Engineering Fasciculin-II to treat Alzheimer's Disease Related Symptoms

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Abstract

Alzheimer's disease is characterized by death of cholinergic neurons, which secrete the neurotransmitter acetylcholine. This results in a loss of the total amount of acetylcholine circulating in neural networks. Modern drugs target acetylcholinesterase so as to inhibit its function, allowing for increased transmission of acetylcholine in cholinergic synapses. The aim is that an increase in cholinergic activity leads to an increase in cognition. However, these drugs only bind temporarily and have consequential problems that make modern AChE inhibitors of little effect.

The main goal of our project is to engineer a snake toxin, fasciculin-II, capable of crossing the blood brain barrier and inhibiting acetylcholinesterase with enough affinity that it increases cholinergic transmission without yielding toxic outcomes. This fasciculin binds to the peripheral acetylcholinesterase found in neuromuscular junctions and causes rapid twitching. Fasciculin-II binds to acetylcholinesterase very tightly with the use of many charged residues and hydrophobic residues and we would like to optimize this interaction so that it may serve a therapeutic purpose in patients of AD.

1 Introduction

Alzheimer's (AD) is the 6th leading cause of death in the United States[1]. Researchers are still struggling the understand more about the disease's pathology and there are many theories about the onset of AD. But we do know that AD is a common type of dementia with symptoms such as cognitive degeneration, memory loss, impaired judgement, altered behavior and personality change. It is a progressive disease that begins with forgetfulness that can be commonly dismissed, but it can slowly impair the patient's self efficiency and eventually leads to death. There is no cure for the disease yet, but symptoms can be controlled and deterioration can be slowed but the quality of life will be greatly affected.

Researchers have observed that cholinergic neuronal death is common among the affected, which results in decreased production of acetylcholine, a neurotransmitter[2]. In healthy individuals, neurons relay signal to muscles cells at the synaptic cleft with the help of acetylcholine (Figure 1). Once the signal is passed, the neurotransmitter must be degraded in order for the next signal to be relayed correctly. In AD patients, the amounts of acetylcholine circulating the neural networks are low. Without acetylcholine, neurons of an

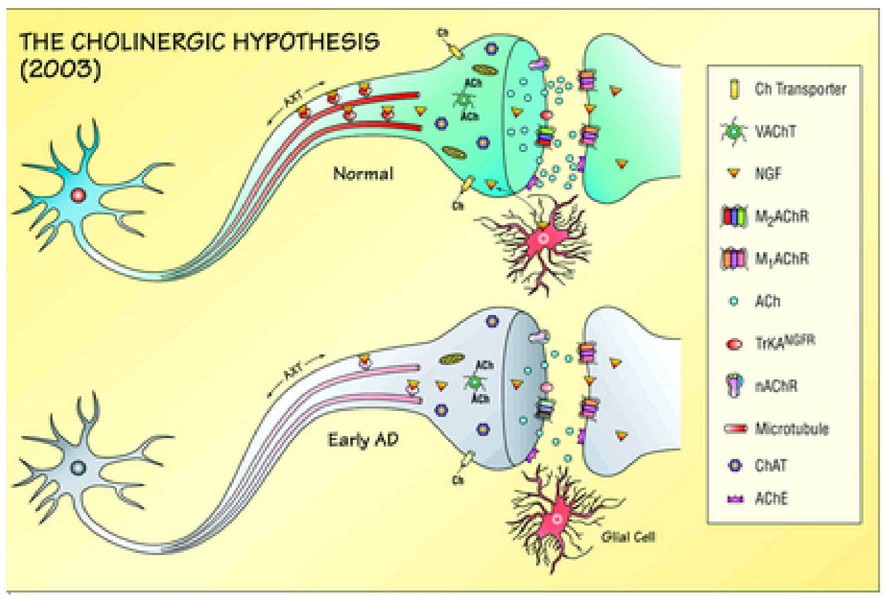

Figure 1: Cholinergic hypothesis between normal and AD patients[3].

AD patient will slowly lose their ability to function because neurons cannot communicate with muscle cells[2]. Therefore, raising the levels of acetylcholine is the target of current AD treatments.

One way to raise the level of acetylcholine is to inhibit a protein named acetylcholinesterase (AChE). It is a hydrolase that degrades acetylcholine at the neuron and muscle synapses and is mainly found in synaptic cleft. From the Protein Data Bank (PDB) protein structure of AChE, it has an deep pocket active site only big enough for acetylcholine to enter [4]. AChE is highly efficient and destroys 25,000 acetylcholine molecules per second[3]. This makes a good target to raise the level of acetylcholine and strengthen the signals at the synapses. Many drugs used to inhibit acetylcholinesterase in AD patients are derivatives of toxins and poisons. Inhibiting AChE is potentially dangerous; drugs are given in titration phase until desired results for tolerability and safety reasons are achieved[5]; this makes it difficult for patient care. They can also cause adverse side effects because they affect both the peripheral AChE and central nervous system esterases. Moreover, these drugs only bind temporarily to AChE and have consequential problems that make modern AChE inhibitors have little effect. Our project addresses these problems with a potential drug that binds more specifically and irreversibly.

The main goal of our project is to engineer a small peptide similar to a snake toxin, fasciculin-II, capable of crossing the blood brain barrier and inhibiting acetylcholinesterase with enough affinity that it increases cholinergic transmission without yielding toxic outcomes. Fasciculin binds to the peripheral acetylcholinesterase found in neuromuscular junctions and causes rapid twitching[8]. Fasciculin-II binds to acetylcholinesterase very tightly with the use of many charged residues and hydrophobic residues and we would like to optimize this interaction so that it may serve a therapeutic purpose in patients of AD.

2 Experimental Approach

2.1 Introduction

We chose to perform both comparative and rational design due to the large library of available sequence and protein structures of acetylcholinesterase and fasciculin-II. The goal of the mutations we plan on performing through comparative design is to make the most lipid-soluble and stable protein possible as that may increase its chances in entering the blood brain barrier and remain in the body. For rational design, we hope to decrease the affinity that fasciculin-II has for acetylcholinesterase so as to not increase the toxicity of acetylcholine in the central nervous system.

2.2 Comparative Design

The first step was to collect the protein sequence of Fasciculin-II (Fas-II) from UniProt (P0C1Z0), identify related sequences using blastp, and collect all sequences with a sequence similarity greater than 40%. A multiple sequence alignment (MSA) was conducted directly on the BLAST results page. Figure 2 shows the MSA as seen when the BLAST alignment was inputted into ESPRIPT.

The mutations we wanted to make were those that increased the lipid-solubility of the protein while increasing the stability of the protein. By increasing the lipid-solubility, we increase the chances of it permeating through the blood-brain barrier. We also increase its chance of being absorbed by neurons. By increasing its stability, we increase its turnover rate and its half-life. Ideally, we want to improve standard patient care by decreasing the number of doctor visits, and if our engineered Fas-II exhibits higher stability, it will circulate in the brain for longer and inhibit AChE function longer.

Four mutations were made to increase stability: A12K, I13T, G18E, and K32G. These were chosen by mutating residues in the Fas-II sequence that differed greatly from the majority of its homologs. Three mutations were made to increase lipid solubiity (increase number of nonpolar residues): T1I and N47G. K32G is also useful for increasing lipid solubility, as well as stability. Figure 2 shows the location of the mutations made through comparative design. Figure 2 also shows regions of conservation and different secondatry structures. Fasciculin-II is composed entirely of βstrands and loops.

Because the crystal structure of Fas-II complexed with human acetylcholinesterase is available (PDB ID: 1B41), we looked at how these six mutations may actually look like [9]. The first mutation we looked at was the A12K mutation that would increase stability. This mutation can be seen in Figure 3a-b. We believe this mutation would increase stability because the lysine is conserved in many of Fas-II's homologs. However, this mutations would cause steric clash between Fas-II and AChE. This may actually assist in our goal to decrease affinity of Fas-II for AChE while also increasing stability.

The next mutation we looked at was the I13T which would also increase stability for the same reason stated for the previously discussed mutation. This mutation can be seen in Figure 4a-b. When mutated, Thr actually increases the number of H-bonds that this position makes from 0 to 2. Ile originally did not make any interactions, but when we mutated it to Thr, 2 H-bonds were formed between the hdyroxyl of Thr and the backbone and side chain

