The Inhibitive Effects of 5 Dietary Compounds on IAPP Fibril Formation

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Bachelor of Science in Biochemistry

Acknowledgements

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Thank you to all of the graduate students that have assisted me in this project, including Kevin Hartman (for helping design the project and prepare samples) and Samuel Kotler (for advising me on my presentation of data and research).

Thank you to all of the members of the Ramamoorthy lab for your kindness, support, and many fun outings we've had together. You've all made working in a research laboratory such a rewarding experience.

Lastly, thank you to my friends and family. I wouldn't be able to accomplish my goals without everything that you do for me every day.

Contents

Abstract

Amyloid peptides such as Islet Amyloid Polypeptide (IAPP) have the tendency to misfold into large fibrils of stacked beta sheets. The fibrils, which then interrupt and disrupt cell membranes, have been shown to correlate with the development of diseases such as Alzheimer's disease, Parkinson's disease and Type II diabetes. Specifically, the growth of IAPP fibrils has been correlated with the development of Type II diabetes by the mechanism of β-cell death. As a means of a potential medicinal treatment, a growing number of studies are addressing whether certain substances can prevent these fibrils from forming. While some of the first studies focused on the effectiveness of insulin and antibodies, recent research has primarily focused on the effectiveness of various dietary compounds. This thesis will present data on the effects of these compounds on IAPP fibril formation as well as an original fluorescence study involving 5 different compounds.

Introduction

Diabetes

Diabetes is an illness that is characterized by the body's inability to regulate glucose levels. Two different types of diabetes have been identified. Type I, or insulin-dependent diabetes mellitus (IDDM), is a result of the body's inability to produce insulin, a hormone that instructs various parts of the body to compensate for an elevated blood sugar level. Type II, or non-insulin-dependent diabetes mellitus (T2DM), is characterized by the loss of ability for the body's ability to receive and process insulin signals regardless of the amount of insulin produced. An estimated 20 million American suffer from Diabetes, the majority (about 90%) suffering from type II. The disease directly presents several debilitating symptoms including increased thirst and weight loss, changes in vision and diabetic coma brought on by dangerously high blood sugar levels. In addition, those with diabetes are at an elevated risk to develop stroke, hypertension and heart disease. It has become one of the top causes of death in the United States and one of the biggest national health concerns in recent decades. [1]

IAPP peptides and Type II Diabetes

Type II Diabetes is part of a class of more than 20 diseases including Alzheimer's disease and Parkinson's disease that are known as the "amyloidogenic diseases." This group of diseases is characterized by conformational changes in various peptides that then accumulate outside cell membranes. Although these peptides do not share sequence homology, they all form characteristic fibril morphologies heavy in β-sheet residues. [1]

For Type II diabetes, the peptide responsible for this mechanism of disease is Islet Amyloid Polypeptide, or IAPP, the basic molecular structure of which is shown in Figure 1. [6] IAPP is produced by β-cells found in the Pancreas, and when it misfolds, it can create toxic compounds, accumulate with other IAPP fibrils and eventually lead to β-cell death. The death of β-cells along with the decreased ability for insulin reception is thought to contribute to the pathology of Type II Diabetes. Interestingly, it's been found that while IAPP found in humans and other larger mammals such as monkeys and cats is amyloidogenic and toxic, IAPP found in rodents such as rats and mice is not and therefore does not present the same toxicity. [1 ,2, 3]

Figure 1. Amino acid sequence of IAPP.

Figure 2 summarizes the mechanism of formation of IAPP fibrils both in solution and in the presence of lipid membranes. It is a multi-step process, with IAPP first slowly transitioning from a randomly formed coil to a conglomeration of oligemers heavy in β- sheet structure and then transitioning at a faster rate to the β-sheet fibrils. The membrane oriented

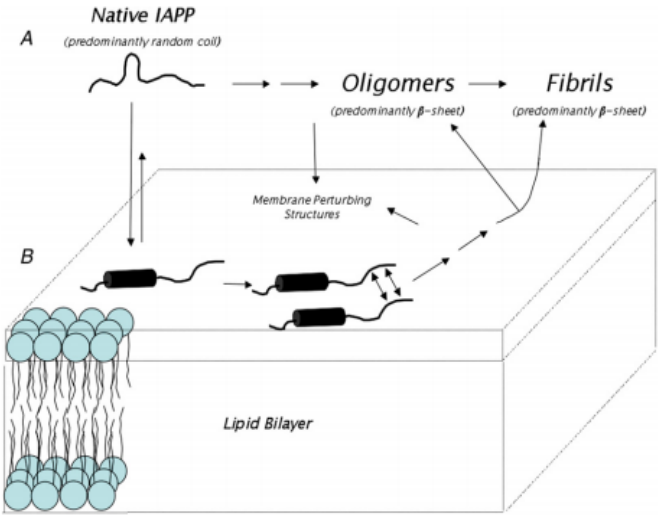

Figure 2. Aggregation of IAPP: A) in solution and B) in the presence of membranes.

conglomeration of fibrils then proceeds through an α-helix structure which is thought to mediate the need of residues hydrogen bonding to the peptide backbone when bound to the membrane due to the lower dielectric constant as compared to the peptide in solution. [1]

While the precise mechanism for the disruption of cell membranes by IAPP fibrils is not completely understood, two main mechanisms have been proposed: pore formation in the cell membranes and a permeation effect of peptide bundles which leads to membrane instability. [1,2, 3] Past studies have indicated that the membrane pore is composed of 5 different subunits reminiscent of pores formed by similar amyloid peptides such as Amyloid-Beta. However, other studies have not come up with conclusive evidence for pore formation, but have suggested alternative mechanisms of membrane disruption. These include the congregation of spherical, β- sheet heavy IAPP oligomers which seem to introduce small defects on the surface of the membrane which accumulate on the membrane over time (similar to the mechanism of a detergent disrupting the membrane). [1, 3] This second method could present a number of indirect effects of IAPP fibril formation on β-cell death, including an increased formation of reactive oxygen and nitrogen species, abnormalities in cell redox systems, a loss of peptide function after aggregation and the hyper-phosphorylation of proteins which contributes to aggregation. [2]

Inhibition of IAPP Fibril Formation

Recent data has provided ample evidence for the inhibitive effects of several different types of compounds on IAPP fibril formation. One major class of these compounds is called polyphenols that are characterized by the presence of one of more aromatic phenolic rings. They are found in high concentrations in foods and beverages such as wine, tea, nuts, berries, cocoa, and a wide variety of other plants. They can be subdivided into vitamins, phenolic acids, flavinoids and other miscellaneous polyphenols. [2]

