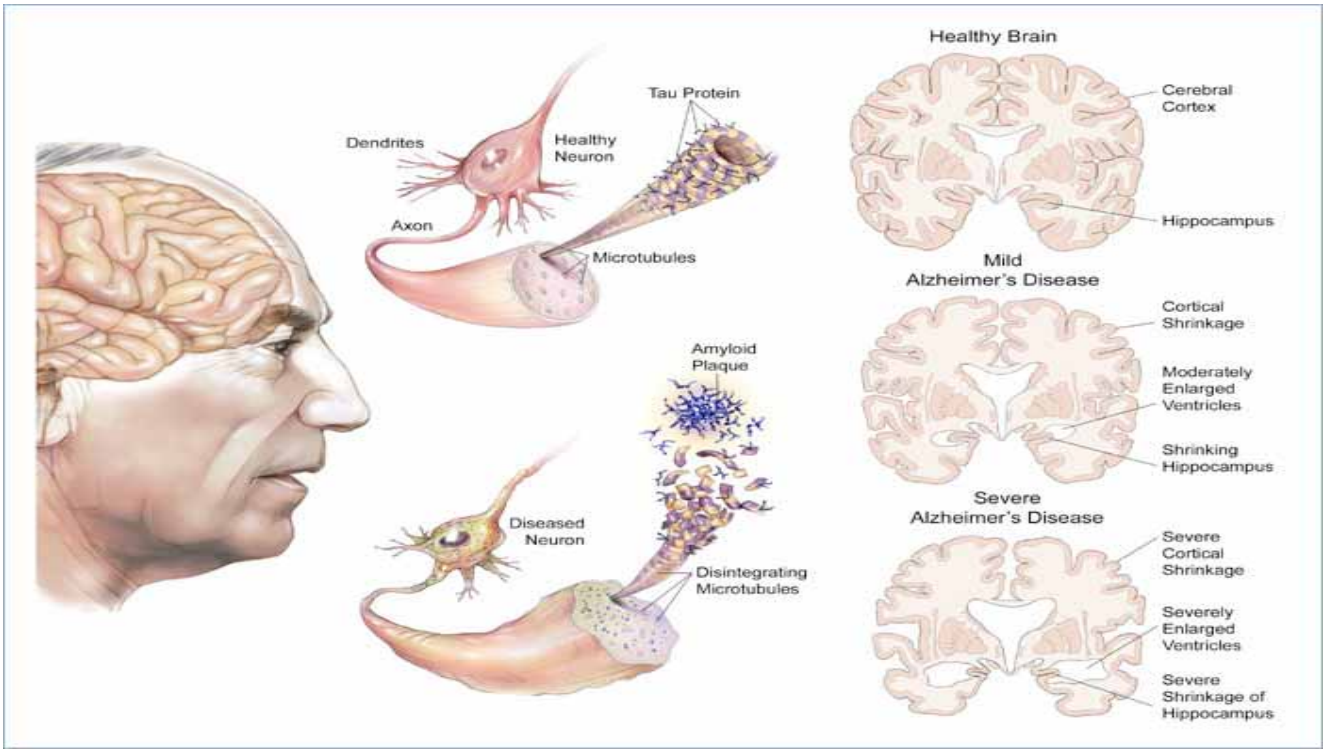
Screening of New Peptides for Neuroprotection Against Alzheimer's Disease



Krystal Xylina Vera

Harlem Children Society

Dr. Sat Bhattacharya

Roswell Park Cancer Institute

Mentor: Dr Thomas Nicotera and Dr Mustapha Bourhim

"*The seat of the soul and the control of voluntary movement - in fact, of nervous functions in general, - are to be sought in the heart. The brain is an organ of minor importance…And of course, the brain is not responsible for any of the sensations at all. The correct view is that the seat and source of sensation is the region of the heart*"