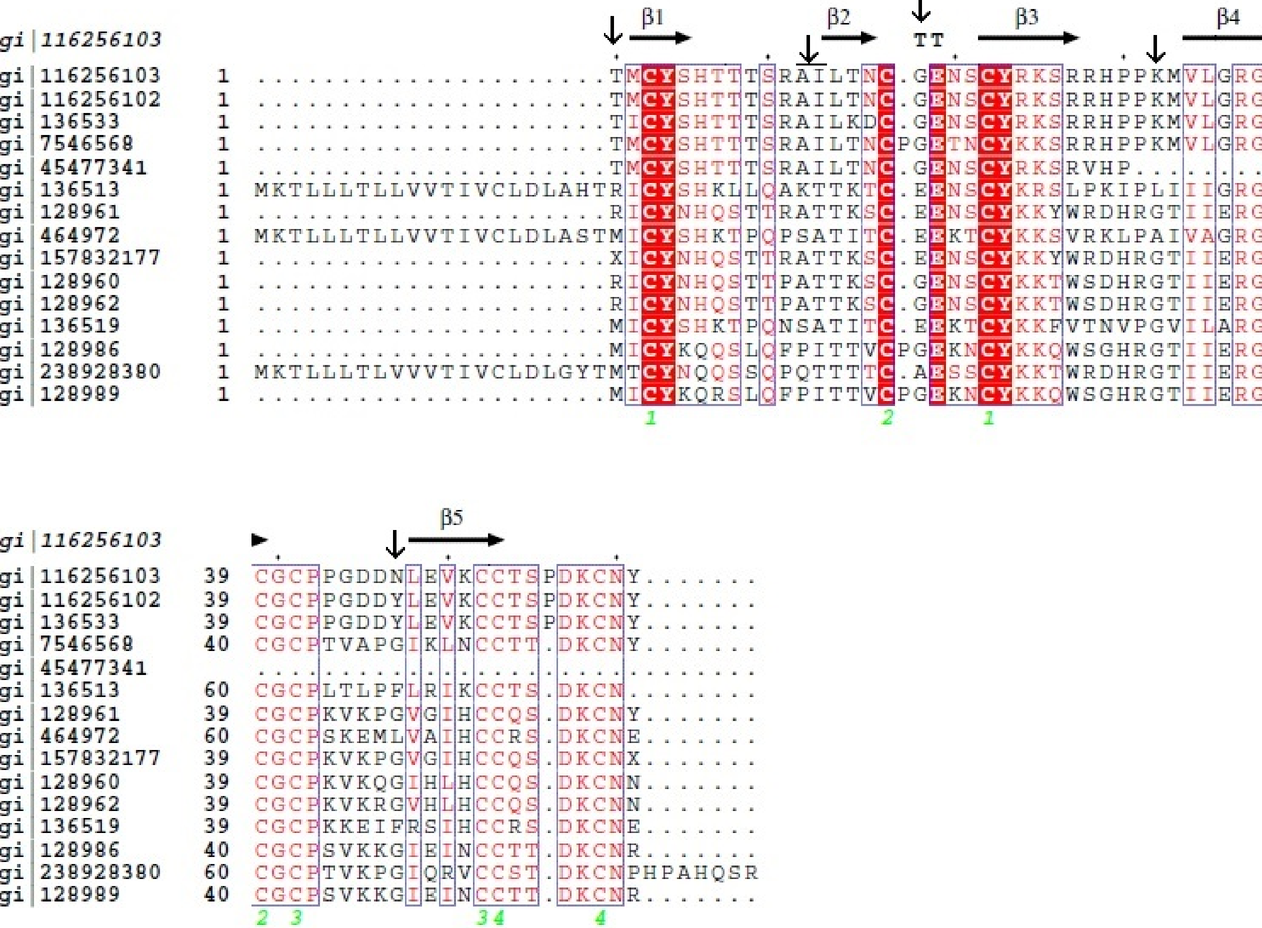

Figure 2: Figure generated with ESPRIPT. This figure shows a multiple sequence alignment of between Fas-II and its homologs. We first used blastp with the protein sequence of Fas-II, selected all sequences with greater than 40% similarity, ran an alignment on the BLAST server, and downloaded the ALN file. This ALN file was uploaded onto the ESPRIPT server and this image was outputted. The arrows above were added afterwards. Some arrows highlight two consecutive residues that are considered for mutation; these have a line above them. There are 6 total mutations: A12K, I13T, G18E, K32G, T1I, and N47G.

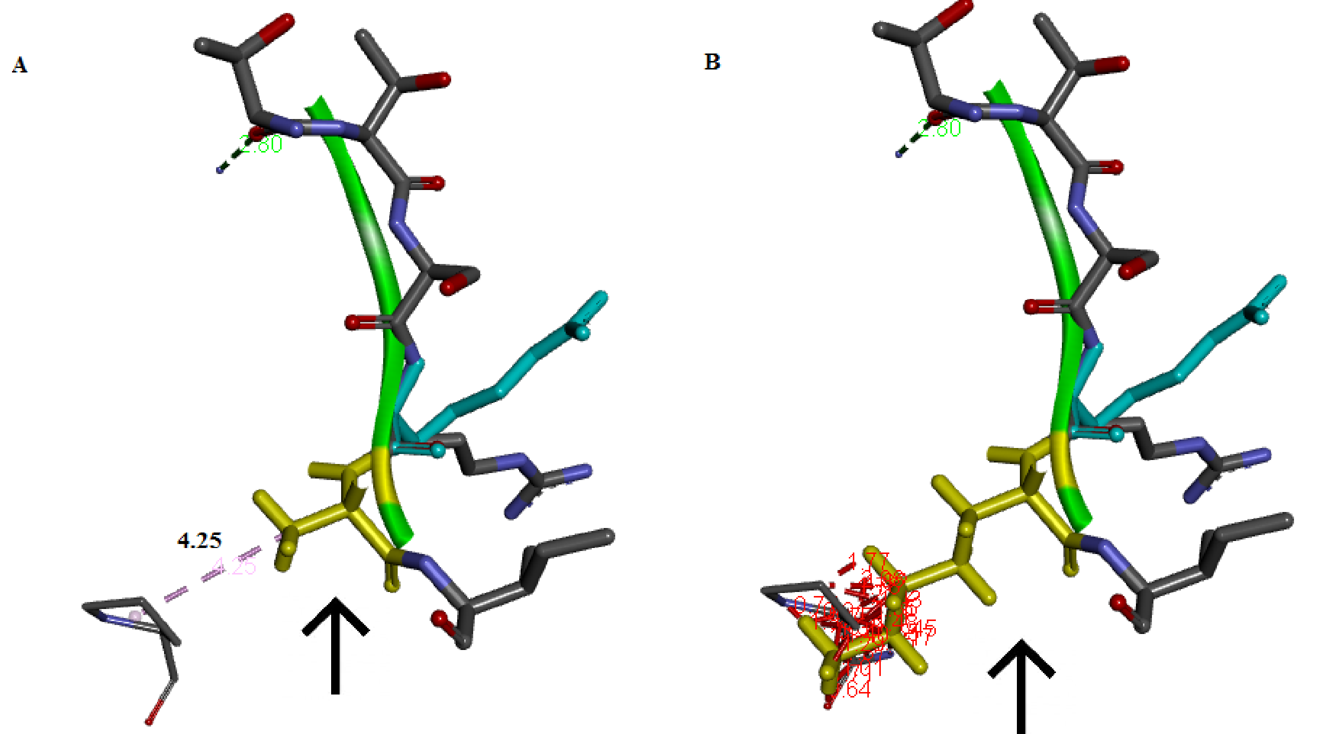

Figure 3: A) The black arrow points to a highlighted yellow residue, representing the alanine residue before it is mutated. This alanine makes a pi-interaction with a residue on AChE. B) When alanine is mutated to lysine, it causes steric clash. This mutation can be located by the black arrow, which points to the mutated lysine. Because of all of the steric clash, this mutation may decrease affinity for AChE, which is additionally a main end goal of our engineering project. The cluster of red dashes indicates the aforementioned steric clash and also exhibit distances less than 1 Angstrom.

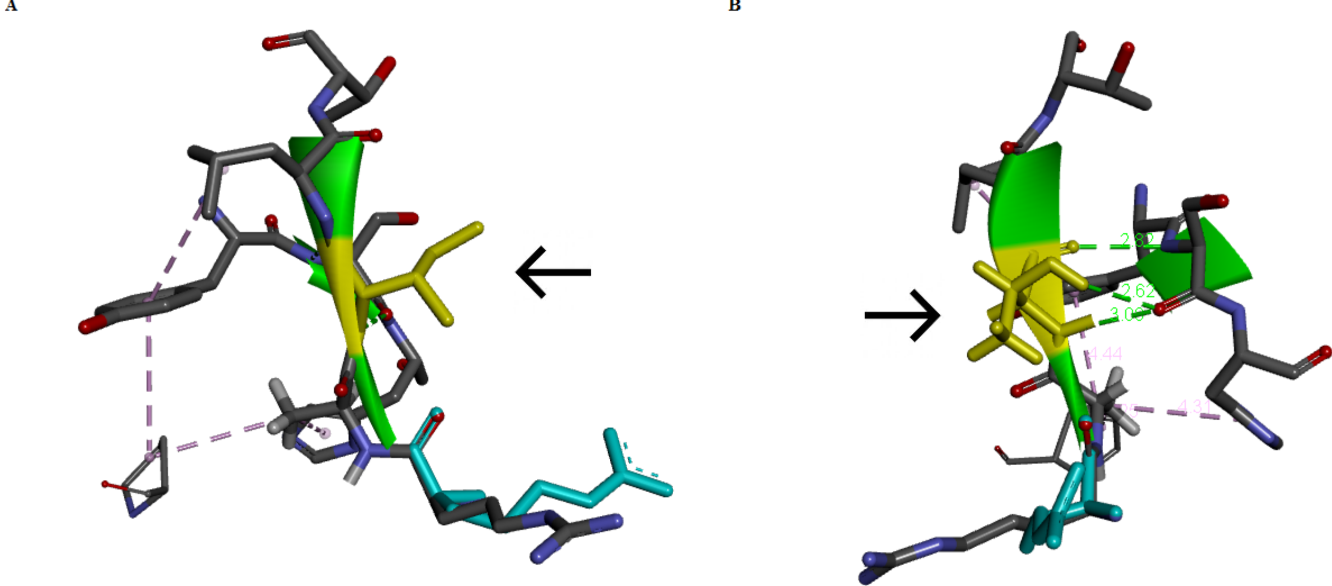

Figure 4: A) The black arrow points to a highlighted yellow residue representing an isoleucine residue. This residue makes no interactions either inter- or intra-molecularly. B) When mutated to threonine, the hydroxyl of threonine makes several hydrogen-bond interactions with othe residues found in Fas-II. This shows increased stability within the protein. Because Ile never made any original interactions, we aren't losing any key interactions.

of another residue on another beta-strand. We believe that this will increase the stability of Fas-II.

The third mutation we looked at was the G18E mutation that we believe would increase stability. However, as seen in Figure 5b, the mutated Glutamate adds steric clash between its side chain and the backbone carbonyl of the adjacent residue. This would not increase stability. Because of this artifact, we did not include this mutation in our final experimental approach because it would only decrease stability and increase polarity, two things we do not aim for in our final engineered Fas-II protein.