Five specific compounds will be examined in the fluorescence assay study later in this paper for their restrictive effects on IAPP membrane disruption. The first of these is Curcumin, which is derived from Tumeric, an Indian spice. It is composed of two relatively polar aromatic head groups connected by a linker. Figure 3 shows how Curcumin interacts with amyloid peptides in order to prevent aggregation (This particular study used Amyloid- Beta peptides as the example, although the model can be considered analogous for all amyloid peptides). Data suggests that activity highly depends on the specific parameters of the R_1, R_2, and R_3 subgroups, or else Curcumin's activity is severely disrupted. [4]

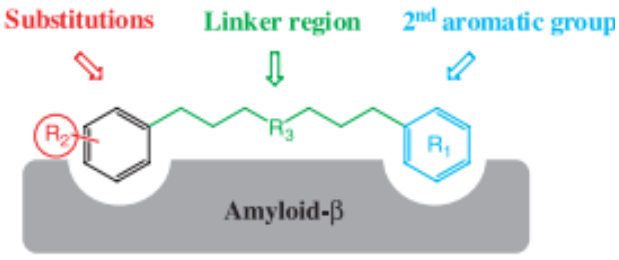

Figure 3. Interaction of Curcumin with amyloid peptides

Curcumin appears to induce alterations in membrane structure, which in turn suggests that it may modulate the membrane assembly of IAPP. Since it appears to be the most effective in a 1:1 ratio with peptide, it suggests that it may interact with the monomeric or early stage aggregation of IAPP. Figure 4 illustrates the structural changes through a Circular Dichroism spectrum of IAPP when incubated with Curcumin- it was determined that Curcumin modulates IAPP by unfolding the α-helix. [6]

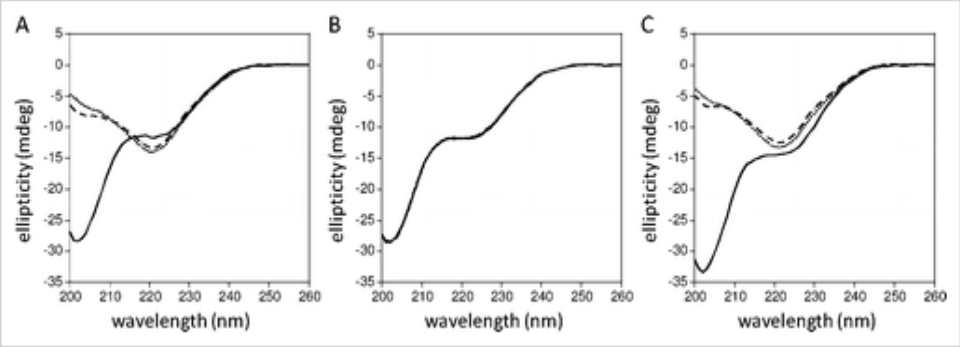

Figure 4. A) IAPP only. B) IAPP plus Curcumin in ethanol [IAPP: Curcumin ratio is 1:1 (mol:mol)]. C) IAPP plus the same amount of ethanol present in panel B. Spectra were taken immediately after sample preparation (solid line), after 1 day of incubation (dashed line) and after 5 days of incubation (dotted line)

The second molecule studied was EGCG, a compound derived from green tea. Recent data suggests that not only is EGCG able to inhibit IAPP membrane disruption, but is also one of the few compounds known to be able to disaggregate IAPP peptides. When fluorescence assays were performed by adding EGCG to IAPP samples, a dramatic drop in fluorescence followed by a gradual drop in fluorescence was observed. Figure 5 shows several TEM images taken during a particular study that demonstrates the ability of EGCG to disaggregate IAPP. The reduction of IAPP fibrils is visibly noticeable and is also reflected in the fluorescence assay. [5]

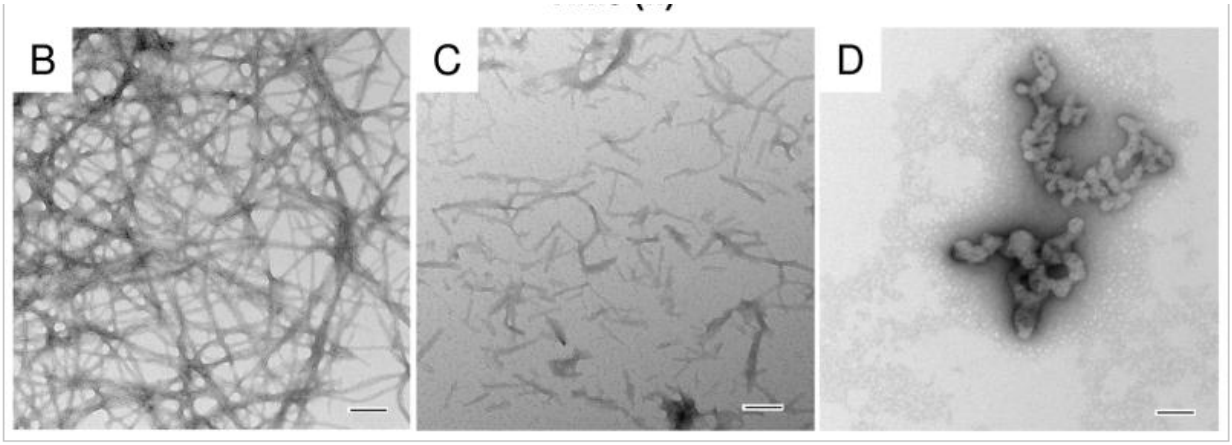

Figure 5. B) IAPP before the addition of EGCG. C) IAPP after approximately 50 hours after the addition of EGCG. D) IAPP after approximately 200 hours after the addition of EGCG.