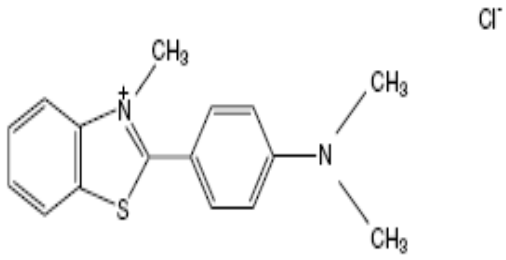
--Aristotle, De motu animalium, 4BCE

Aristotle was, of course, mistaken. It is now known that the brain is, in fact, the control center for the human body. The myriad of voluntary and involuntary functions that your body performs everyday originates in the brain, although the standard physical functions performed by the brain are rarely, if ever, cogitated on by the usual person. These are lower level functions, not really under our control. As such, not much concern is given to afflictions or conditions of the brain that may affect these underlying physical processes. The kind of "event" required to affect the portions of the brain that deal with those types of functions would undoubtedly leave one in the kind of state not prone to pondering ones current condition. Those types of conditions, the result of blunt trauma to the gross structures in the brain, pale in comparison to afflictions of a different sort; afflictions which affect the minute, the seemingly limitless, uncountable chemical interactions among individual neurons and neurotransmitters. The type of condition that afflicts the higher order functions of the brain, things like consciousness, reasoning, and abstraction are the ones that individuals have perhaps come to be most apprehensive of. It is one thing to lose some physical mobility, or some sort of physical attribute, but to lose your ability to think? To reason? Those are, it could be argued, the very things that are required to make us human. Losing control of those abilities, especially a disease whose methods of action are poorly understood, whose risk pool is ill-defined, and whose prognosis is grim to say the least that is the kind of thing that would truly give one pause.

Furthermore, we come to the latest research in the field. New technologies and discoveries in the fields of biology, neurology, and biochemistry have given us valuable insight in the process that Alzheimer's disease affects, the processes by which it effects them, and how if at all these processes could be prevented, reversed or even eliminated There are several approaches to alter the progression of Alzheimer disease but the most rational strategy to treat the disease would involve retarding, halting or even reversing the

process that leads to an increase in production of Aβ. In our laboratory, the strategy developed focuses on the compound that are anti-aggregates and/or antifibrillogenic. It has already shown that Colostrinin (CLN), a mixture of proline rich- polypeptides, prevents aggregation of amyloid peptide (1-40) *in vitro* (D. Schuster et al, 2005). Recently, Bourhim *et al.* 2005 (in press) reported that ThT (Thioflavin T) could be used as a fluorescence reporter to measure the relative amount of β-amyloind aggregation.

Structure of thioflavin T



ThT is known to bind onto β-Sheet containing proteins and to florescence only in the bound configuration. Unbound ThT does not fluoresce. Consequently, it can be used to monitor the effect of drugs in the capacity to ameliorate β - Amyloid aggregation. I will be using this ThT fluorescence method to test several polypeptides derived from CLN for the potential neuroprotective effect. The selected potent peptides will be explored *en vitro*.

Understanding Alzheimer's disease

In figure 1, it has been estimated that there is a neurological disorder that has affected about 4.5 million elderly individuals. This known disorder is called Alzheimer disease, which is a progressive brain disorder that gradually destroys an individual's memory and thought process such as learning, reasoning and making judgment. Alzheimer's disease can initially start from forgetting where you last place your keys to forgetting whom your close relatives and who they are. Forgetting a lifetime memory can also ruin a person's identity. Alzheimer is mostly affected in later years of life like around the age of 60. It is extremely rare for a younger generation to have Alzheimer disease. As an individual continue to age, Alzheimer disease increases and becomes more severe.

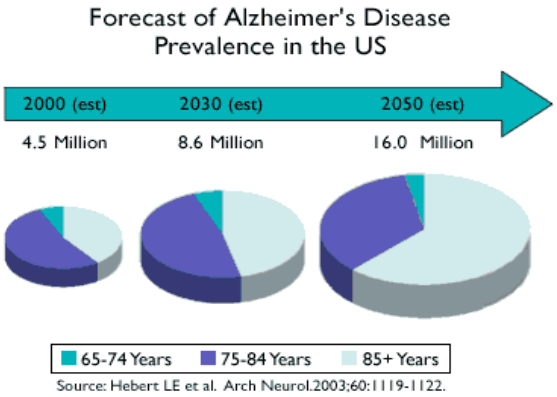


Figure 1: Shows how Alzheimer's disease is rapidly progressing due to aging. In the year 2000, it has been estimated that 4.5 million individual in the age group from 65-74 years old has been affected.

There are three stages to Alzheimer's disease. According to figure 2, it shows the mild stage, the moderate stage and the severe stage. Within each of these stages, there are symptoms that may occur and become visible. These symptoms were determined by an examination called Mini Mental State Examination (MMSE). This examination is used in diagnosis in all three stages. An individual in the mild stage, states "they are actually having trouble making sense of the world around them"