The fourth mutation we looked at was the K32G mutation. We believed this would increase stability as well as increase lipid-solubility. This mutation can be seen in Figure 6a-b. Figure 6a shows lysine making an abundant number of interactions. When mutated to glycine (Figure 6b), all of these interactions are lost. This mutation also slightly changes the structural arrangement of the neighboring residues located on the opposite side of the mutated residues. We do not believe that this mutation will increase stability, but because it may increase lipid-solubility, we did not believe it necessary to scrap this mutation from the experiment. In fact, Figure 6b looks more compact which may suggest a more global rearrangement that could in fact increase stability.

The fifth mutation we looked at was a T1I mutation that we believed would increase lipid-solubility. This mutation can be seen in Figures 7a-b. This mutation may decrease solubility because of adding a very hydrophobic side chain like Ile. This mutation does not seem to drastically change the structure of Fas-II nor does it significantly decrease the

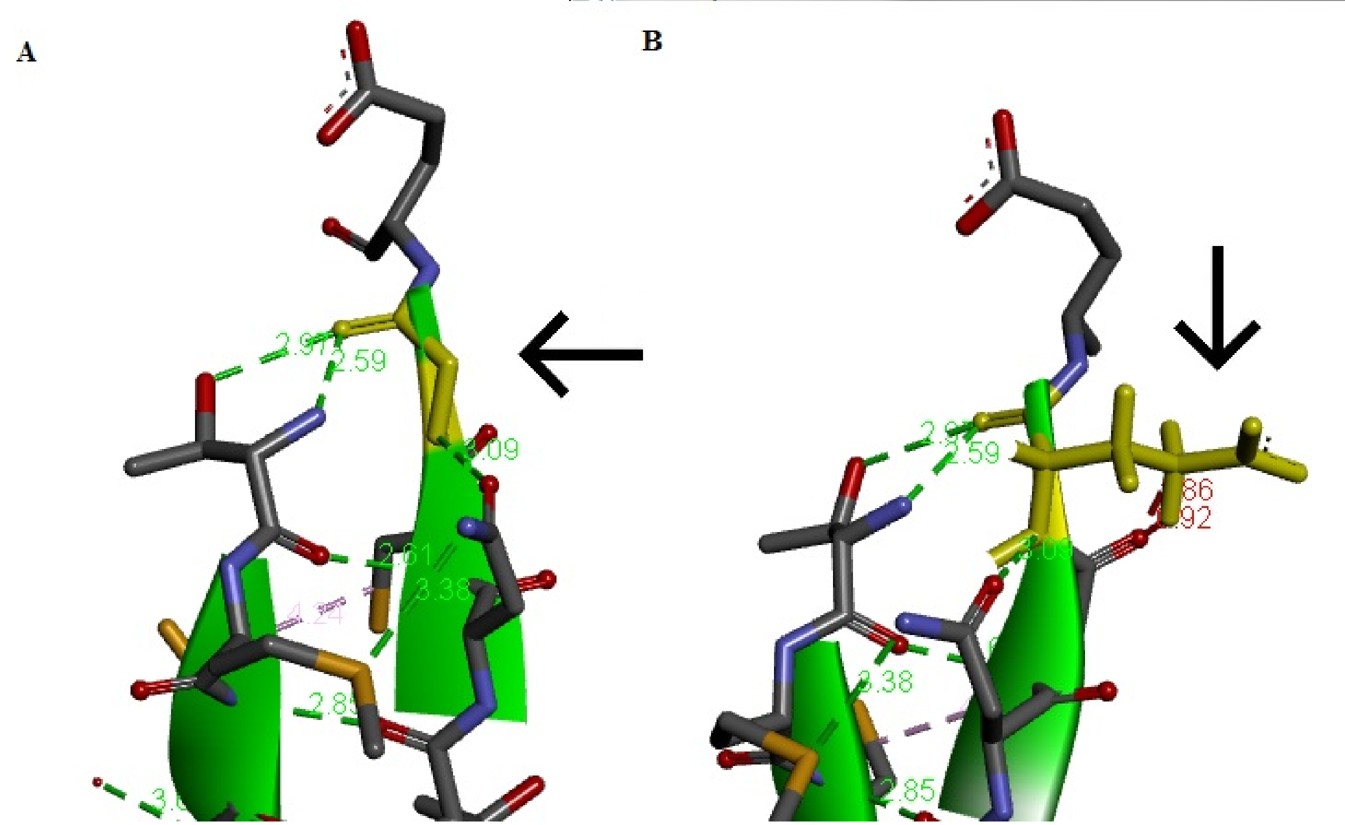

Figure 5: A) The black arrow points to a highlighted yellow glycine residue which does not mak any interactions except for the backbone interacions seen behind the side chain. B) When mutated to Glu, we do observe some steric clash between Glu and the backbone carbonyl of the adjacent residue. This may not increase stability.

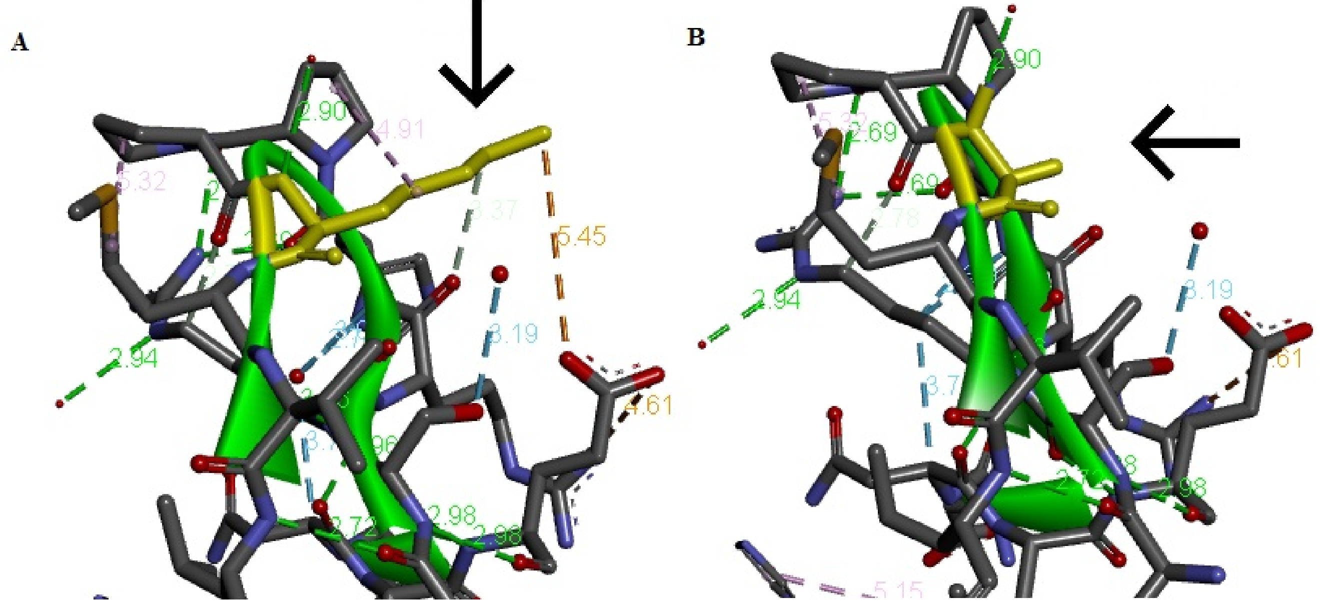

Figure 6: A) The black arrow points to a highlighted yellow lysine that makes several intra-molecular interactions with proximal residues. B) When mutated to glycine, all of these intra-molecular interactions are lost. There are also some structural rearrangements observed. On the opposite side of the side chain, there seems to be a shift in the position of 2-3 neighboring residues. This is most likely due to the loss of the interactions that lysine provided.

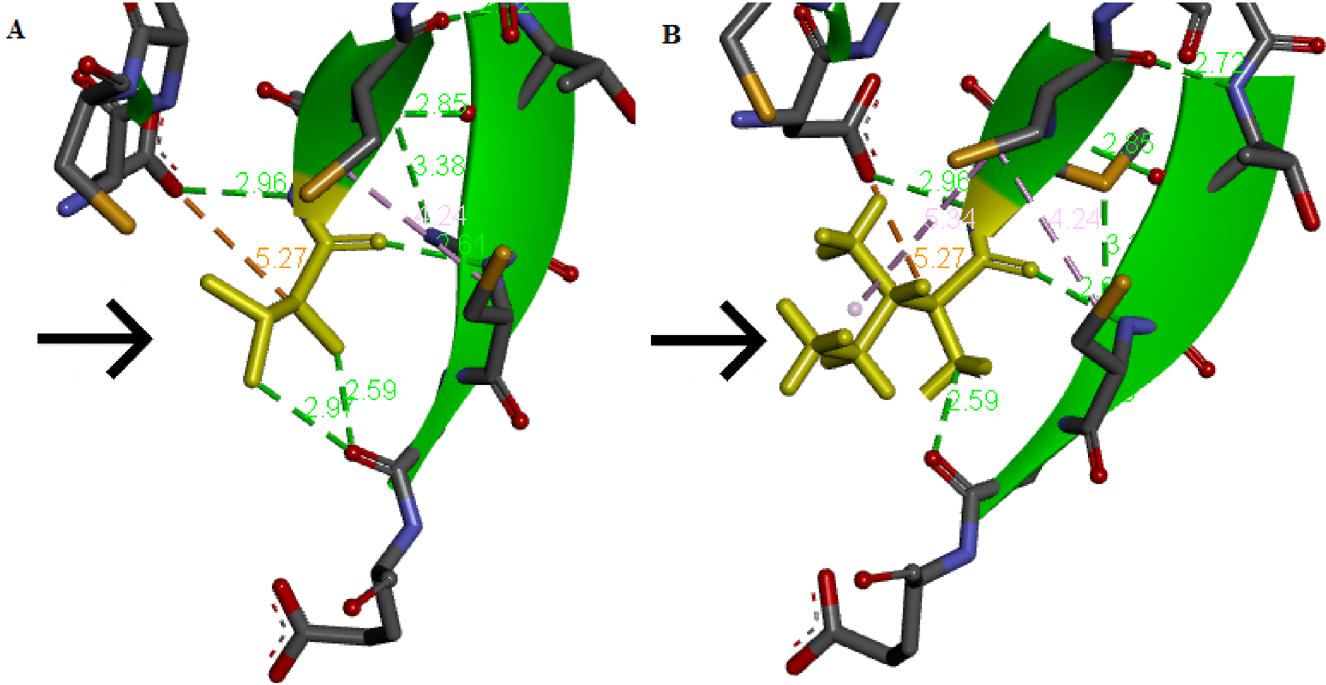

Figure 7: A) The black arrow points to a highlighted yellow threonine. This threonine makes several important interactions between a backbone carbonyl and a carboxyl group. B) When mutated to isolecuive, we see a loss of one H-bonds and possible steric tension between the Ile side chain and the carboxyl group.

number of interactions. There may be some steric clash that may endanger the stability of the protein but the software did not detect it enough to output it (no red-dashed lines as seen in Figure 2, indicating steric clash).