The third molecule studied was Congo Red, a synthetic dye used to test for Amyloidosis. Congo Red, along with Thioflavin T, is known for being a dye that selectively bonds to amyloid fibrils and not monomers or oligomers. Previous studies show that Congo Red also preferably binds to amyloidal human IAPP over non-amyloidal rodent IAPP which demonstrates its role in the IAPP inhibiting mechanism. A recent study with Congo Red as a binding agent recently challenged the notion that rodent amyloid peptides did not form fibrils and was not toxic. It was found that, while Congo Red could specifically bind human Amyloid- Beta peptides in all buffers tested, it could only bind rat Amyloid Beta in Tris- HCl buffer. This suggests that rat Amyloid Beta may form fibrils under certain conditions. [7]

The fourth molecule studied was Resveratrol, a compound derived from red wine. Recent data suggests the Resveratrol acts to maintain the set amount of β-sheet content present in the

structure and to prevent monomers from forming more β-sheet content. Monomeric amyloid peptides were found to be much more present in the presence of Resveratrol than a sample of amyloid peptide alone. From a thermodynamic standpoint, this suggests that Resveratrol acts to stabilize the monomeric form of IAPP and therefore prevent fibrilization. [8]

The final compound studied was Rifampicin, an antibiotic. Rifampicin has been found to prevent IAPP fibril formation in concentrations as low as 3 μM (which was also found to be the highest non-toxic concentration.) However, there is not conclusive evidence that Rifampicin is able to inhibit the formation of toxic IAPP oligomers or to protect against cell death. In fact, Rifampicin was even found to aggrevate cell death at increasing concentrations. There are two possible explanations given for this trend. The first was that the toxicity produced from Rifampicin itself outweighed the inhibitive effects on IAPP fibril formation. However, the discrepancies between the effective concentration of Rifampicin to inhibit fibril formation (between 3 and 10 μM) and the toxic concentration (> 12.5 μM) calls doubt this explanation. An alternative theory is that IAPP fibrils are able to derive toxic properties from the oligomers that Rifampicin is unable to affect. Lack of data for this explanation suggests that the toxic oligomer and fibril formation mechanisms are distinct. Since Rifampicin was unable to prevent cell death, it would probably be the least likely of the compounds mentioned used to prevent β-cell death. [9]

Experiment

Methods

The model membranes used were LUV liposomes composed of 1:1 1-palmitoyl-2-oleoyl-snglycero-3-phosphocholine: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG/POPC) lipids. The lipid stock solutions were prepared with a chloroform solvent, which was evaporated with Nitrogen gas. The lipid films were then dried in a vacuum oven for 24 hours. Half of the lipids used were prepared with Phosphate/ NaCL buffer and half were prepared with Thioflavin T dye at a final concentration of 10 mg/mL for each of the lipid solutions. The LUV vesicles were then prepared using mini extruder from Avanti lipids, and the LUVs were separated from lipid aggregations that had formed post extrusion through size exclusion chromatography. These LUV fractions were then stabilized by freezing in liquid nitrogen and thawing in boiling water a total of 5 times for each fraction.

Figure 6 shows the typical kinetic curve of IAPP fibril formation as observed by fluorescence assays. It is characterized by a lag phase dominated by the presence of monomers and a sigmoidal transition to fibril formation through oligomer intermediates. [3]

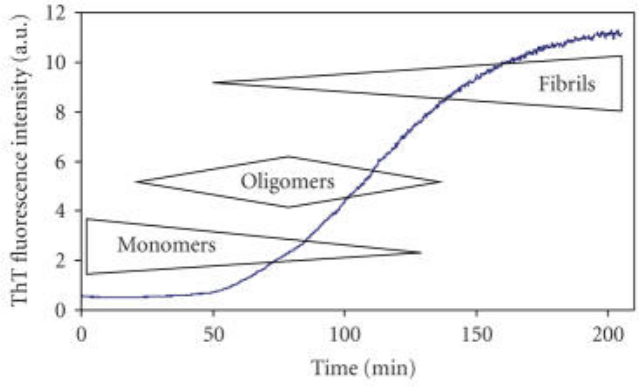

Figure 6. Typical Kinetic Curve of IAPP fibril formation

Five different dietary compounds were used in this experiment to test their effects of IAPP fibril formation: Curcumin, Congo Red, EGCG, Resveretrol and Rifampicin. All of these compounds were prepared in 1 mM stock solutions with Phosphate/ NaCL buffer. The peptide solution was prepared by dissolving purified IAPP in dimethyl sulfoxide (DMSO).

84 wells total were used for the dye leakage experiment in two trials. For the first trial, the wells were prepared with a mixture of Phosphate/ NaCL buffer, Lipids (.287 mg/mL of LUV with dye, .152 mg/mL of LUV with buffer), IAPP, the dietary compounds and the remaining volume with Phosphate/ NaCL buffer for a total volume of 70 µL per well. 4 wells were used as a control with IAPP but with no compounds. The compounds were present at concentrations of 5 µM, 10 µM, 20 µM and 50 µM, and 4 wells were used for each concentration. For the second trial, 4 wells were used with just lipids, and 4 wells for each concentration for each compound without IAPP were used.

The assay was carried out with a fluorescence reader at an excitation wavelength of 494 nm and an emission wavelength of 520 nm. Background fluorescence measurement was taken with the vesicles and compounds for 30 minutes. IAPP was then added and the fluorescence data was then taken for the lag time of fibril formation for a total of 30 hours for trial 1 and 16 hours for trial 2. A maximum fluorescence value was then obtained by adding detergent and measuring fluorescence for one hour for trial 1 and 20 minutes for trial 2.

The data is shown in terms of time (in kilo-seconds) and the ratio of each fluorescence measurement to the first of the average of the four wells used for each measurement in order to account for differences in initial fluorescence values. Each graph shows the plots for fractions containing IAPP peptide and the different concentrations of compound, and the controls of IAPP with lipids, the compound with lipids, (with the same concentration as the non-control fraction) and the lipids by themselves. These last two controls were taken for 16 hours as opposed to the 30 hours for the first control and IAPP plus compound, so the maximum fluorescence value for the controls is noted at 16 hours. The IAPP control and the samples of IAPP plus each of the five compounds each yielded a higher fluorescence ratio than the lipid control, which alludes to fibril formation in all of these samples.