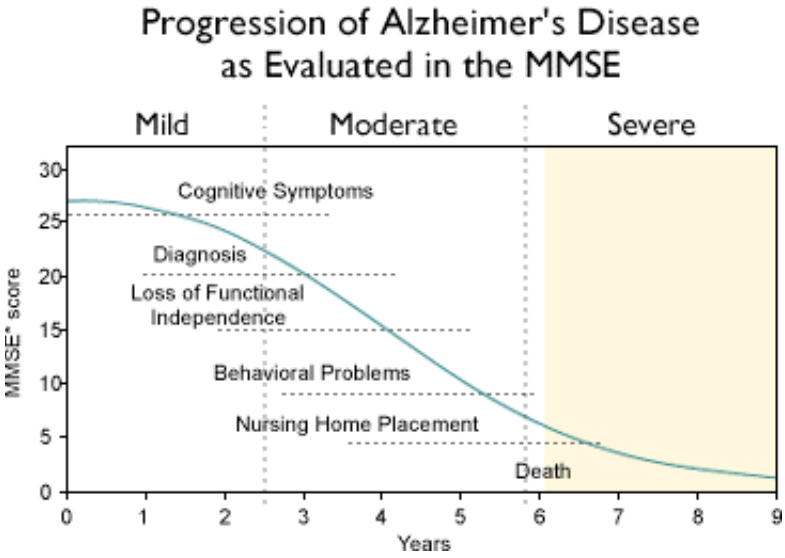


Figure 2: The graph below show's that there are three stages to Alzheimer's disease. There is a mild, moderate and a severe stage. Each stage show certain symptoms that can take place in relation to the number of years an individual would go through if they were to have Alzheimer disease

(Alzheimers Online). In other words, thing that are performed on a daily basis is gradually being forgotten.

To be considered in the moderate stage means that the individual cognitive process has worsen through time. Also," signs and symptoms become more pronounced and behavioral problems can occur" (Alzheimers Online). This occurs within 2.5-6 years. After a 6 year duration, if an individual continues with Alzheimer's disease, it may come down to a point where its severe. This process is known in the severe stage. The severe stage is when "all sense of self seems to vanish" (Alzheimers Online). In other words, their brain cells, such as the neurons, have been damaged and their senses have completely disappeared. This is also the stage where the individual needs to be cared for because they can come close to death.

What exactly happens to cause Alzheimer's disease?

Alzheimer's disease is a build up of amyloid plaques (a general term for protein fragments that are normally produced in the body). My study in the lab is testing new peptides that can break down this amyloid-beta which "is a fragments of a protein snipped from another protein called amyloid precursor protein (APP)". Because of the accumulation or aggregation of Amyloid beta (Aβ), this is what causes the Alzheimer's disease. Because of the aggregation of Aβ, this can cause harm to the neuron cells

Overview of the Experiment

Aβ can be produced *in vitro*. The Amyloid-β(1-40) was obtained from a company called Biosource International Inc. It was given in a quantity of 1 mg of Ultra pure form of Aβ{1-40) with a molecular weight of 4331. To create the β-Sheets, I obtained a micro-centrifuge tube and pipette 1mL of base (100mM NaOH) into the tube. Also, pipette 1μL of Aβ into the tube and vortex the solution. This solution is known as the Aβ aggregation, also known as the β-Sheets that causes Alzheimer's disease. In my lab, we were trying to find "useful means to study mechanisms of β-amyloid-mediated cell toxicity and to devise strategies for protection by reducing the β-amyloid-load either through prevention of fibril formation or by turning over existing deposits (LeVine, 1993)" (Bourhim *et al.*

2005 (in press)). One common technique that is used to monitor the protein fibrils is the Thioflavin T (ThT) fluorescent. We want to experiment that ThT is responding correctly.It is known that, "ThT fluorescence is not one of the 'defining' criteria for amyloid fibrils, it is relatively well accepted as an indicator of the presence of amyloid fibrils" (Nillson, 2004). ThT is responsible to bind on to the β-sheet. After we prove that such thing is happening then we can continue on to testing the neuropeptides and see if these peptides are breaking the β-sheets apart and avoid from getting Alzheimer's disease.

Pricedure:

The Tris solution and the ThT have to be prepared. To do so, it was as follow:

Preparing Tris

50mM Tris ----------→ 100mM Tris

The question that I ask myself is how many grams of Tris Hydrochloride I need when working with 100mM of Tris. The formula I used is:

Molarity = moles/ volume

We have Molarity and volume, so I will be solving for moles. The formula is written as:

Moles = Molarity x moles.

Mole is also known as grams/ Formula weight. We are trying to then figure out how many grams of the Tris Hydrochloride should I put into my solution. So now the formula is rewritten as:

Grams = Molarity x Volume x Formula Weight

With the formula given, I can now plug in what I have so far. Molarity equals 100mM; I want to make 500mL of my Tris solution, so my volume equals 500mL, and the Formula Weight of the Tris Hydrochloride equals 157.60g.The formula is now as follow:

$$\text{Grams} = 500\text{mL} \times 100\text{mM} \times 157.60$$

To answer my problem, I need to make sure my units are all the same so that when solving, I'm able to cancel out the units to be left with only grams. So after all the conversions were made, my equation is now as follow:

$$\text{Grams} = 0.5\text{L} \times 0.1^{\text{g/fw}}/\text{L} \times 157.60$$

Formula weight and liters are canceled which only leaves behind the grams, which is exactly what we are solving for.