The sixth and final mutation we looked at was a N47G mutation that we believed would increase lipid solubulity. This mutation can be seen in Figure 8a-b. Based on the structure, this mutation does not seem to affect any interactions because asaparagine did not originally make any contacts with its side chains. All the backbone interactions throughout this mutation are conserved and no significant structural change is observed. We believe this mutation will indeed increase the hydrophobicity of our final engineered Fas-II.

Because we eliminated the G18E mutation, we only have 5 mutations to consider. We definitely believe the A12K, the I13T, and the N47G (Figures 3, 4, and 8) will be included because they do not have grave consequences that we can see based on the crystal structure and only provide beneficial effects. The K32G and the T1I do not entirely output the best product and so we will have to incorporate them in seperate samples in order to determine which final mutant protein is indeed the greatest. This final mutant protein will then be used for further engineering in our rational design component.

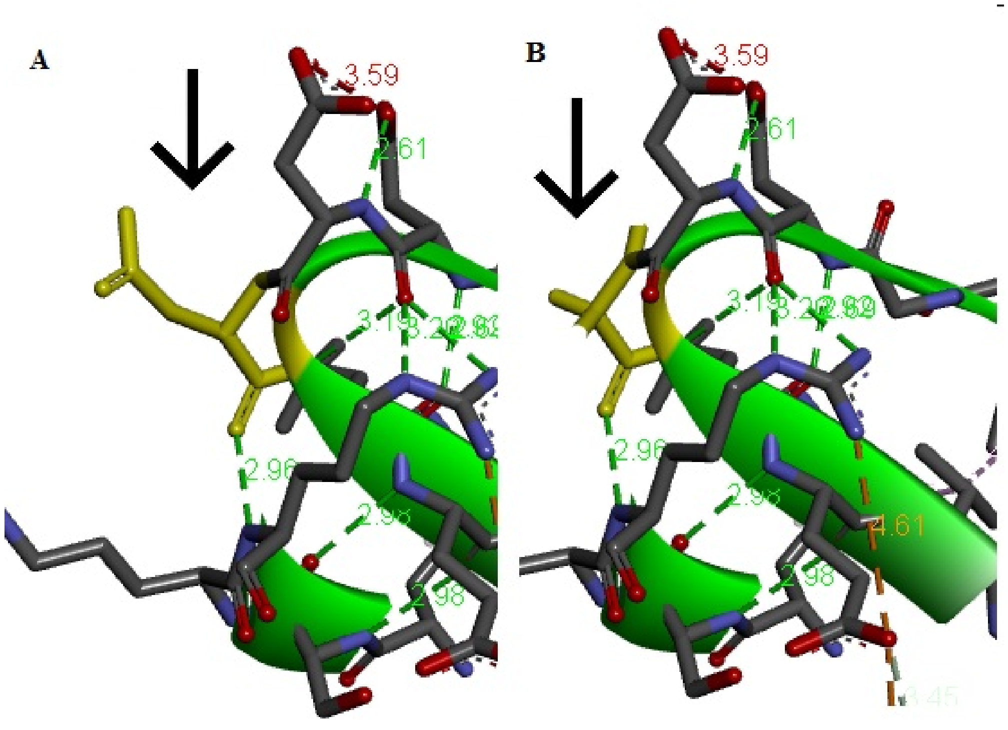

Figure 8: A) The black arrow points to a highlighted yellow Asparagine. This asaparigine only makes backbone interactions. B) When mutated to a glycine, the backbone interaction is maintained. No drastics structural rearrangement is noticed.

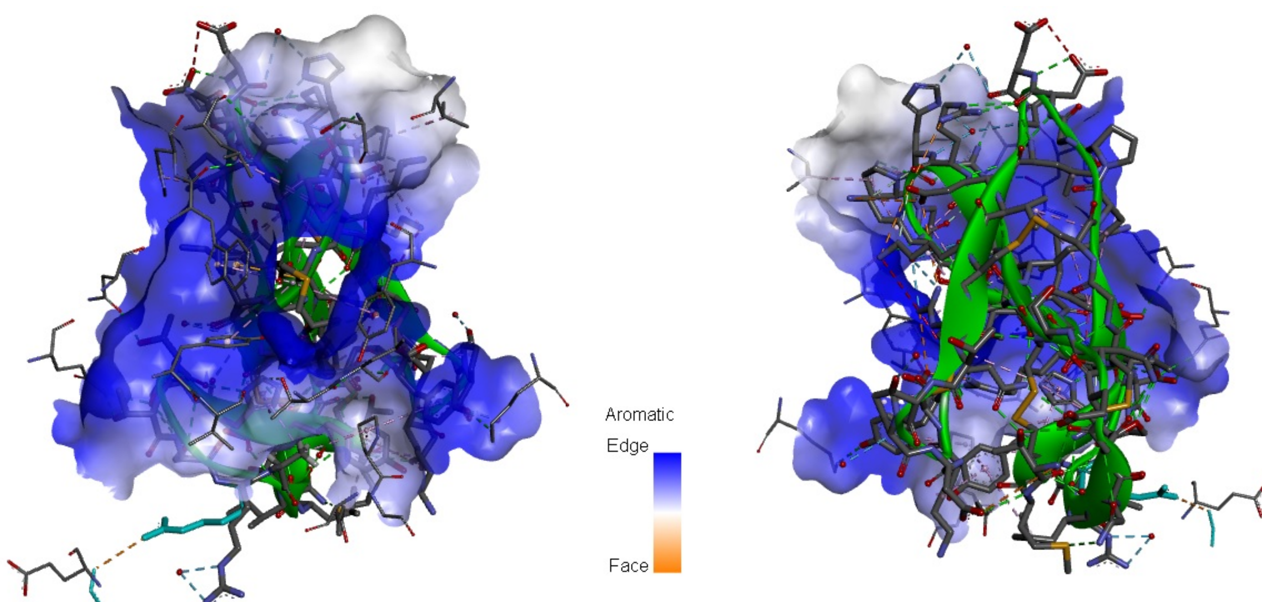

Figure 9: Generated through Accelrys Discovery Studio (ADS). This figure shows two views of the surface-surface interactions between Fas-II and AChE. The legend in the middle suggests that regions of dark blue indicate regions of high amounts of pi-edge interactions while regions of yellow indicate regions of high amounts of pi-face interactions. Any white area indicates no pi-interactions or very little. The green structure which composes most of the image is the Fas-II and only certain residues from AChE can be seen; the visible residues are all those that interact directly with Fas-II. As can be seen in this figure, most of the interactions between Fas-II and AChE are composed of pi-edge interactions.

2.3 Rational Design

Because fasciculin-II binds with high affinity to acetylcholinesterase, mutations that decrease binding between fasciculin-II and acetylcholinesterase were chosen so that cholinergic toxicity would not occur in patients of AD. Through rational design, we looked at various interactions between AChE and Fas-II and determined several mutations that would decrease the affinity between the two proteins. Two assays would be performed: an *in vitro* assay to test for lower rates of acetylcholinesterase activity and an *in vivo* assay with AD APP knock-in mouse models to test for improvements in cognition when injected with our blood-brain barrier soluble fasciculin. The AD APP knock-in mice will be explained shortly.

Before we began to make mutations, we wanted to understand the surface-surface interactions between the two proteins, Fas-II and AChE. This would give us better insight as to what type of interactions were most abundant and therefore most significant in creating the high affinity for Fas-II to AChE. We also wanted to get a better idea of the location of most of these residues so that we could minimize our search map.

Figure 9 shows that the abundance of interactions come from edge-edge pi-interactions between aromatic residues and other carbon residues. This gave us the idea that to decrease affinity we would want to target those residues that made pi-edge interactions with residues on AChE.

