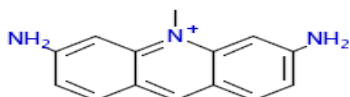


Acriflavinium Hydrochloride, A Possible Alternative to ThT for the Detection of Protein Fibrils

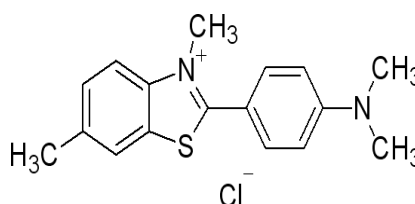
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ABSTRACT



Acriflavinium Hydrochloride



Thioflavin T (ThT)

Fluorescent studies were made with acriflavinium hydrochloride in an attempt to elucidate the possibility of it acting as a probe of protein fibrils, especially interesting is whether or not it can detect alpha-synuclein fibrils. ThT is the currently used probe for detection of alpha-synuclein fibrils. Results with acriflavinium hydrochloride proved to be promising, as changes in fluorescence were observed in multiple experiments when alpha-synuclein fibrils were present. In addition to experiments that attempted to determine whether acriflavinium hydrochloride could be used as a probe for fibrils, an experiment was run to try and determine where the interaction between acriflavinium hydrochloride and alpha-synuclein takes place. From the first run it appears that the interaction may be occurring at the C-terminal of the protein.

Parkinson's disease (PD) is the second most common neurodegenerative disease, with Alzheimer's being the first. PD affects about 1-2% of the population over the age of 65.¹ Although the exact cause of the disease still remains unknown the fibrillation of the protein, alpha-synuclein, has been implicated as a possible critical step in the development of Parkinson's disease (PD).² With either, one of the intermediate species on the pathway to fibril formation, or the fibrils themselves having a toxic effect which

¹ Dusa, A.; Kaylor, J.; Edridge, S.; Bodner, N.; Hong, D.; Fink, A.L. *Biochemistry*. **2006**, 45, 2752-2760

² Uversky, V.N.; Fink, A.L. *Proteins*. **2002**, 1, 153-186

results in the death of dopaminergic neurons located in the substantia nigra. Therefore the study of the formation of alpha-synuclein fibrils has been an important research area. Alpha-synuclein is a natively unfolded protein made of 140 amino acids and currently has an unknown function.³ However, alpha-synuclein may have some role in vesicular transport, and the monomer has been shown to contain some antioxidant properties for unsaturated lipids in vesicles.³

Alpha-synuclein is an important protein to study because of its association to PD, and the ability to probe for fibrils plays a crucial role in the research of the protein. Currently the chemical that is used to monitor the formation of alpha-synuclein fibrils is Thioflavin T (ThT). ThT is a compound that is known to emit strong fluorescence when bound to amyloid fibrils.⁴ The monitoring of fibril formation has been a vital part of the research to find potential novel drug treatments for PD, which treat the disease rather than the symptoms. During a screening of chemicals, for the inhibition of fibril formation, the compound acriflavinium hydrochloride gave a graph that was different from anything seen with any previous chemical. Described here are the experiments that were run to try and determine the reason for the odd results that were initially seen with acriflavinium hydrochloride.

Initial experiments were run monitoring the change in fluorescence over time using a 96 well plate reader (Fluoroskan acent CF, Labsystems Inc.). The excitation and emission wavelengths were and nm respectively. The plate was shaken at rpm with a diameter of mm at °C. Fluorescence intensities were recorded for hours at intervals of . These first experiments with the compound revealed some interesting aspects of