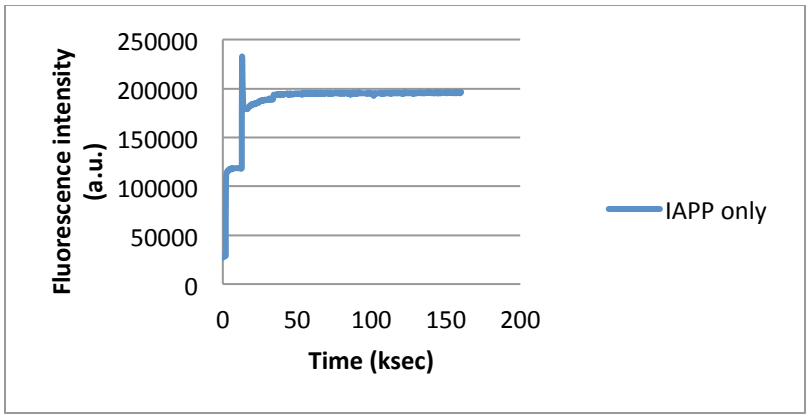

Figure 8A- IAPP control curve without adjustment

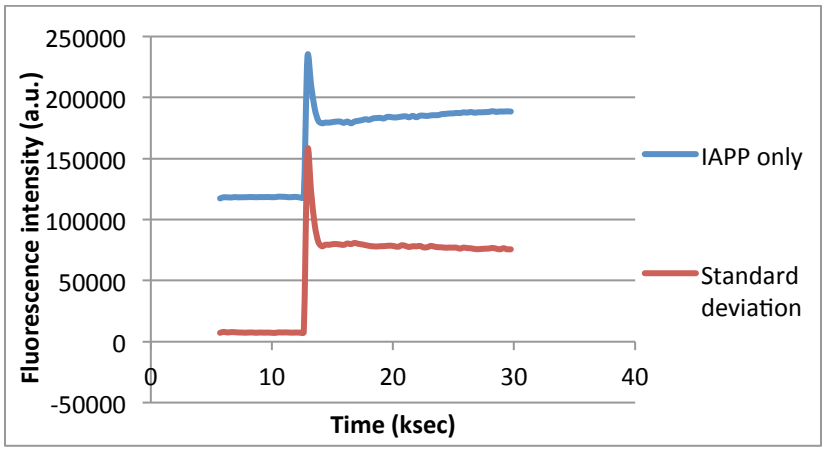

Figure 8B- IAPP versus Standard Deviation for problematic area around 13 kilo-seconds

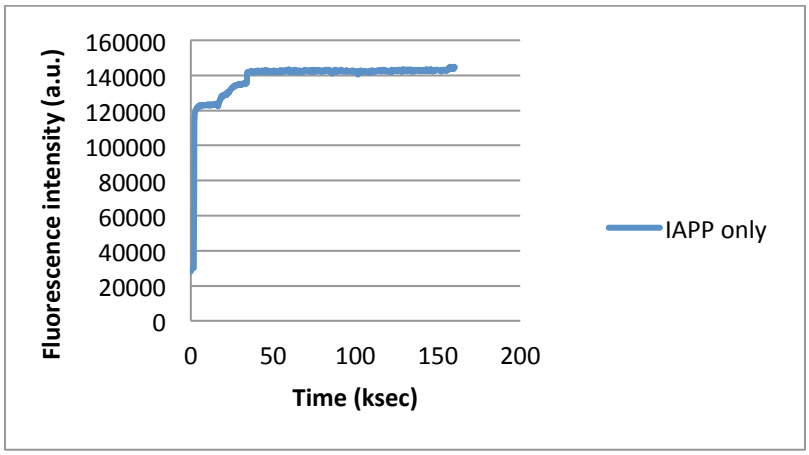

Figure 8C- IAPP control curve with adjustment to IAPP

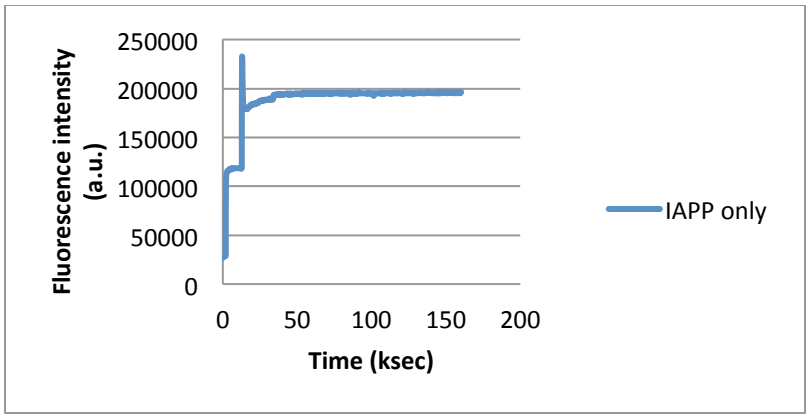

Figure 8A- IAPP control curve without adjustment

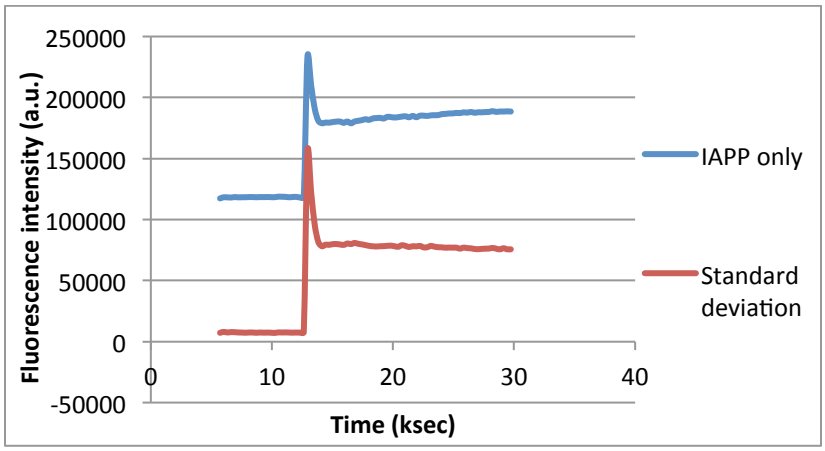

Figure 8B- IAPP versus Standard Deviation for problematic area around 13 kilo-seconds

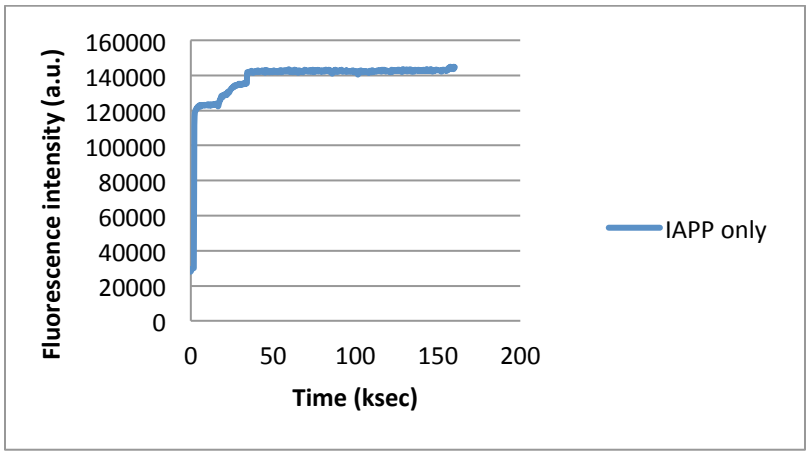

Figure 8C- IAPP control curve with adjustment to IAPP

Curcumin

Figures 8 A-D show the plot of the fluorescence ratios versus time for samples containing Curcumin as well as for the controls. It was observed that all the IAPP + Curcumin samples had less fluorescence upon the insertion of the IAPP peptides than the IAPP control, with the 5 μM sample fluorescence signal reduced by approximately 4%, the 10 μM sample reduced by approximately 40% and the 20 μM and 50 μM samples both reduced by approximately 68%. This suggests that Curcumin prevents IAPP fibrils from forming and penetrating the cell membrane with a possible maximum reduction potential.