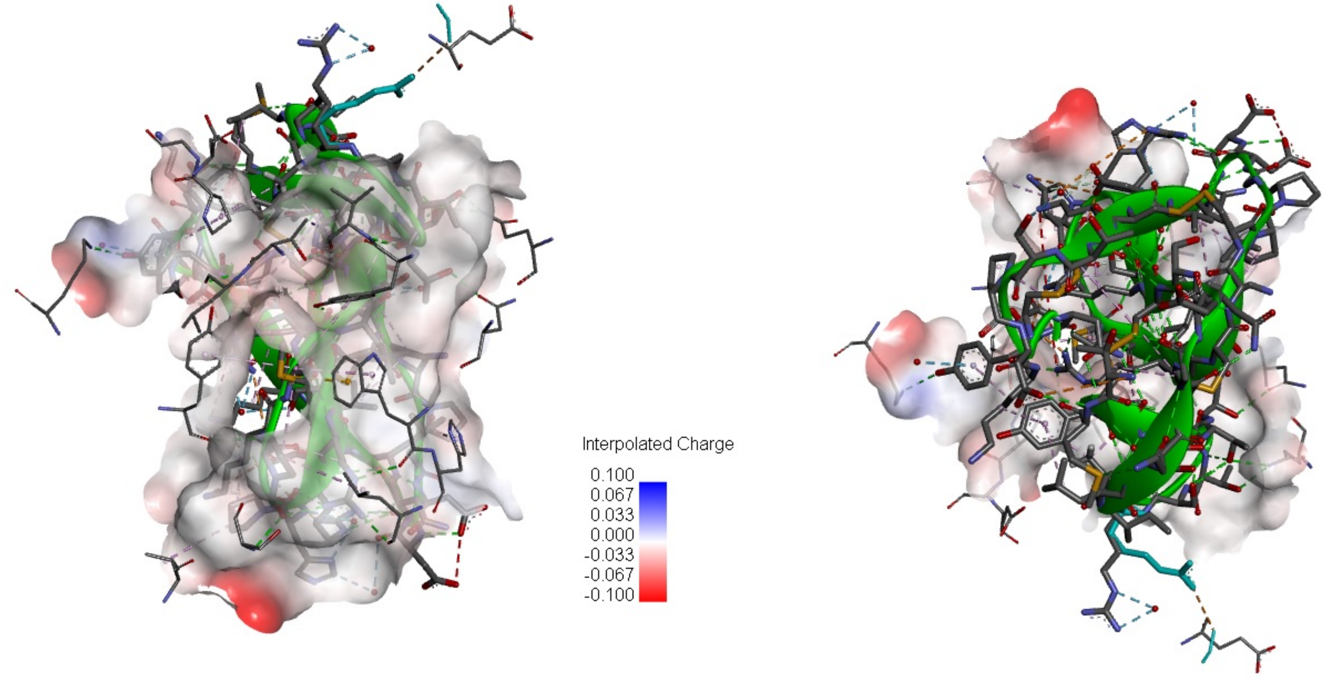

Figure 10: Generated through ADS. This figure shows two view of the surface-surface interactions between Fas-II and AChE. The legend in the middle suggests that regions of blue and red are highly charged whereas regions of white are mostly neutrally charged. The green strcuture which composes most of the image is the Fas-II and only certain residues from AChE can be seen; the visible residues are all those that interact direcrly with Fas-II. As can be seen from this figure, most of the interactions between Fas-II and AChE are neutrally charged.

The next thing we wanted to understand was the amount of charged-interactions between AChE and Fas-II. As can be seen in Figure 10, most of the intreactions are neutrally charged, which makes sense given most of the interactions are pi-interactions occuring between hydrophobic and aromatic residues. This insight suggests that we will be targeting mostly carbon side chains. This gives us slight relief as many hydrophobic side chains are of relatively the same size and are abundant in the amino acid library. This also suggests that we will not have to compensate charged-interactions, mostly pi-interactions and hydrogen bonds.

Lastly, we wanted to look at the hydrophobicity levels at the protein-protein interaction site. Figure 11 shows the hydrophobicity levels as predicted by ADS. The image generated suggested that the levels of hydrophobicity are not as we expected. Because of the large amount of pi-edge interactions seen at the surface of the AChE and Fas-II interactions, we expected a large amount of hydrophobicity but here we see neutral and charged levels. This suggests that we will be looking at a mixture of charged and uncharged particles.

These different surface parameters shows high degrees of pi-interactions, a conservative amount of charged interactions at the edges of the proteins, and very few hydrophobic inter-

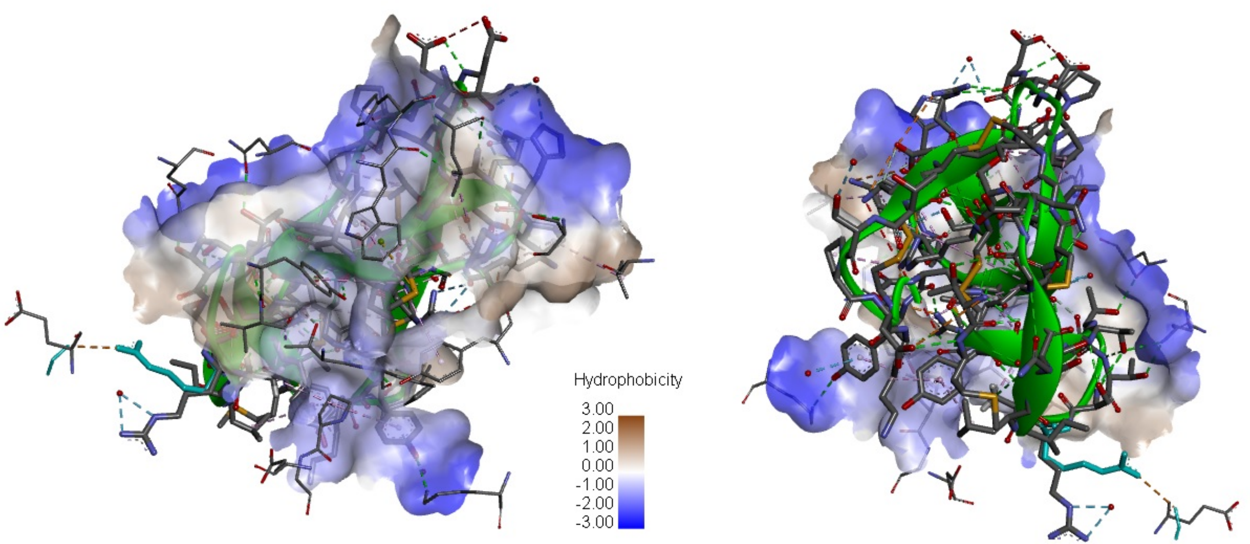

Figure 11: Generated through ADS. This figure shows two view of the surface-surface interactions between Fas-II and AChE. The legend in the middle suggests that regions of brown are areas of high hydrophobicity and levels of blue are regions of low hydrophobicity. The green strcuture which composes most of the image is the Fas-II and only certain residues from AChE can be seen; the visible residues are all those that interact direcrly with Fas-II. As can be seen from this image, most of the residues at the protein-protein surface are not hydrophobic. There are areas at the edges that are low in hydrophobicity. These areas contain charged residues, such as arginine.

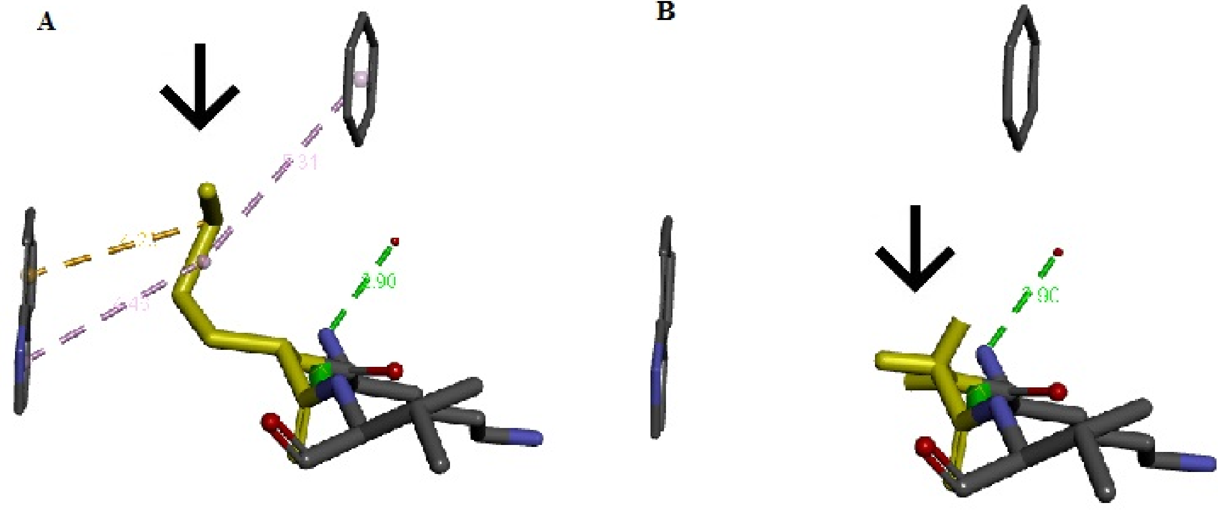

Figure 12: Generated with ADS. A) The black arrow points to a highlighted yellow residue corresponding to a methionine residue. This residue makes a pi-edge bond in an intramolecular interaction as well as a pi-edge interaction with a tryptophan on AChE. This is an example of polar atoms making pi-edge interactions with hydrophobic residues. B) When methionine is mutated to valine, both of the pi-bonds are lost. This means we decrease affinity of the interactions between Fas-II and AChE, but it also means we decrease the stability of Fas-II. However, by mutating Met to Val we also increase lipid solubility.

actions. Because of this we decided to pursue residues that had many pi-stacking interactions, possibly between polar atoms and/or carbon atoms and aromatic residues.

The first residues we looked at was Met33 in Fas-II and Trp279 in AChE. These two residues arranged themselves in an unusual stacking interaction causing for there to be a pi-sulfur and a pi-alkyl interaction. Figure 12 shows this interaction. In order to get rid of these pi-interactions, we mutated the Met33 of Fas-II to a valine. This new mutation can also be seen in Figure 12. This mutation leads to a decrease in Fas-II and AChE affinity, a decrease in Fas-II stability, and an increase in lipid solubility. The first consequence is caused by the loss of the two pi-alkyl bonds between Met and Trp when Met is mutated to Val. The second consequence is caused by the loss of the pi-alkyl interaction between Met and the phenylalanine located in Fas-II decreasing intramolecular interactions. The third consequence is caused by mutating a polar residue (methionine) to valine, making it less soluble and more likely to cross the lipid bilayer.