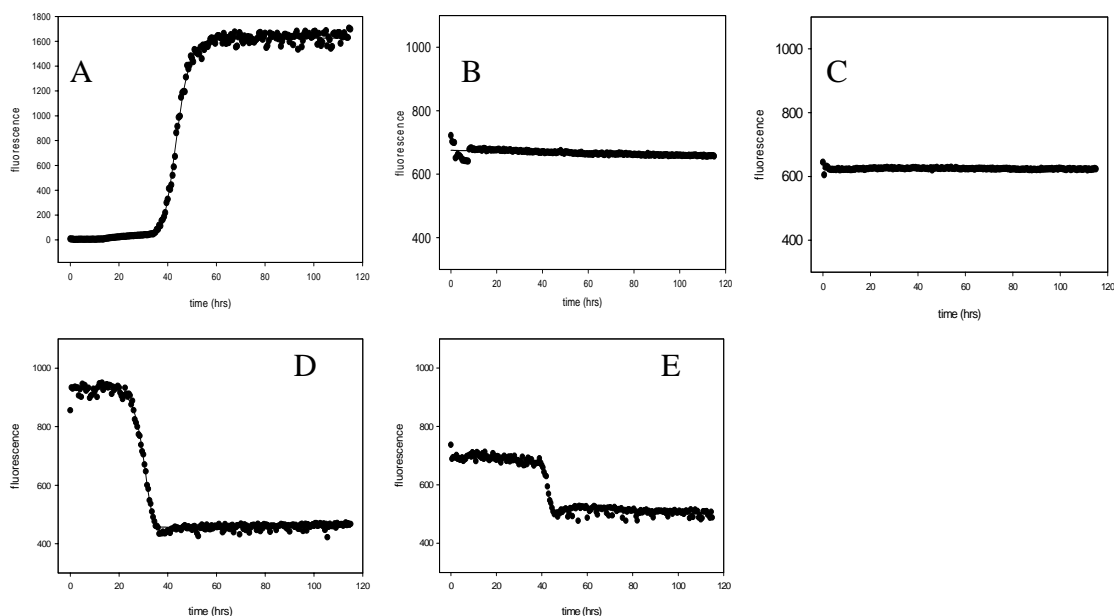


Figure 1. Fluorescence readings over time of samples. Each sample was prepared using 10mM phosphate buffer and contained 100mM NaCl. In addition sample (A) had α -synuclein and ThT, sample (B) contained acriflavinium hydrochloride, sample (C) contained acriflavinium and ThT, sample (D) contained α -synuclein and acriflavinium, sample (E) contained α -synuclein, acriflavinium, and ThT.

³ Zhu, M.; Qin, Z.; Hu, D.; Munishkina, L.A.; Fink, A.L. *Biochemistry*. **2006**, 45, 8135-8142

⁴ Ban, T.; Hamada, D.; Hasegawa, K.; Naiki, H.; Goto, Y. *J. Biol. Chem.* **2003**, 278, 16462-16465

Acriflavinium hydrochloride. From the readings acriflavinium hydrochloride is shown to have fluorescence without protein fibrils present (Figure 1B). Also, it is apparent that there is no significant quenching caused by the presence of ThT (Figure 1C), therefore the results of the first screening was not due to ThT. The reproducibility of the odd shaped curve when screening acriflavinium hydrochloride is a good indication that there is some sort of interaction between the fibrils and acriflavinium hydrochloride. The fact that the fluorescence is increased in the presence of protein may suggest that acriflavinium hydrochloride interacts with the monomer causing an increase in fluorescence, and then when fibrils form this somehow causes a quenching of the signal. The last interesting aspect of the data obtained is the smaller range of fluorescence values when there is both ThT and acriflavinium hydrochloride in the presence of protein. This may suggest that there is some sort of competition between the two compounds for binding.

The next set of experiments that were run had the purpose of reaffirming the fact that acriflavinium hydrochlorides fluorescence does indeed change in the presence of alpha-synuclein fibrils. The experimental setup was taking preexisting fibrils provided by the lab and diluting them in some 10mM phosphate buffer. The fibrils were then added 2 μ L at a time to a solution of 1mL 0.5 μ M acriflavinium hydrochloride in 10mM phosphate buffer. Before the first addition and after each 2 μ L a fluorescence reading was taken using a FluoroMax-3 spectrofluorometer (Jobin Yvon Horiba). Emission spectra were collected from 460 to 600nm after exciting at 450nm. From the spectrum a clear