$$\text{Grams} = 0.5 \times 0.1 \times 157.60$$

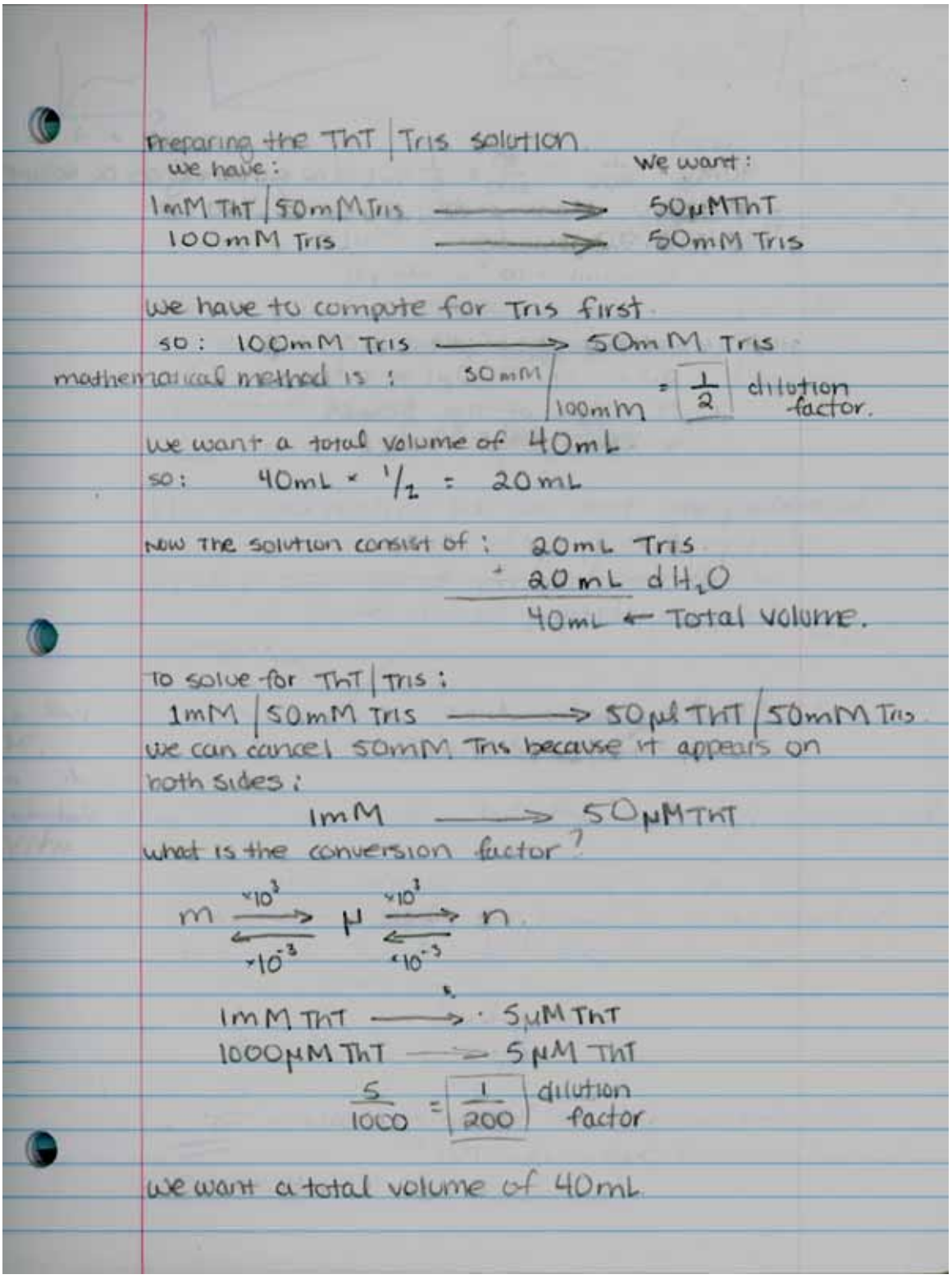
The answer to the above problem is:

$$\text{Grams} = 7.88\text{g}$$

The Tris Hydrochloride was measured with a balance scale. It was then put into a jar that only had 450mL out of 500mL of distilled water (ddH_2O). The reason there was only 450mL of water is so that the pH level of the Tris solution can me be tested. I wanted my pH level to be around 7.4. After the pH level was taken, I added the rest of the distilled water to measure up 500mL.

