was monitored using a plate reader with an excitation wavelength of 485nm and an emission wavelength of 540nm.

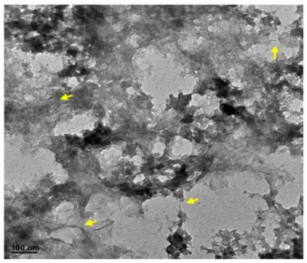
Fluorescence Measurements: Fluorescence microscopy was used to visually confirm and image fibrillar fluorescence.

Imaging Fibrils: Fibrils were imaged on a TEM (Transmission Electron Microscope) to confirm that the addition of Z-compounds used did not alter the chemical or organic structure of the amyloid fibrils.

Rottlerin Assay: A rottlerin assay was performed to determine whether or not the Z-compounds could monitor fibril disassembly. The protein rottlerin was treated with either Thioflavin T or a Z-compound, then pipetted at time intervals and monitored with a plate reader at an excitation wavelength of 485nm and an emission wavelength of 540nm.

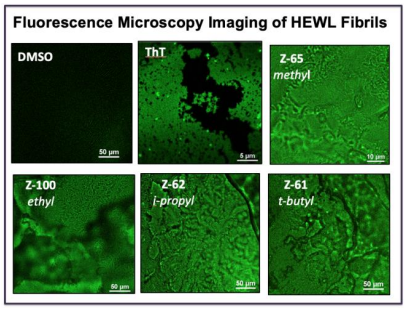
DATA 1

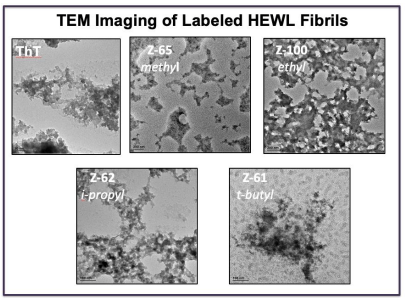
HEWL Amyloid Fibril Formation:



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

HEWL Amyloid Fibril Fluorescent Imaging:





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.

HEWL Amyloid Fibril Imaging:

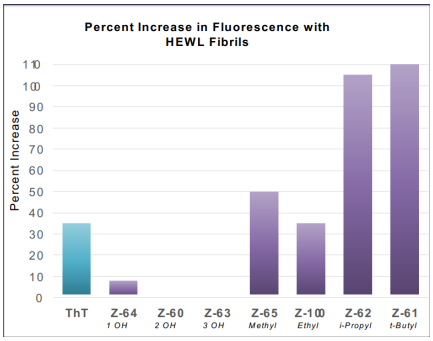


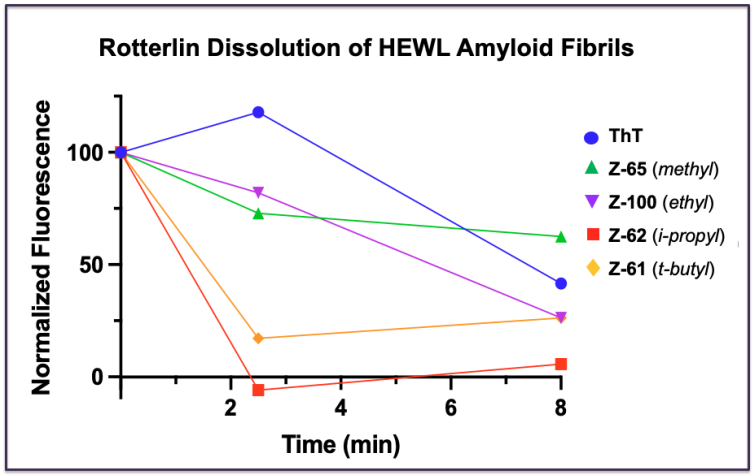
Table 1. Fluorescence Values of Z compounds and ThT with HEWL Fibrils

	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.