The behavior of the Curcumin controls was also noted. Since no IAPP was present in these samples, it would be expected that there would be a baseline fluorescence level observed similar to the lipid control samples, with the fluorescence only increasing after 16 hours when detergent was added and a maximum fluorescence value was obtained. The fluorescence exhibited by the Curcumin sample at 5 μM largely follows this pattern despite a small increase in fluorescence. However, the samples at 10 μM, 20 μM and 50 μM all exhibited a steady rise in fluorescence over the 16 hour period before leveling off. The 10 μM and 50 μM controls both actually leveled off at a fluorescence ration larger than that of the IAPP control. A possible conclusion may be that, while Curcumin can interact with IAPP to prevent penetrative fibril formation, Curcumin in isolation may have membrane penetrating properties (as noted in the introduction section.)

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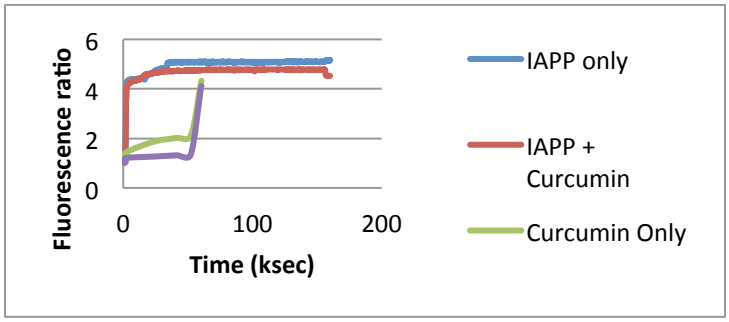

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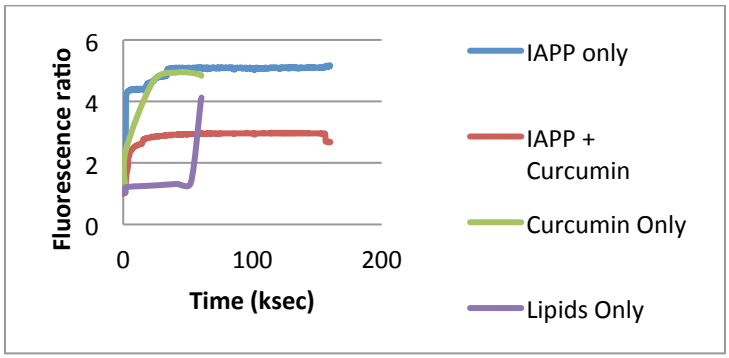

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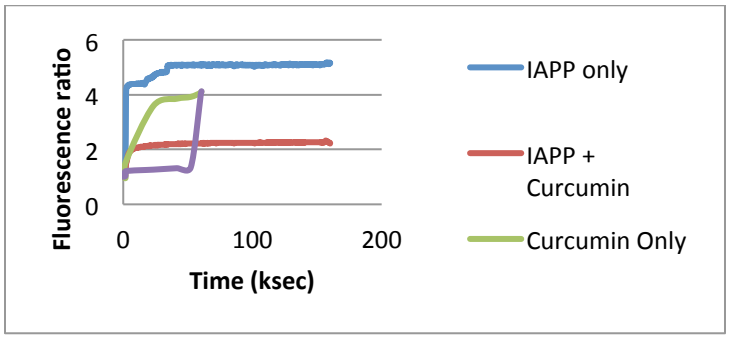

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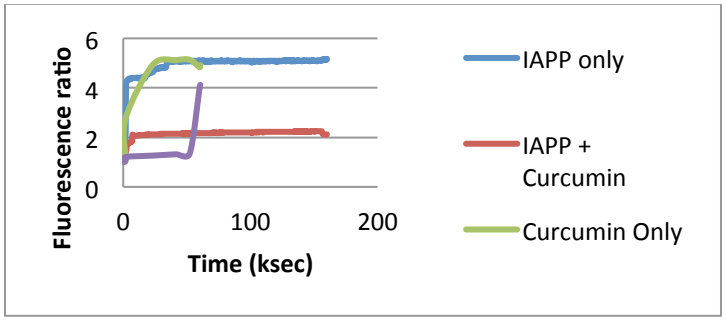


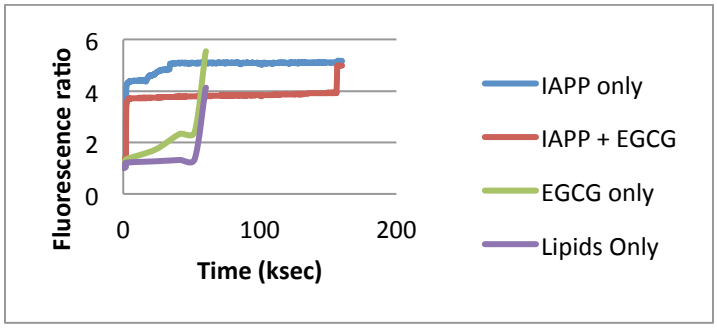
Figure 8. Fluorescence ratio vs time for Curcumin at A) 5 µM B) 10 µM C) 20 µM and D) 50 µM

EGCG

Figures 9 a-d show the plot of the fluorescence ratios versus time for samples containing EGCG as well as for the controls. It was first observed that both the IAPP control and the IAPP + EGCG samples had a higher fluorescence signal than the lipid control, which alludes to the formation of IAPP fibrils in both of these samples. It was also observed that all the IAPP + EGCG samples had less fluorescence upon the insertion of the IAPP peptides than the IAPP control, with the 5 μM sample fluorescence signal reduced by approximately 22%, the 10 μM sample reduced by approximately 36%, the 20 μM sample reduced by approximately 38% and the 50 μM sample reduced by approximately 44%. This suggests that EGCG prevents IAPP fibrils from forming and penetrating the cell membrane. Since the fluorescence decreased across increasing concentrations of EGCG, it is unclear whether EGCG would have a maximum reduction potential and at what concentration it would occur.

The EGCG controls did not steadily increase in fluorescence to the extent of the Curcumin controls. However, an interesting trend to note is that the fluorescence increase over the 16 hour period decreased slightly from the first three controls to the 50 μM control. The mechanism behind this observation is not completely clear, but since the decrease is minimal, it is most likely due to random changes in the distribution of EGCG in the sample well.