** Note** - to reduce acid, add base buffer (naOH). To reduce base, add acid buffer (HCl).

PreparingThT/Tris



Preparing the ThT | Tris solution

we have: We want:

1mM ThT | 50mM Tris ⟶ 50μM ThT

100mM Tris ⟶ 50mM Tris

we have to compute for Tris first

so: 100mM Tris ⟶ 50mM Tris

mathematical method is: 50mM / 100mM = 1/2 dilution factor.

we want a total volume of 40mL

so: 40mL × 1/2 = 20mL

now the solution consist of: 20mL Tris

+ 20mL dH_2O

40mL ← Total volume.

to solve for ThT | Tris:

1mM | 50mM Tris ⟶ 50μl ThT | 50mM Tris

we can cancel 50mM Tris because it appears on both sides:

1mM ⟶ 50μM ThT

what is the conversion factor?

m $\xrightarrow{\times 10^3}$ μ $\xrightarrow{\times 10^3}$ n

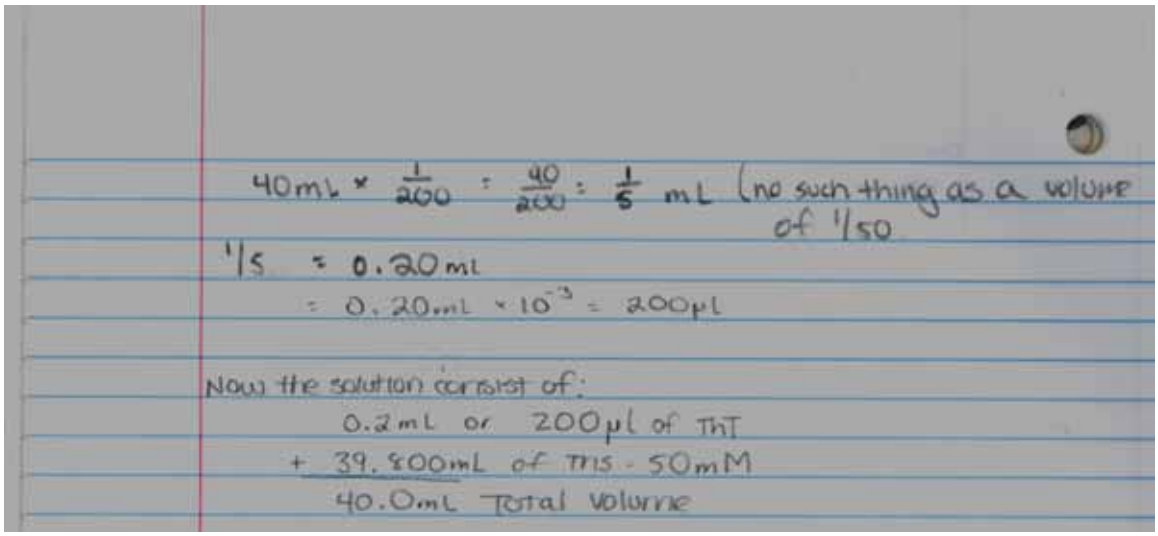
m $\xleftarrow{\times 10^{-3}}$ μ $\xleftarrow{\times 10^{-3}}$ n

1mM ThT ⟶ 5μM ThT

1000μM ThT ⟶ 5μM ThT

5/1000 = 1/200 dilution factor

we want a total volume of 40mL



Method:

The following chart was used to create the samples. We will then read the Fluorescence Intensity to each triplicate.

Aβ in µg	0µg	10µg	20µg	30µg	40µg	50µg
Solution of Aβ 1ml/mg	0µl	2µl	4µl	6µl	8µl	10µl
50mM Tris	10µl	8µl	6µl	4µl	2µl	0µl
5µM ThT / 50mM Tris	90µl	90µl	90µl	90µl	90µl	90µl

Results:

		200ng	400ng	600ng	800ng	1000ng
	1	2	3	4	5	6
A	151.298	311.388	188.596	312.831	368.216	550.138
B	174.486	337.688	285.094	362.258	417.287	477.334
C	109.576	279.613	271.852	479.627	451.143	428.973
D	294.014	306.118	290.125	393.738	414.35	537.852
E	229.448	366.803	508.348	463.771	563.917	565.701
F	375.465	384.718	314.501	451.349	490.72	445.009

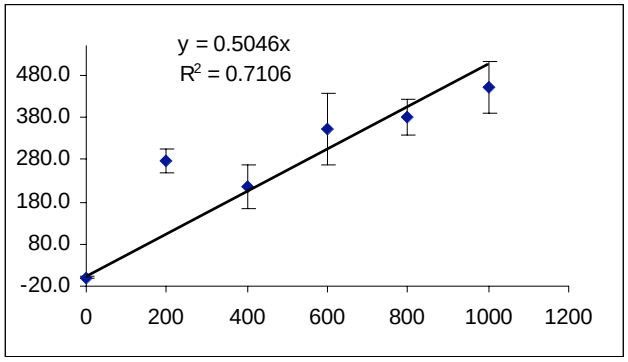
The results above are read according to the wells they were in respectively. So for an example, if you look into a 96 wells plate you will see that in wells 1A, the computer read 151.298mV. In well 1B, the computer read 174.486mV and so on. According to our results, our highlighted area (ThT alone) is too high above normal. It is only a buffer, which means that the measurement readings should be estimated around 15-55mV. Whenever I had high readings, I predicted that there was some background that should not be there. For my purpose, I added additional ThT control into wells G1-G6 and H1-H6 and read its results. With additional readings to the ThT, my absorbance came out to be more reliable according to my results below. .