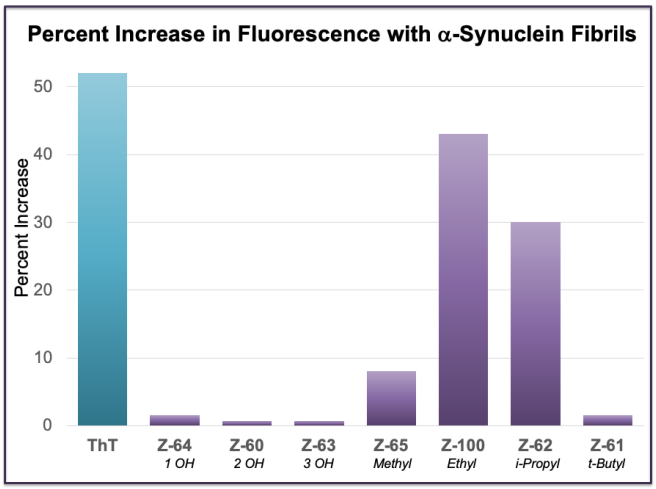
DATA 2

Rottlerin Assay
Monitoring HEWL Fibril Dissolution:



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 in the absence of fibrils.

Probing α-Synuclein Amyloid Fibrils:



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

DATA Analysis 1

HEWL Amyloid Fibril Formation: After a 120-hour incubation period (5 days), the formed precipitates were suspended and imaged using TEM, where varying spread-out groups of fibrils were observed.

HEWL Amyloid Fibril Fluorescent Imaging: I observed a 35% increase in ThT fluorescence in the presence of HEWL fibrils. All Z compounds were fluorescent, while the hydrophobic groups showed a notable increase in fluorescence. Compared with ThT, Z-61 and Z-62 (with larger alkyl groups) performed about 3 times better, Z-65 about 4 times better, and Z-100 performed as well as ThT. The hydrophobic compounds showed limited or no fluorescence.

HEWL Amyloid Fibril Imaging: Fluorescent microscopy confirmed fluorescence, showing that the best-performing compounds had strongly fluorescent fibrils. TEM confirmed the presence of fibrils, with some differences in fibril shape.

DATA Analysis 2

Rottlerin Assay

Monitoring HEWL Fibril Dissolution: All four hydrophobic compounds and ThT were able to show a drop in fluorescence at 2 and 8 minutes after adding HEWL amyloid fibrils. The best compound, based on the compound that decreased most rapidly and completely, was Z-62. The small variations above and below 100 could be due to a lack of replicates for this experiment.

Probing α-Synuclein Amyloid Fibrils: Compared with both ThT performance and overall HEWL amyloid fluorescence, Z-62 performed worse; Z-100 was most similar to ThT. 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 HEWL and a-synuclein fibrils.

CONCLUSIONS

- For HEWL fibrils, my hypothesis was confirmed, with the four best compounds were Z-61, Z-62, Z-65, and Z-100, the compounds with hydrophobic groups. Z-60, Z-63, and Z-64 had hydrophilic groups and showed limited or no fluorescence.

- For a-synuclein fibrils only the lesser hydrophobic compounds (Z-62 and Z-100) showed an increase in fluorescence whereas the more hydrophobic compounds (Z-61 and Z-65) were not as effective. This could be explained by the fact that a-synuclein fibrils are known to be less hydrophobic than HEWL fibrils, and only partially confirms my hypothesis.

- Overall, compounds Z-61 and Z-65 were specific for HEWL fibrils, which could be important for lysozyme-based diseases, and Z-100 was the best overall.

- In the future, I would like to conduct more rottlerin assays, experiment with more amyloid fibrils, and 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 BiophysicaActa. 2011, 1810, 809–814.
6. Kumar S, Ravi VK, Swaminathan R. Suppression of lysozyme aggregation at alkaline pH by tri-N-acetylchitotriose, Biochimica et BiophysicaActa. 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.