We then discovered a proline at position 78 on AChE making three important pi-interactions between Ala12, Tyr4, and Tyr61. These interactions can be seen below in Figure 13. In order to decrease the effect of these three pi-interactions, we mutated the Tyr4 into an alanine to eliminate a pi-alkyl bond. The loss of this bond can be seen in Figure 13B below. The bottom facing arrow is pointing in Figure 13B to the mutated Alanine causing for a loss of that pi-alkyl bond. We chose to mutate the Tyr4 rather than any other residue because we believe the proline is structurally essential for the AChE protein. We also did not want to

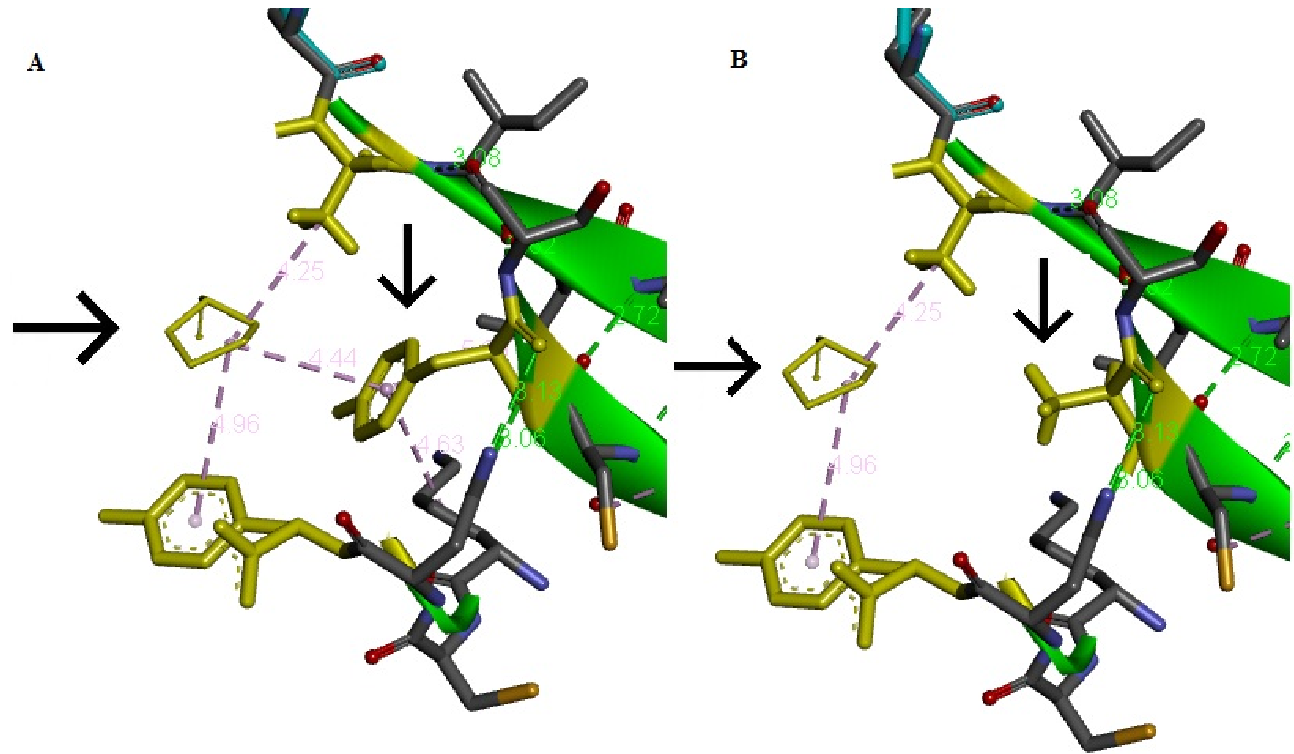

Figure 13: Generated through ADS. A) The black arrow in this image corresponds to P78 on AChE. This proline makes three intermolecular interactions with Fas-II: Ala12, Tyr4, and Tyr62 (ordered from top to bottom). B) This image shows the Tyr4 to Ala4 mtation. This mutation leads to the loss of the pi-alkyl bonds. This bond is the second shortest distace from Proline (4.44 Angstroms).

mutate the shortest distance (Ala12 to Pro78 is 4.25 Angstroms) because that bond may contain the strongest interaction. In contrast, we did not want to mutate the longest distance (Tyr62 to Pro78 is 4.96 Angstroms) because the mutation might not have as strong as an effect. The distance between Tyr4 to Pro78 is 4.44 Angstroms which is a good intermediate between the two allowing for a decrease in affinity without losing significant contacts that may play an important role in the integrity of AChE structure.

Another mutation we are considering is changing Leu48 of Fas-II to an alanine, which eliminated the pi-alkyl bond Between Leu48 and His287 of AChE. Figure 14 shows the before and after image of this mutations. We believe this mutation will decrease affinity of Fas-II with AChE.

Figure 15 shows the next mutation we considered. Thr7 makes two hydrogen bonds with a backbone residue on AChE and a backbone residue on Fas-II. Mutating this residue to to Alanine eliminates its interaction with AChE, decreasing affinity. However, it also loses its intramolecular hydrogen bond decreasing its stability. Thr8 is pointed out to avoid confusion. Our mutation explicitly involves only Thr7.

In a paper by Karlsson et al.[10], they noticed several arginine residues crucial to Fas-II activity. These include Arg11, Arg24, Arg27, Arg28, and Arg36. We decided to solely look

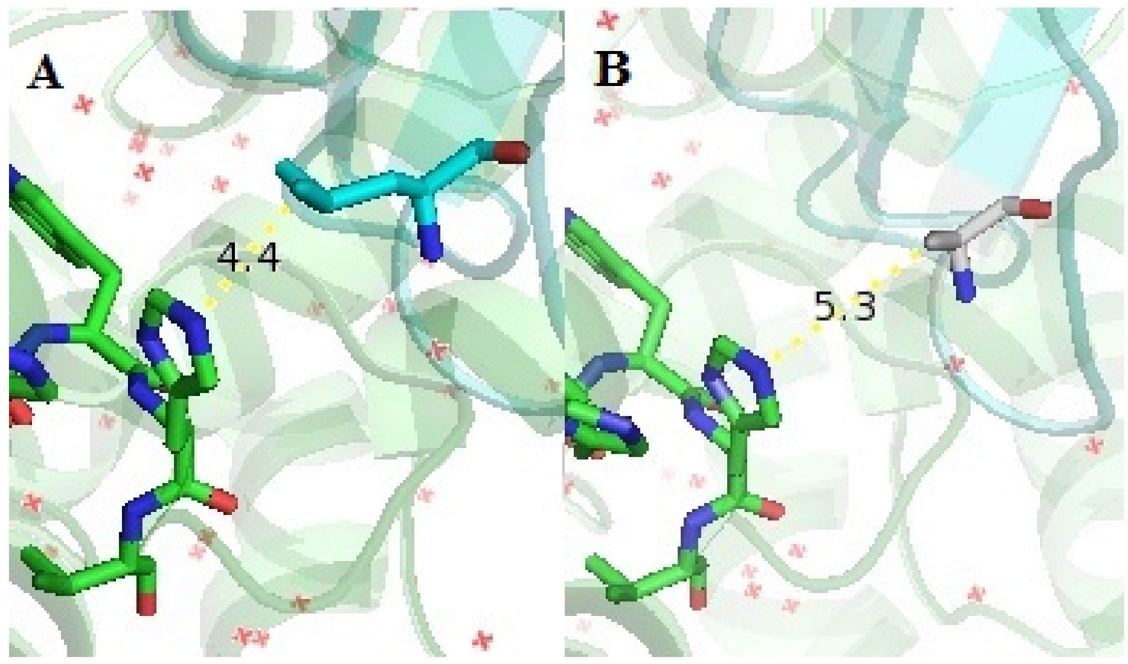

Figure 14: Generated through PyMol. A) The blue residue correpsonds to Leu48. The green residue corresponds to His287 on AChE. The distance between these as generated by PyMol is 4.4 Angstroms which is a reasonable distance to make a pi-edge interaction. B) When mutated to Alanine, the distance goes from 4.4 to 5.3 Angstroms which is too far to make a strong interaction. We believe this mutation will decrease affinity for Fas-II to AChE.

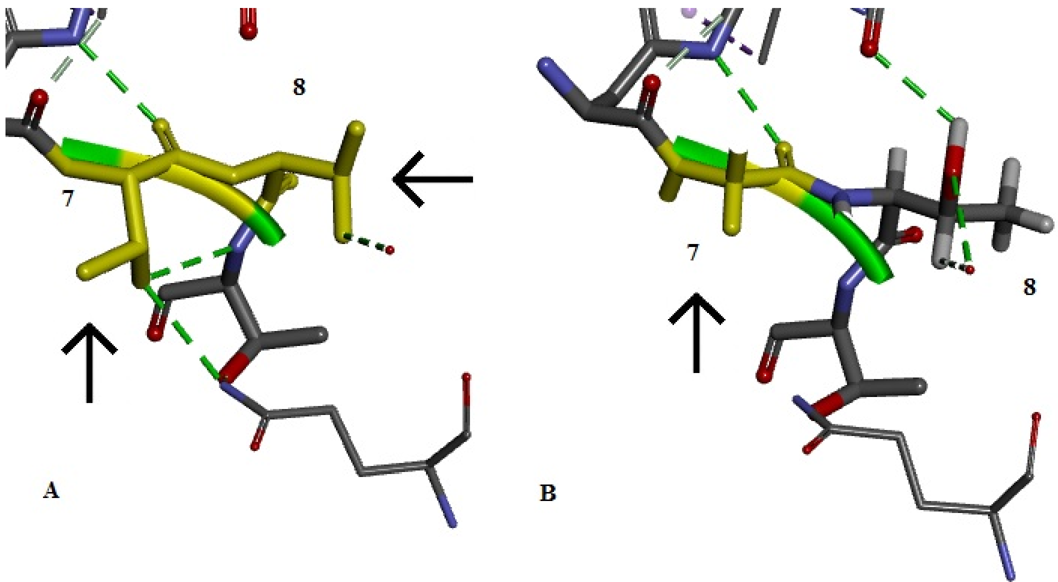

Figure 15: Generated through ADS. A) The upward facing arrow corresponds to Thr7. The left-facing arrow corresponds to Thr8. We are not mutating Thr8. Thr7 makes one inter- and one intra-molecular interaction. B) By mutating this to alanine, we eliminate the both the inter- and intra-molecular interaction, decreasing affinity yet also decreasing Fas-II stability.