		200ng	400ng	600ng	800ng	1000ng
	1	2	3	4	5	6
A	151.298	311.388	188.596	312.831	368.216	550.138
B	174.486	337.688	285.094	362.258	417.287	477.334
C	109.576	279.613	271.852	479.627	451.143	428.973
D	294.014	306.118	290.125	393.738	414.35	537.852
E	229.448	366.803	508.348	463.771	563.917	565.701
F	375.465	384.718	314.501	451.349	490.72	445.009
G	31.715	33.662	31.436	39.746	33.822	252.628
H	26.893	32.328	33.377	28.613	33.457	41.83

From the chart above, everything was then organized unto nano-grams (ng). So, all the readings within 0ng where placed in one row, all the readings for the 200ng were place in another row and so on. The reason everything was organized in such a way because the experiment was done in triplicates incase if one of the readings went wrong. Also, it can be easily seen when finding the average and its standard deviation respectively. Average – Blank was an indicator to use to subtract from each of the average readings. I did not want the absorbance reading for the ThT, but only for Aβ itself. So from the following chart, the average for ThT is 32.5. We take that 32.5 and subtract it to itself because it was known as a control and there was no Aβ in it so our Fluorescence Intensity should equal to 0. For the following readings, the average

0ng	31.715	33.662	31.436	39.746	33.822	26.893	32.328	33.377	28.613	33.457
200ng	311.388	337.688	279.613							
400ng	188.596	285.094	271.852							
600ng	312.831	362.258	479.627							
800ng	368.216	417.287	451.143							
1000ng	550.138	477.334	428.973							

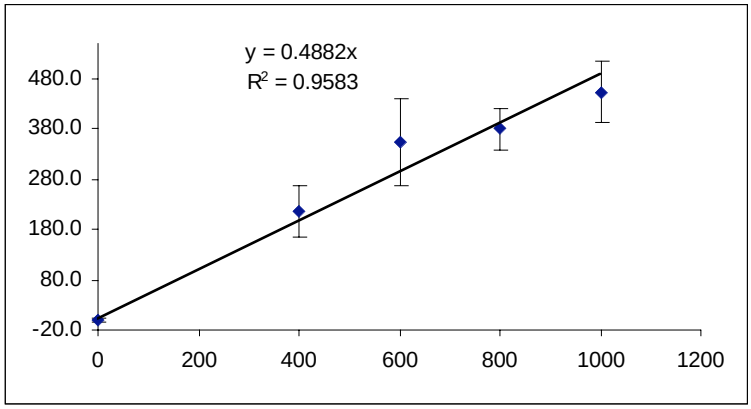
Standard Line for AB- Sheets



Average	StDev	Average - blank
32.5	3.4	0.0
309.6	29.1	277.1
248.5	52.3	216.0
384.9	85.7	352.4
412.2	41.7	379.7
485.5	61.0	453.0

Everything in the "Average – blank was graphed from 200ng – 1000ng with an exception of the 200ng because it throws off our R square value. In the graph above we want our values to be closer to our slope line so that out R-square value can be closer to one. In the graph above, out R square value is known to be 0.7106, which is not the greatest because it is not closer to one. We want to get as far close as possible. I want the R- square value to be closer to 1 as much as possible.

Standard Line for AB- Sheets



I am trying to test new peptides that can decrease and eliminate the β-Sheets and avoid any harm to the neurons. In past studies, it has been hypothesized that CLN is a beta-sheet breaker. My objective is to determine "if CLN can: (1) prevent aggregation of Aβ- peptides (i.e., prophylactic effect) and (2) disrupt existing Aβ Aggregation (i.e. therapeutic effect)" (D. Schuster et al, 2005). For my next experiment, I will use CLN as a positive control because it is known to decrease the aggregation of Aβ; and other peptides that derives from it, such as: TS1, TS2, and TS3. As I am experimenting, I want to keep in mind that I am taking readings for four days including the day I start my experiment follow by three consecutive days. I want to observe the following peptides and see if I observe any decrease of Aβ aggregation in Alzheimer's disease.

Procedure

The following chart is the method used in preparing the samples in a 96 well plate. Also, these samples were done in triplicates. When getting our results, I will average out my reading to get precise fluorescence intensity to each peptide.

	0 nM	2.5 nM	5nM	7.5 nM	10 nM
Aβ- (100ng/ul)	5µl	5µl	5µl	5µl	5µl
Peptides (10nM)	0µl	2.5µl	5µl	7.5µl	10µl
ThT/ Tris	95µl	92.5µl	90µl	87.5µl	85µl

The following table is an example of ho I receive my absorbance readings. I will continue this for the next 3 days and putting it onto a "column graph" which will then compare the peptides over the three day period and see if there was a decrease to the A aggregation.