A)



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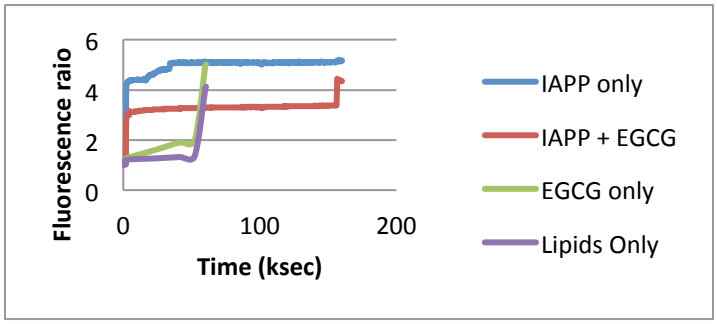

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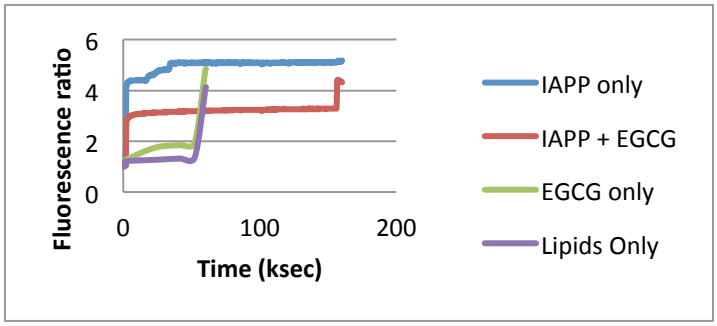

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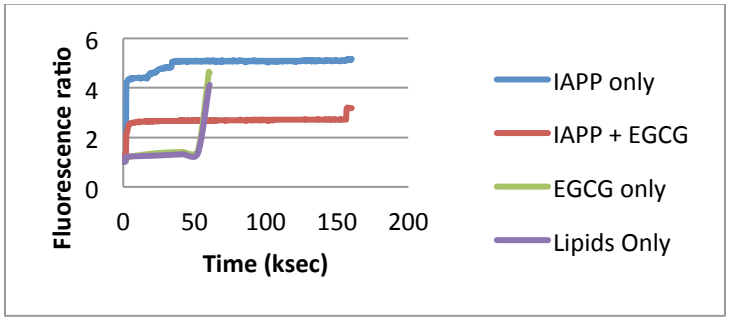
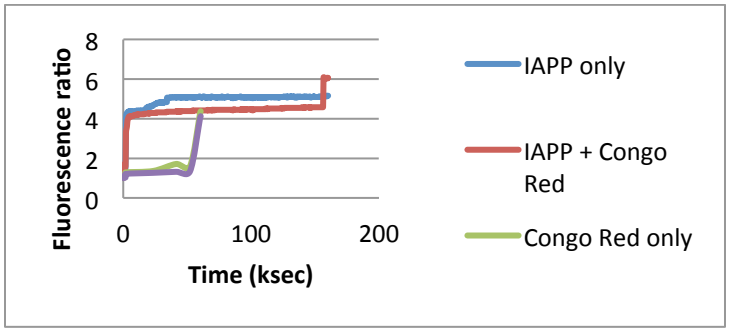


Figure 9. Fluorescence ratio vs time for EGCG at A) 5 µM B) 10 µM C) 20 µM and D) 50 µM

Congo Red

Figures 10 A-D show the plot of the fluorescence ratios versus time for samples containing Congo Red as well as for the controls. It was also observed that all the IAPP + Congo Red samples had less fluorescence upon the insertion of the IAPP peptides than the IAPP control, with the 5 μM sample fluorescence signal reduced by approximately 26%, the 10 μM sample reduced by approximately 36%, the 20 μM sample reduced by approximately 40% and 50 μM both reduced by approximately 64%. This suggests that Congo Red prevents IAPP fibrils from forming and penetrating the cell membrane with a possible maximum reduction potential. Since the fluorescence decreased over all increasing concentrations of Congo Red, it is unclear if there is a maximum reduction potential for Congo Red and at what concentration it would occur. The Congo Red controls had very similar fluorescence profiles to the lipid control which suggests that Congo Red does not have any membrane disrupting properties in isolation.

A)



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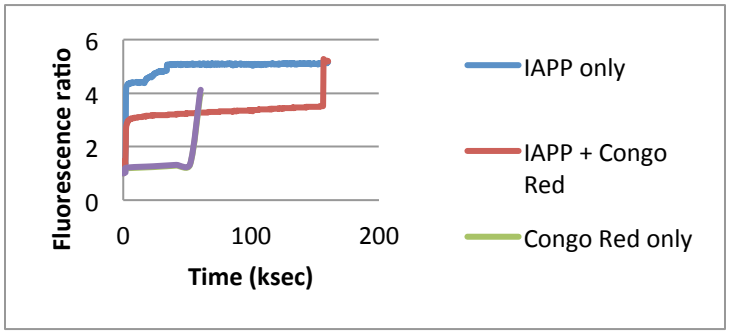

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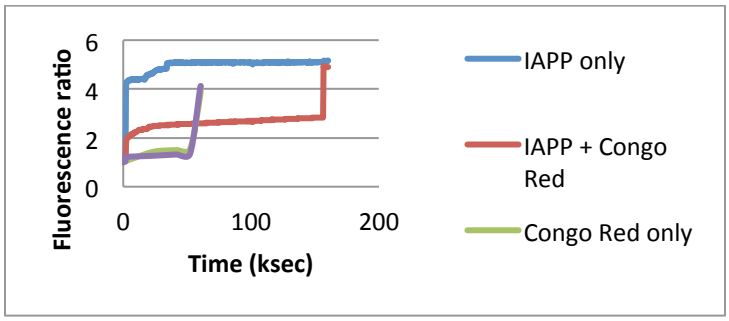

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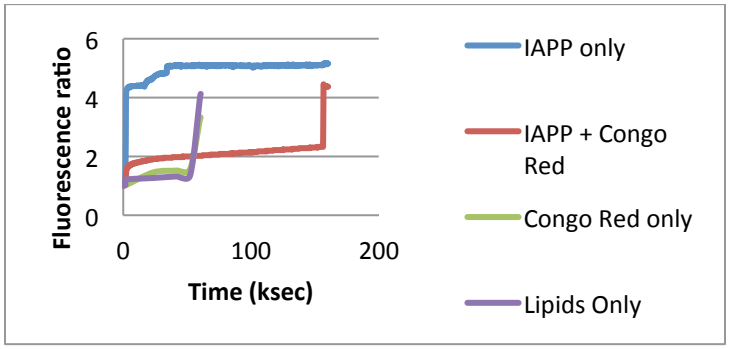