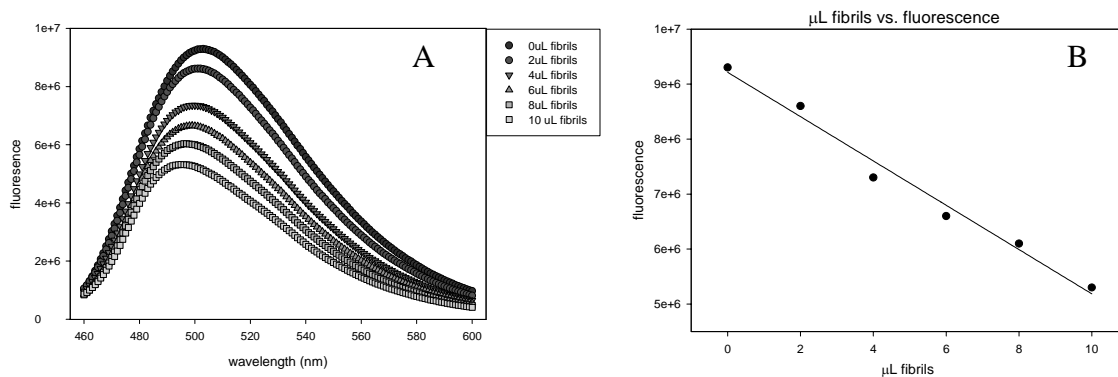


Figure 2. Following the fluorescence of a 0.5 μ M solution of acriflavinium hydrochloride upon the addition of alpha-synuclein fibrils. Spectrum from 460 to 600nm (A), and a plot of max fluorescence versus μ L fibrils added (B).

decrease in the fluorescent reading is apparent. This again is a good indication that there is some interaction between the alpha-synuclein fibrils and acriflavinium hydrochloride.

The last experiments that were run to help determine whether acriflavinium hydrochloride can be used as a probe for alpha-synuclein fibrils was following the formation of fibrils with ThT versus acriflavinium hydrochloride. Two solutions of 1mg/mL alpha-synuclein, 100mM NaCl, in 10mM phosphate buffer were prepared. One solution also contained 50 μ M acriflavinium hydrochloride. Fluorescent readings were taken every few hours on the FluoroMax-3. For the solution without the acriflavinium hydrochloride 10 μ L of solution were added to 1mL of 10 μ M ThT and a reading was

taken. For the solution containing the acriflavinium hydrochloride 10 μ L of the solution were added to 1mL of 10mM phosphate buffer and a reading was taken. From the plot

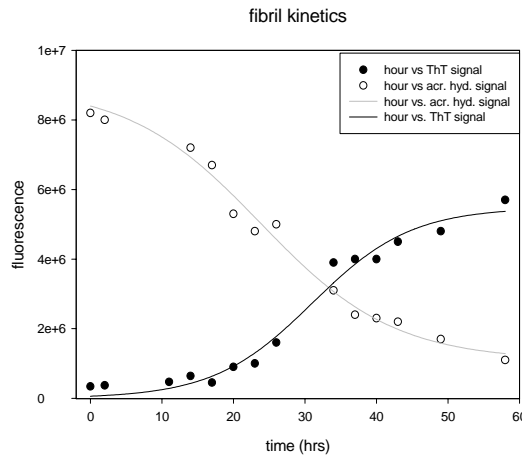


Figure 3. Following the formation of alpha-synuclein fibrils by changes in fluorescence of ThT (black filled circles) and acriflavinium hydrochloride (non-filled circles).

of the max fluorescence versus time we see an almost mirror image when comparing the two lines. Again this indicates that acriflavinium hydrochloride is interacting with fibrils and can be used to identify fibril formation.