Day 0 Reading

Day 0 Reading											
1	2	3	4	5	6	7	8	9	10	11	12
214.596	513.978	288.941	398.306	403.145	430.659	397.432	513.978	511.243	498.853	280.296	388.523
286.669	561.844	418.456	438.512	492.259	492.435	375.817	561.844	412.588	443.067	418.764	386.573
169.962	586.449	374.839	341.111	455.673	525.466	602.47	586.449	301.027	409.726	347.417	308.163
210.594	349.222	449.138	455.117	501.086	579.623	456.34	76.671	481.314	649.609	567.98	108.79
215.153	358.545	524.488	316.611	517.718	542.006	408.489	514.762	621.295	544.972	588.441	447.136
288.004	396.969	454.942	454.034	466.229	455.591	459.539	500.316	598.851	560.095	514.528	428.386
79.403	98.612	88.911	89.58	33.912	363.867	229.945	129.006	294.835	271.481	301.115	32.879
117.912	84.858	180.295	212.208	388.984	37.412	108.201	41.436	94.7	31.538	28.712	#Sat

After the three-day period of readings, I put everything onto a column graph and it showed a decrease. The following graphs are as follow to each peptide

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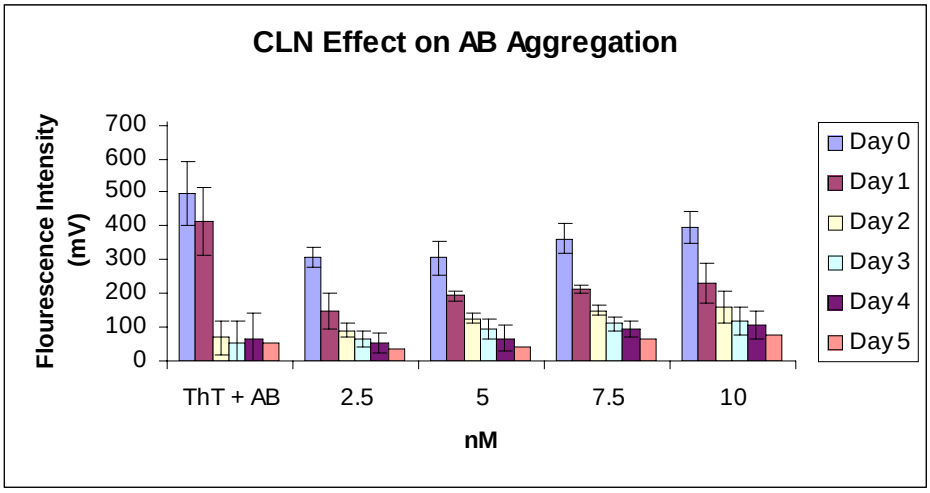