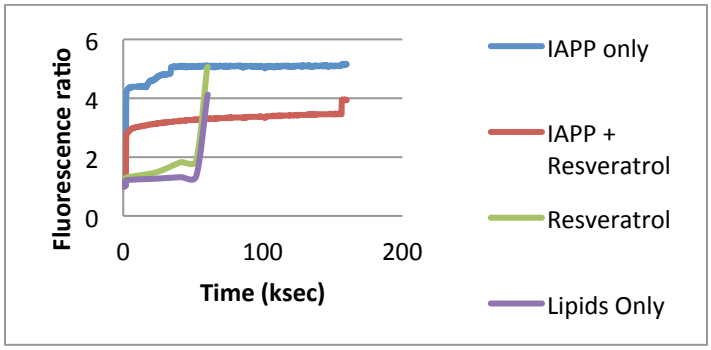
Figure 10. Fluorescence ratio vs time for EGCG at A) 5 µM B) 10 µM C) 20 µM and D) 50 µM

Resveratrol

Figures 11 A-D show the plot of the fluorescence ratios versus time for samples containing Resveratrol as well as for the controls. It was first observed that both the IAPP control and the IAPP + Resveratrol samples had a higher fluorescence signal ratio than the lipid control, which alludes to the formation of IAPP fibrils in both of these samples. It was also observed that all the IAPP + Resveratrol samples had less fluorescence upon the insertion of the IAPP peptides than the IAPP control, with the 5 μM sample fluorescence signal reduced by approximately 30%, the 10 μM sample reduced by approximately 42%, the 20 μM sample reduced by approximately 56% and 50 μM both reduced by approximately 46%. This suggests that Resveratrol prevents IAPP fibrils from forming and penetrating the cell membrane with a possible maximum reduction potential.

It is unclear why the fluorescence ratio of the 50 μM sample increased slightly from the 20 μM sample, but as the increase was small enough to not be considered of great statistical significance. A possible explanation would be premature IAPP fibril formation during the process of preparing the assay before the 30 hour observation period began, thus raising the initial observed fluorescence for this period. The Resveratrol controls had very similar fluorescence profiles to the lipid control which suggests that Resveratrol does not have any membrane disrupting properties in isolation.

A)



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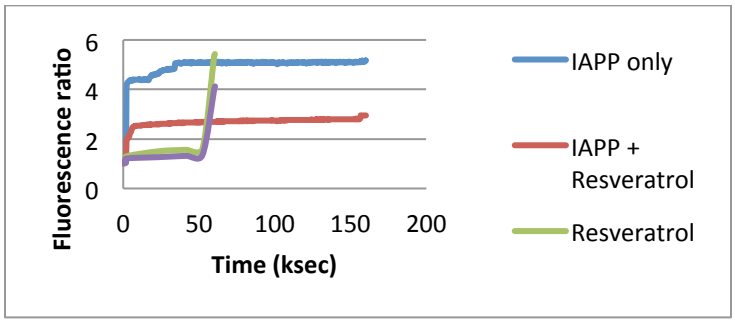

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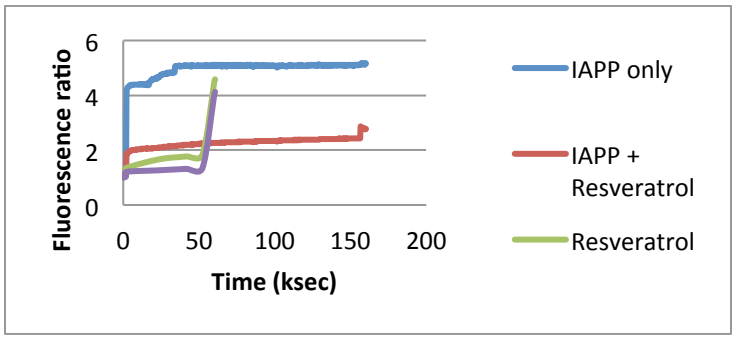


D)

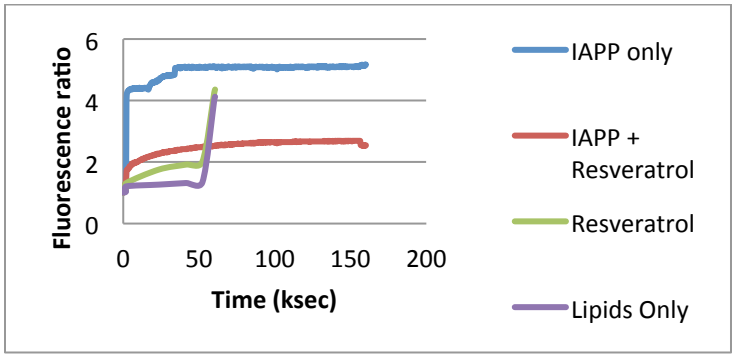


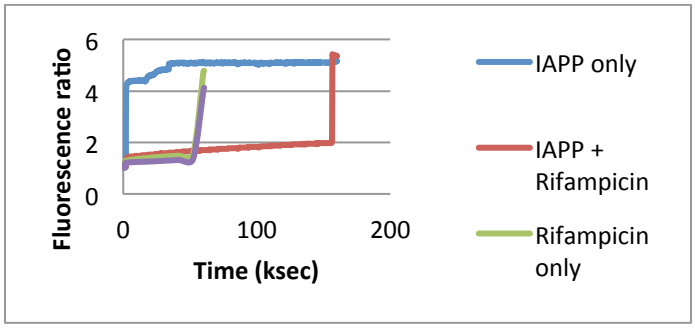
Figure 11. Fluorescence ratio vs time for Resveratrol at A) 5 µM B) 10 µM C) 20 µM and D) 50 µM

Rifampicin

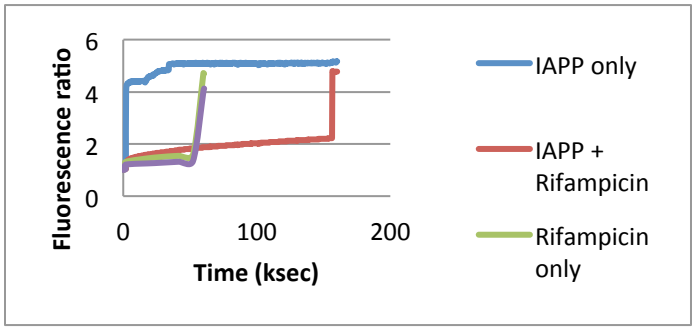
Figures 12 A-D show the plot of the fluorescence ratios versus time for samples containing Rifampicin as well as for the controls. It was first observed that both the IAPP control and the IAPP + Rifampicin samples had a higher fluorescence signal ratio than the lipid control, which alludes to the formation of IAPP fibrils in both of these samples. It was also observed that all the IAPP + Rifampicin samples had less fluorescence upon the insertion of the IAPP peptides than the IAPP control, with the 5 μM sample fluorescence signal reduced by approximately 30%, the 10 μM sample reduced by approximately 42%, the 20 μM sample reduced by approximately 56% and 50 μM both reduced by approximately 46%. This suggests that Rifampicin prevents IAPP fibrils from forming and penetrating the cell membrane with a possible maximum reduction potential.