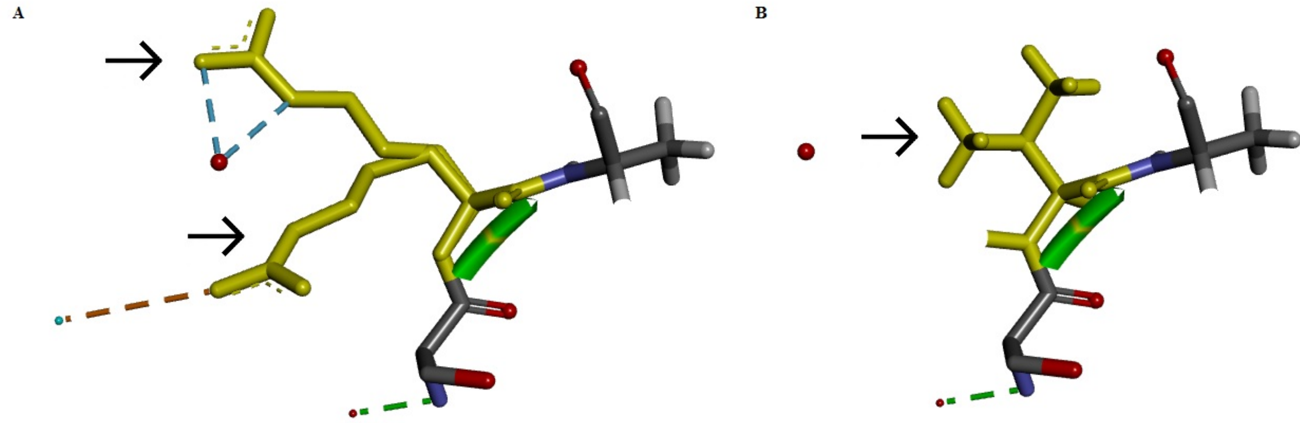

Figure 16: Generated with ADS. A) The black arrows are pointed to the highlighted yellow arginines. Both arginines are located at position 11. Their duplication may be an artifact of this residue exhibiting some kinetic movement. We believe this arginine can either flip in or out, depending on whether it binds to a water molecule or a polar atom on a side chain of AChE. B) The black arrow points to a highlighted yellow residue corresponding to the mutation we made from arginine to valine. This valine neither interacts with the water molecule, increasing hydrophobicity, or the polar side chain of AChE, decreasing affinity of Fas-II for AChE.

at Arg11 (Figure 16) because it was on the outer edge of the protein, allowing us a chance to increase lipid solubulity and decrease affinity. The paper also reported that mutating the other residues would lead to a decrease in affinity greater than 50%, which we believe would be too great of a decrease for our purposes. Arg11 on Fas-II was placed twice in this protein complex. We believe that it may occur due to arginine possessing some type of dynamic movement because it is at the outer edge of the surface interactions between Fas-II and AChE and could potentially flip between interacting with a water molecule and a charged residue on AChE (green). Figure 16 does not show the entire AChE residue but instead shows the presence of a polar atom belonging to a side chain on AChE. In the inward facing conformation, arginine makes a contact with this polar side chain. In the outward facing conformation, arginie makes a contact with a water molecule. If we mutate this to a valine, we increase its hydrophobicity, eliminating its hydrogen bond interactions with water molecules. We also may decrease the dynamics as the valine will tend to face towards the center of Fas-II because valine is more hydrophobic. This mutation would also increase lipid solubulity by eliminating an arginine at the outer edge of the protein.

In total, we came up with four mutations that made key interactions between Fas-II and AChE. We tried to keep as many intramolecular interactions in tact so that we wouldn't compromise the stability of Fas-II, but as noted, there were some we considered worthwhile. Figure 18 shows the comparison between wildtype and mutant protein sequences. All of the changes are idniated in the lower sequence by a red mark. As you can see, all mutations involve eliminating a long side chain to a short side chain so that key contacts would be lost

TMCYSHTTTSRAILTNCGENSCYRKSRRHPPKMVLGRGCGCPPGDDNLEVKCCTSPDKCN Y
TMCASHATTSVAILTNCGENSCYRKSRRHPPKGVLGRGCGCPPGDDNAEVKCCTSPDKCN Y

Figure 17: This image shows the top wildtype sequence of the protein and the bottom engineered sequence of fasciculin-II. Four mutations were made; most consisted of reducing the size of the residue so that pi-interactions could not be made between Fas-II and AChE. This explains why all mutations involve mutating to cine and alanine.

to the AChE protein.

2.4 *In Vitro* and *In Vivo* Assays

Each mutation was performed individually through site-directed mutagenesis with primers designed synthetically through IDT (Integrated DNA Technologies). Since we are using the ApoB fusion transport system (explained in Section 3), we used the cloning method used in their original publication[11]. We cloned our engineered Fas-II in frame into a pcDNA3.1–myc–His vector. The His-tag is replaced by the human ApoB gene. At the N-terminus of the protein, the secretory leader sequence of preprotrypsin was cloned in-frame to allow for secretion. The entire construct containing the secretory signal, the Fas-II gene, and the ApoB gene (in order from N- to C- terminus) was cloned into a third generation self-inactivating LV vector driven by the β-actin promoter found in chickens[11]. Mice were injected with these viral cells and their brains were later sectioned and analyzed using similar methods as seen in the Verma *et al,* paper but with different antibodies specific for our Fas-II.

In order to test if our engineered toxin will block the activity of the enzyme, we will be using the Ellman method for testing the enzymatic activity of acetylcholinesterase. This is the most commonly used method in the field of acetylcholinesterase. This method takes advantage of the byproducts of the enzyme's reaction with acetylcholine to produce a colorimetric difference in the solution. When acetylcholinesterase is active, it will cleave the ester bond between the acetyl group and the choline. The choline then reacts with the Ellman reagent, also known as DTNB, to produce a chemical that releases 412nm light. This reaction is very well understood and reliable, so we would looks at our engineered inhibitor in solution with acetylcholinesterase and if there is a decrease is reflected light, that means there is a decrease in the activity of acetylcholinesterase [12].

Sigma-Aldrich has a kit that can screen for enzymatic activity of acetylcholinesterase in a high-throughput assay of 100 wells for a colorimetric difference. It works on both human and mouse strains of acetylcholinesterases and functions at room temperature. With this kit, we would be able to screen a multitude of engineered protein inhibitors before we even moved onto in vivo approaches [12].

The *in vivo* assay will include testing APP-transgenic mice in a Morris Water Maze test. This test involved learning and remembering where a platform is underwater so that mice do not have to swim. This is dependent on learning and visual cues. APP-transgenic mice are often used in studies involving Alzheimer's Disease. APP is the human gene responsible for coding the Aβprotein, which is what research believes causes the plaques found in the brain.

Originally, AD mouse models simply overexpressed this APP gene, but they could not find any evidence of plaque formation or cognitive defects. However, when they mutated this gene at several positions, they found they were able to produce plaque formation and cognitive defects [13]. The APP transgenic mice will be our negative control, while mice without this APP defect will be our positive control. We will then test different combinations of mutated Fas-II along with our blood-brain barrier transport system to determine if any significant change can be recorded. The blood-brain barrier transport system is discussed in the following section.

3 Hurdles

The major hurdle of this approach is passing our protein through the blood-brain barrier (BBB). The BBB exists to protect the brain from a variety of noxious agents. However, this also prevents therapeutic macromolecules from entering the brain. In order for our therapeutic protein to actually treat Alzheimer's, we have to employ a method to safely facilitate the delivery of our protein, without damaging the brain. Additionally, we have to be able to control this method along with the concentration of therapeutic proteins in the brain. There are many current methods on going through the BBB, however, there are 3 main methods that we are currently investigating to cross the BBB.

The first is the use of nanobiotechnology, where nanoparticles (NPs) are used to facilitate crossing the BBB. One particular NP that has been proven to be able to pass the BBB are liposomes. A research group from the University of Milano-Bicocca from Italy, showed that amyloid-β-targeting liposomes linked to an anti-transferrin receptor antibody, had the ability to pass the BBB. The second is using exosomes, which are cell-derived vesicles present in all biological fluids. These exosomes transports RNA and proteins, leading many experts to believe in their potential for crossing the BBB. Specifically, a group from the University of Oxford has shown that exosomes can cross the BBB and deliver siRNA and proteins to neurons in the brain by systematic injections[18]. This particular group showed that the delivery of siRNA-loaded exosomes resulted in the mRNA and protein knockdown of a specific gene in mice. To incorporate this method into our project, we would load exosomes with our peptide and once it crosses the BBB, we would measure the concentrations of the peptide in the mice brain.

However, the method we are most interested in is using cell-surface receptors. The BBB only allows molecules up to around 400-500 Da to pass through, and in order for proteins to pass through, they have to undergo a process called receptor-mediated transcytosis.The type of BBB receptors we are interested in are called low-density lipoprotein receptors (LDLP), which are cell-surface receptors that bind to apolipoproteins[18]. When LDLPs binds to apolipoproteins, transcytosis occurs and the apolipoproteins can be taken up by neurons in the brain. The general strategy of this method is basically fusing the binding domain of a prominent apolipoprotein to our small peptide, and when the apolipoprotein binds to the LDLP, transcytosis occurs, allowing the peptide to pass through the BBB where it is released into the neurons in the brain. The next section will talk about specifically how we would exert this method for our protein.