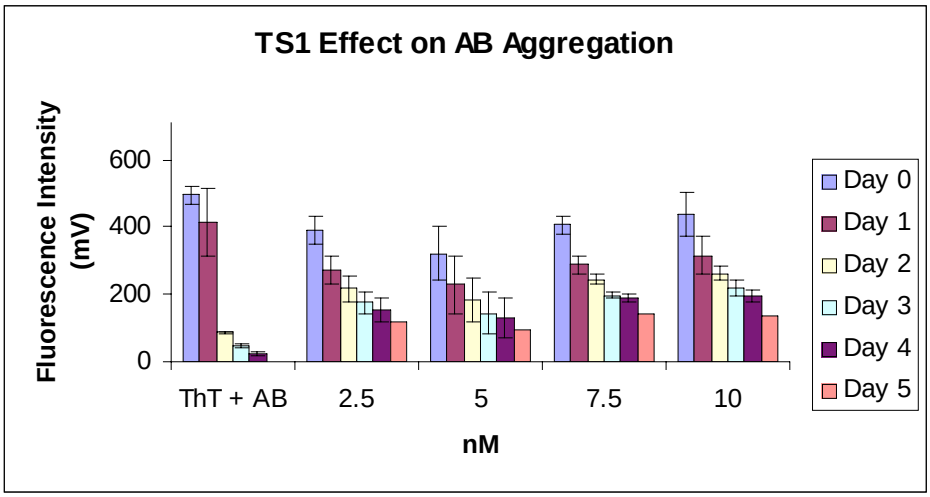

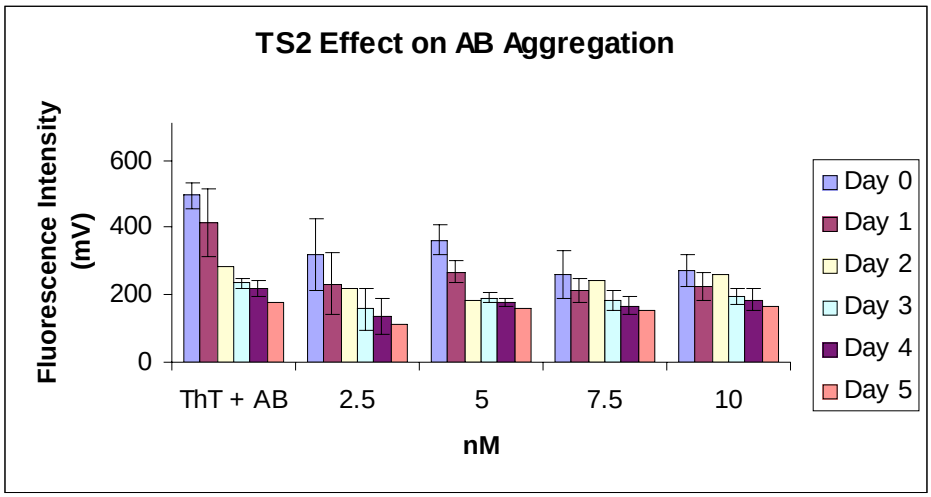

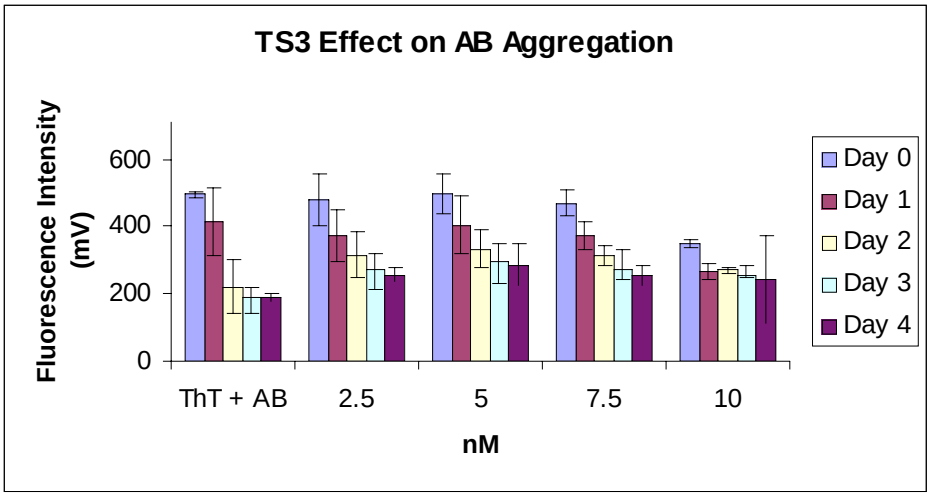

Results

The graphs that are displayed above tell us that there has been a decrease but it is not the best results that we expected. If you see the ThT + Aβ, this is known to be our controls. As a control, there should not be much of a decrease, in other words, their readings for each day should have roughly have been the same especially since we did not add any peptides to it. This is an experiment that I will have to be repeated in the nearer future and figure out exactly what is making my control decrease by so much. My peptides are seasonable and it supports my hypothesis but not when our controls are also doing the same thing. Something must have gone wrong wit our control and that is what I need to analyze more strongly on.

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Personal Experience

Since the summer of 2006, I have been working at Roswell Park Cancer Institute, in Buffalo, NY. It was a difficult process for me to land an intern in Buffalo because not many places take undergraduate students, without any recommendations. Most institutes look for graduate students to perform laboratory work. I was one of the lucky ones to have to find an intern at Roswell Park Cancer Institute. Dr Sat Bhattacharya and I were searching for institutes that were willing to work with an undergraduate student. Because of Dr. Bhattacharya's intelligence and remarkable effort, he was able to consult with Craig Johnson who is the Director of Graduate Admissions and Summer Research Programs. Mr. Craig Johnson was more than willing to help me find someone who would be more than happy to take me into his or her laboratory. I then was transferred over to my supervisor Dr. Thomas Nicotera who works in the Department of Cancer Chemo prevention.

Dr. Thomas Nicotera, and Dr. Mustapha Bourhim, were mentoring me around the laboratory and introduced me to the project on "Screening New Peptides Neuro-protection Against Alzheimer's Disease". With a very short period of time of my summer remaining, I did not think a lot can be accomplished especially while I was in school. Scheduling to go to work was a problem for me because I had to find a way to squeeze it into my schedule between classes. It was too much to go through especially with public transportation. I took me an hour to get to work and hour to get back onto campus. But everything came out for the best and Mr. Craig Johnson heard of my success throughout the lab that he considered an offer for me to continue working for the lab. I was please and confused all at the same time because I would not know how to manage my time as an employee and as a student. It was given a lot of thought but I was comfortable with where I am working and also comfortable being in the lab that I took the position as a "Research Apprentice". I am so excited that I can hold this position and progress through my research.