It is unclear why the fluorescence ratio of the 20 μM and 50 μM samples increased from the 5 μM and 10 μM samples. A possible explanation would be premature IAPP fibril formation during the process of preparing the assay before the 30 hour observation period began, thus raising the initial observed fluorescence for this period. The 2 spikes observed during the 30 hour observation period for the 20 μM sample could also be evidence of fibril formation despite the presence of Rifampicin. Since the increase (almost 100% at the maximum fluorescence before detergent was added) is large enough to be considered of statistical significance, it is unclear if there is a maximum reduction potential for Rifampicin. The Rifampicin control samples had very similar fluorescence profiles to the lipid control which suggests that Rifampicin does not have any membrane disrupting properties in isolation.

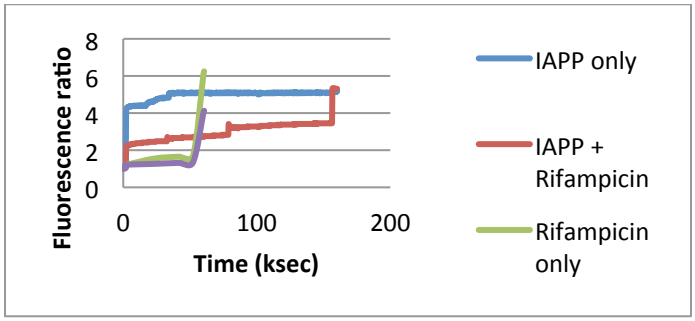
A)



B)



C)



D)

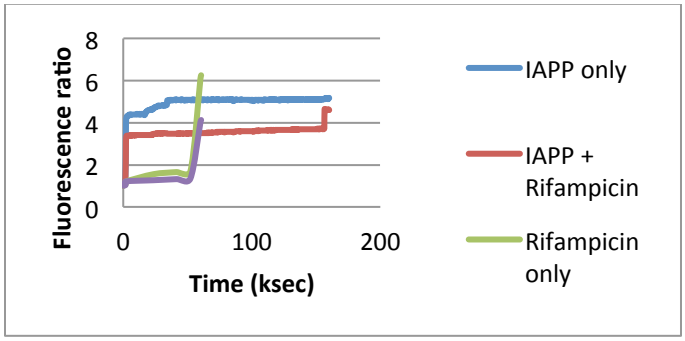


Figure 12. Fluorescence ratio vs time for Rifampicin at A) 5 µM B) 10 µM C) 20 µM and D) 50 µM

Conclusion

Each of the five constituents studied appear to inhibit IAPP fibril formation with varying mechanisms and degrees of effectiveness. As the prevalence of Type II diabetes continues to be an epidemic in this country, it is imperative that the medical research community determine effective methods of treatment and prevention. In theory, these compounds have the potential to help alleviate the processes that lead to development of this disease. However, it is also clear that an inhibition of fibril formation does not necessarily correlate with a decrease in β- cell death and toxicity. Further studies should be taken to investigate the relationship between these compounds and the prevalence of disease. If such a life threatening disease could be significantly prevented with simple changes in diet without the potential additional toxicity of medical intervention, a gradual decline in disease rates for Type II diabetes, heart disease and stroke may be possible in the near future.

References

[1] Jayasinghe, S., & Langen, R. (n.d.). Membrane interaction of islet amyloid polypeptide. (2007). *Biochimica et Biophysica Acta*, *1768*(8), 2002-9.

[2] Porat, Y., Abramowitz, A., & Gazit, E. (n.d.). Inhibition of amyloid fibril formation by polyphenols: Structural similarity and aromatic interactions as a common inhibition mechanism. (2006). *Chem Biol Drug Des*, *67*, 27-37.

[3] Khemtemourianrian, L., Killian, J., Hoppener, J., & Engel, M. (n.d.). Recent insights in islet amyloid polypeptide-induced membrane disruption and its role in β-cell death in type 2 diabetes mellitus. (2008). *Experimental Diabetes Research*, 1-9.

[4] Reinke, A., & Gestwicki, J. (n.d.). Structure–activity relationships of amyloid beta-aggregation inhibitors based on curcumin: Influence of linker length and flexibility. (2007). *Chem Biol Drug Des*, *70*, 206-215.

[5] Meng, F., Abedini, A., Plesner, A., Verchere, C., & Raleigh, D. (n.d.). Article the flavanol (−)-epigallocatechin 3-gallate inhibits amyloid formation by islet amyloid polypeptide, disaggregates amyloid fibrils, and protects cultured cells against iapp-induced toxicity. (2010). *Biochemistry*, *49*(37), 8127-8133.

[6] Lui, G., Gaines, J., Robbins, K., & Lazo, N. (n.d.). Kinetic profile of amyloid formation in the presence of an aromatic inhibitor by nuclear magnetic resonance. (2012). *ACS Med. Chem. Lett.*, *3*(10), 856-859

[7] Milton, N., & Harris, J. (n.d.). Fibril formation and toxicity of the non-amyloidogenic rat amylin peptide. (2013).*Micron*, *44*, 246-253.

[8] Jiang, P., Li, W., Shea, J., & Mu, Y. (n.d.). Resveratrol inhibits the formation of multiple-layered β-sheet oligomers of the human islet amyloid polypeptide segment 22–27. (2011). *Biophysics Journal*,*100*(6), 1550-8.

[9] Meier, J., Kayed, R., Lin, C., Gurlo, T., Haataja, L., Jayasinghe, S., Langen, R., & Glabe, C. (n.d.). Inhibition of human iapp fibril formation does not prevent β-cell death: evidence for distinct actions of oligomers and fibrils of human iapp. (2006).*American Journal of Physiology*, *291*(6), 1317-1324.