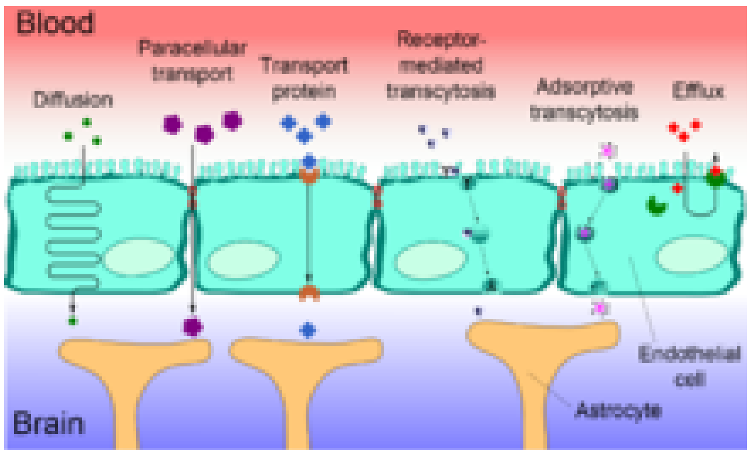

Figure 18: The blood brain barrier and the options for transport across its membrane. From left to right, there is simple diffusion of small lipid soluble molecules or water, paracellular transport that squeezes molecules between the tight junctions between the endothelial cells, receptor mediated which has binding activate a transport across the cell, and adsorptive transport of molecules in a liposome [14].

4 Permeability through the Blood-Brain Barrier

A major problem for Alzheimer's disease treatment is that only small molecule drugs can be used to enter the brain. Small molecule drugs are useful but it is a restriction on the diversity on inhibiting enzymes. The other option for treatment is using therapeutic proteins to inhibit the acetylcholinesterase, but the FAS-II protein is far too large to pass through the blood brain barrier. Initially, we wanted to engineer the FAS-II protein to be small enough to squeeze through the blood brain barrier, but since this seemed impossible, we looked into drug transport systems. In order to take advantage of the drug transport systems, we will be using our final optimized sequence of FAS-II after mutations and activity assays have been conducted. First, it is important to understand what the blood brain barrier is. The blood brain barrier (BBB) contains a highly selective membrane that mediates what can pass through into the brain. This membrane can withstand a very large potential which will not allow most large molecules into the brain. Concentration gradient does not play a role in its permeability. Despite its important role of keeping the brain from coming into contact with dangerous neurotoxins, it does still need to supply the brain with nutrients and basic building blocks for life. Therefore, it is still permeable to water, oxygen, and small lipid soluble molecules. Anything else that needs to get to the brain has a specific receptor that will carry the molecule across the membrane and into the central nervous system. Shown in Figure 1, the BBB consists of Endothelial cells that can facilitate the diffusion into the brain [14] [15].

Initially, we sought out to engineer the FAS-II neurotoxin to be small enough to diffuse across the endothelial cells; however, molecules that are able to do that need to be on the order of 400 Da or less. Since the average molecular weight of a single amino acid is around 110Da, we would only be able to create a three amino acid long peptide. This could not incorporate the necessary activity on the acetylcholinesterase that would prove this drug's

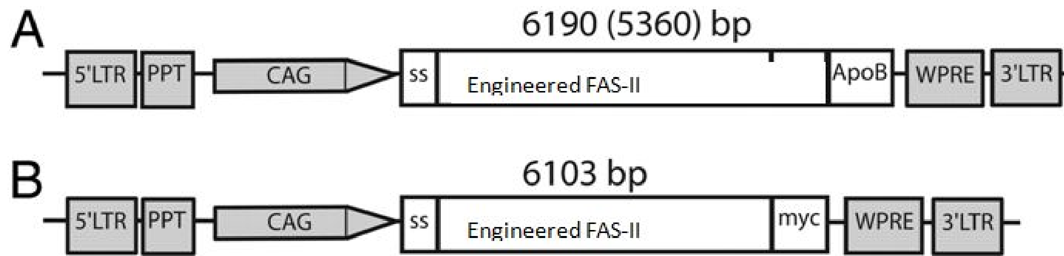

Figure 19: The construct made by Spencer and Verma in order to take advantage of the LDL transcytosis of lipid soluble molecules too large for diffusion. This is a lentivirus vector that contains a self-inactivating vector that is controlled by the chicken β-actin/globin (CAG) promoter. The PPT stands for poly-purine tract. The ss region is a secretory signal that gets cleaved off after secretion. It also contains a myc epitope tag for purification purposes. The ApoB is the protein domain that binds to the LDLR receptor and stimulates transcytosis. Part B contains no ApoB for negative control purposes. Their gene of interest is Glucocerebrosidase or eGFP [16].

usefulness. A three amino acid long peptide would also not bind to the entire active site of the acetylcholinesterase [15]. Since this was out of the question, we did further research into drug transport systems. One that seems very plausible for our project is the research done by Brian Spencer and Inder Verma. They developed the construct shown in Figure 2 that produces a fusion protein that can successfully transport your therapeutic protein across the BBB. In this construct, there is a chicken β-actin/globin promoter labeled in Figure 2 as CAG. Our gene of interest, our engineered FAS-II can be inserted in place of their Glucocerebrosidase gene. The myc tag is important for purifying the fusion protein and the ApoB is the apolipoprotein B. This protein is responsible for binding to the low-density lipoprotein receptor or LDLR and triggering transcytosis of the protein across the endothelial cells[16] .

The LDLR family consists of ten types of receptors commonly found on the cell surface of the endothelial cells. Normally, these receptors bind to ApoB or ApoE to take in lipids to be degraded in the lysosomes. The receptor is recycled as it returns to the cell surface and the Apolipoprotein is sent to the opposite side of the cell to be absorbed into the central nervous system. Using this construct, we would plan to fuse our engineered FAS-II protein to the ApoB domain. Fusion of these two proteins would allow for ApoB domain to bind to the LDLR and transport the whole fusion protein across the endothelial cells and into the central nervous system. This construct has worked before with bigger proteins, so this should work just as well for our protein of interest [**?**].

Initially, we want to translate our fusion protein in vitro in a mammalian cell culture, purify it using column chromatography taking advantage of the myc tag, and inject the therapeutic protein into the test mice. This would be ideal for mass production of our fusion therapeutic protein as a drug. However, if we find that this injection process degrades the protein too quickly requiring too many consecutive, we would attempt to PEGylate the fusion protein or attempt to infect the mice directly with the lentivirus vector so their own cells would be producing the fusion protein.

Table 1. Comparison of pharmacological characteristics of 5 acetylcholinesterase inhibitors

	Tacrine	Donepezil	Rivastigmine	Galantamine	Huperzine A
Structure					
Target enzymes	AChE and BuChE	AChE	AChE and BuChE	AChE	AChE
Recommended dosage	160 mg/day (four times daily)	10 mg/day (once daily)	9.5 mg/24 h patch (once daily) 12 mg/day (twice daily)	24 mg/day (twice daily)	0.4 mg/day (twice daily)
Plasma half-life	2-4 h	About 70 h	About 3 h (patch) About 1 h (capsule)	About 7 h	About 60 h
Period of disease treatment	—	All stages of Alzheimer's disease	Mild to moderate Alzheimer's disease	Mild to moderate Alzheimer's disease	Mild to moderate Alzheimer's disease
AChE IC_{50} (nM)	190	22	48,000	800	47
BuChE IC_{50} (nM)	47	4.1	54,000	73,000	30
Adverse reactions	Hepatotoxicity	Diarrhea, nausea	Diarrhea, nausea	Nausea, weight loss	Nausea

Figure 20: Image taken from a review by Ling et al, [].

We have found that this is the best method for protein delivery to the central nervous system and mass production of our therapeutic protein drug. The rate in which the FAS-II protein can diffuse into the brain would still be controlled based on concentration and recycling rate of the receptors, but this should actually benefit our Alzheimer's disease drug. Regulation of our engineered neurotoxin should limit the activity of acetylcholinesterase but not knock it out fully. This is an important discovery towards solving the problem that is Alzheimer's disease.

5 Innovation and Comparison to Current Acetylcholinesterase Inhibitors

As we mentioned previously, Alzheimer's Disease is characterized by decreased acetylcholine transmission in brain areas associated with memory and learning [17]. Since 1993, many drugs have been approved by the FDA, most of which are acetylcholinesterase inhibitors. Figure 14 below shows five of the most common drugs used.

Tacrine is rarely used because of low efficacy and a multitude of harmful side effects. Donepzil's effects last longer, is better tolerated, and has fewer side effects. Rivastigmine is fast-acting, minimizes drug interactions, and drastically improves memory. Galantamine not only improves cognition but protects neurons from the toxicity caused by the ameloid-beta aggregates. Huperzine A is the only drug that can cross the blood-brain barrier and also provides protection for neurons; it is also more selective and has a longer inhibiton period. However, all of these drugs are only able to treat mild to moderate AD [**?**].

We hypothesize that engineering a peptide that is highly selective towards AChE will increase the efficacy of the acetylcholinesterase inhibition and thus increase cognition in patients of Alzheimer's disease. Fasciculin-II is already a highly efficient inhibitor, and we believe that by optimizing its affinity we will lower cholinergic toxicity and the side effects that are consequential to those high levels. We also believe that the half-life of this peptide will remain long enough to increase patient comfort during the injections.

Fasciculin-II is relatively small and can thus be expressed in inexpensive organisms like *E. coli*. The sequence is also readily available allowing for site-directed mutagenesis to be achievable through primer design.

6 Conclusion

Our goal is to design a small peptide based on fasciculin II that can permeate the blood brain barrier and enter synaptic clefts in the brain. We use protein engineering techniques such as comparative and rational design to predict mutations. Our peptide should be able to bind to acetylcholinesterase and deactivate the protein. The end goal will be to retain acetylcholine neurotransmitters to strengthen neuralgic signals between the brain and muscles.

Although there are proteins that naturally inhibits acetylcholinesterase, these proteins are not ideal for treatment for several reasons. Firstly, they bind nonspecifically to both esterases in the periphery and those in the central nervous system, causing unpleasant side effects. Secondly, they bind to AChE temporarily, making their reactions hard to predict. Thirdly, these proteins are also derived from animal sources, they can trigger an immune (antibody) response which can lead the drug inefficiency as the body adapts to the protein.

There is currently no cure for Alzheimer's. We can only raise standard patient care and help with their quality of life. The disease slowly breaks down its victims, and the process is painful and debilitating. Until we can find a cure, we can only help those by making their life easier.

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