The second to the last experiment that was run included using a different protein and acriflavinium hydrochloride. The protein used was human insulin, which is also known to produce fibrils. This experiment was to show whether or not acriflavinium hydrochloride could recognize and interact with other protein fibrils or if it was a specific interaction with alpha-synuclein. The data shows similarities to that of alpha-synuclein indicating that acriflavinium hydrochloride can interact with other protein fibril species.

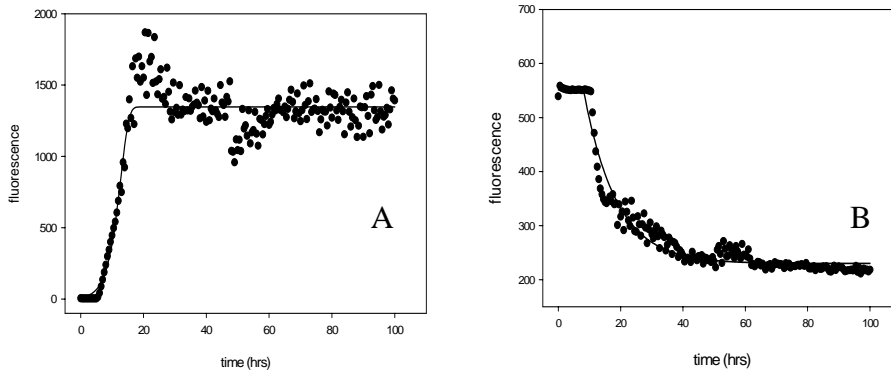


Figure 4. Following fluorescence over time using the Fluoroskan plate reader. Similar conditions for both samples except that A contains ThT and no acriflavinium hydrochloride while B contains acriflavinium hydrochloride but no ThT.

After having ran all these different experiments and coming to the belief that acriflavinium is interacting with alpha-synuclein and can be used as a probe for protein fibrils, the next step is attempting to find how and where the interaction occurs. One preliminary experiment was run with the Fluoroskan and using truncated proteins. Six

samples were ran: wild type alpha-synuclein with and without acriflavinium hydrochloride, N-terminal truncated alpha-synuclein with and without acriflavinium hydrochloride, and C-terminal truncated alpha-synuclein with and with out acriflavinium hydrochloride. Shown are just the results of the C-terminal truncated with and without acriflavinium hydrochloride since the other samples results were similar to those of before. From the results it would appear that without the C-terminal portion of

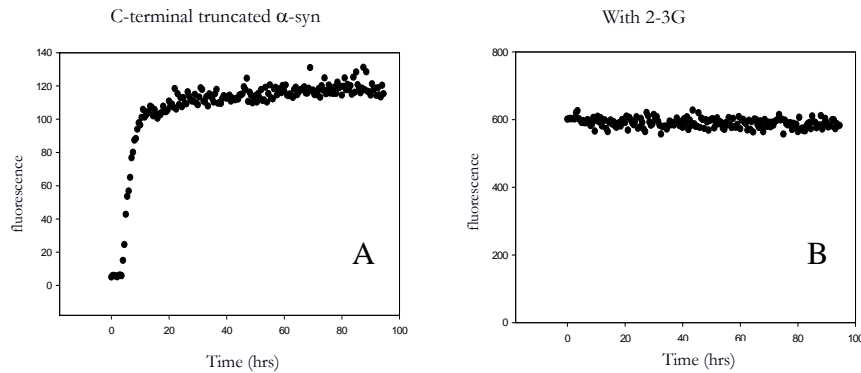


Figure 5. Fluoroskan readings over time of the C-terminal truncated protein with (A) and without (B) acriflavinium hydrochloride.

alpha-synuclein acriflavinium hydrochloride can not interact in the appropriate manner that allows for the quenching of the fluorescence.

The work done on acriflavinium hydrochloride thus far shows the potential for it being used as an alternate to ThT for detection of protein fibrils. Further test may be run to try and understand whether or not acriflavinium hydrochloride is a better probe for protein fibrils than ThT. Also, more test may be ran to try and determine where the interaction is occurring and how it is occurring between acriflavinium hydrochloride and alpha-synuclein.

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