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Insulin based inhibitors of human islet amyloid polypeptide (hIAPP) and their effect on aggregation of hIAPP in the treatment of type II diabetes

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Insulin Based Inhibitors of Human Islet Amyloid Polypeptide (hIAPP) and their Effect on Aggregation of hIAPP in the Treatment of Type II Diabetes

by

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Abstract:

Human islet amyloid polypeptide protein (hIAPP) is secreted by the pancreas along with insulin and is assumed to play a role in pathological development of type II diabetes. It has 37 amino acids in its sequence. Amyloid is formed due to misfolding of the protein, which is cytotoxic to beta cells in the pancreas of type II diabetic patients. The presence of amyloid deposits is also a characteristic feature of a number of other diseases like Alzheimer's disease and Parkinson's disease. Since insulin has been reported to interact with hIAPP and block amyloid formation, fragments of insulin were synthesized and the inhibitory effects were studied against an hIAPP analog in the presence of phospholipid vesicles. While these sequences might inhibit amyloid formation, preliminary results indicate that they actually enhance membrane damage, as measured by the increased leakage of carboxyfluoroscein dye from membrane model vesicles.

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Chapter 1. Introduction

1.1 Background and Amino acids

The field of biochemistry has been very diversified in the past decade or so, in order to understand the mysterious and new diseases that are coming at a very fast rate. To solve this problem we need to have a better understanding of the complex nature of human cells and their interactions with each other and exo-chemicals to which they are exposed. This issue can only be solved by understanding the basic mechanism of every chemical reaction in the cells.

To understand what is going on in the cells, we need to study the basic foundation of life, the amino acids. Amino acids are short carbon backboned molecules that have a carboxyl group at one end and an amino group at other end. The difference between each amino acids lies only by varying the side chain present on it, also called R groups. These R groups distinguish the amino acids from each other. The twenty amino acids each have unique characteristics, which depend on the length, structure, solubility, charge, and nature of the R groups. The structures of the amino acids are in Figure 1.

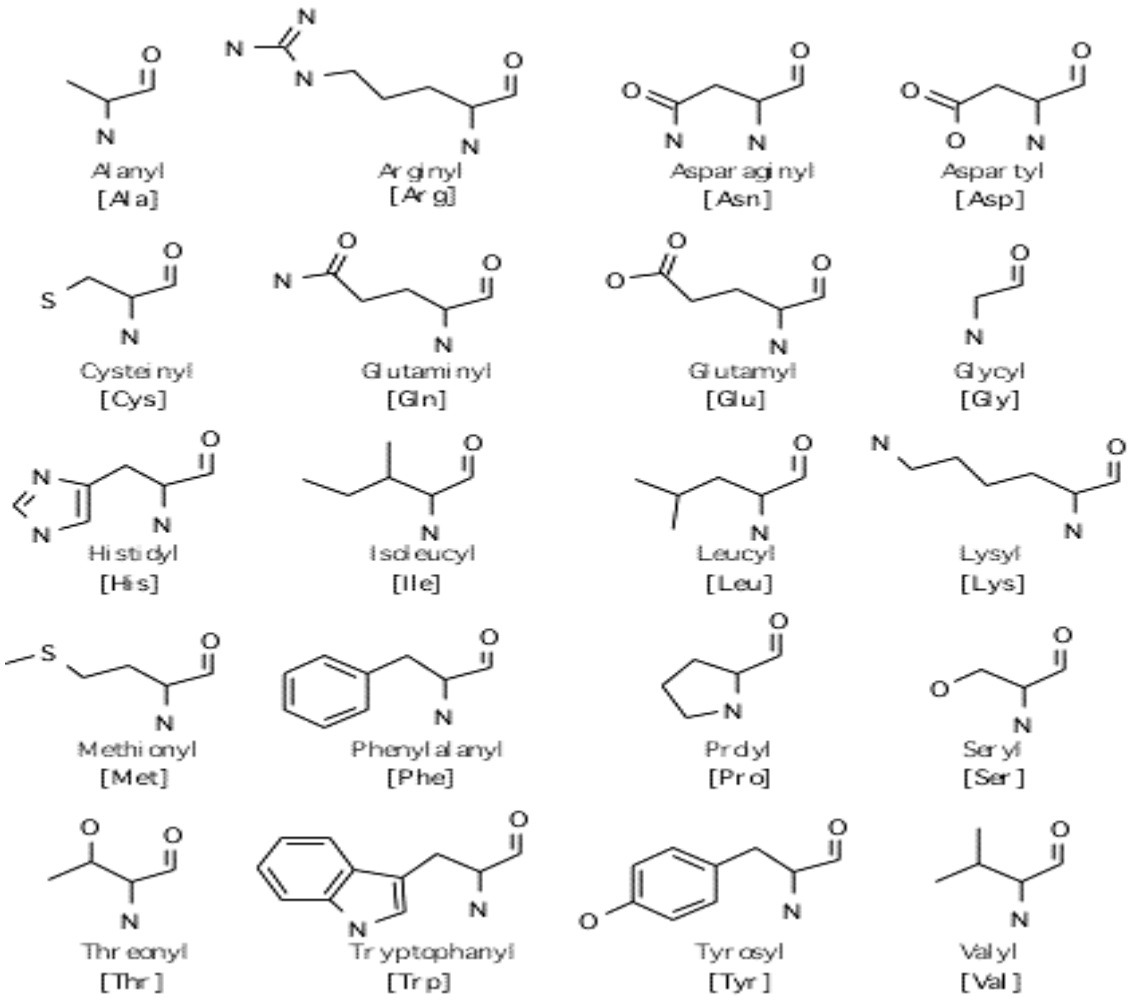


Figure 1. Twenty amino acids with neutral charge and abbreviated by three letters code.[1]

The twenty amino acids shown above undergo a process called a condensation reaction where the amino terminal of one alpha-amino acid reacts with the alpha-carboxyl terminal group of another amino acid, by losing a water molecule and forming an N-C=O bond, called an amide bond. Amino acids attached in this manner, if in a short chain of two to twenty amino acids, are called a peptide chain. If the peptide chain grows longer, then it is called a polypeptide. Polypeptides of high molecular weight perform certain biological functions and are called proteins. A protein is usually a very long linkage of amino acids, from dozens to several

thousands. Its pattern and structural integrity are very important for its biological function. Theories have suggested that even minor changes of less than one percent can cause huge fluctuations in their biological activity and function.

1.2 Protein Structure:

When proteins are explored using a technique called crystallography, their three dimensional structures are revealed, and these structures are classified into four levels: primary, secondary, tertiary, and quaternary (Figure 2). Primary structures of a protein are simple sequences that are linearly arranged in space, forming the backbone. Secondary structure is where the backbone is twisted spatially, forming helical or pleated segments. Tertiary structure is the compact structure of any protein including backbone and side chain arrangements, and in this form it's functional (function of the protein is associated with tertiary structure). Quaternary structure occurs in some of the proteins that consist of two or more polypeptide units. Each and every protein and polypeptide have a unique and specific three-dimensional structure associated with them. This uniqueness comes from the arrangement of amino acids in their peptide chain. Their conformation allows them to express their functions and activity. Moreover, many proteins have an inhibition or activation region where it is sterically hindered, allowing only specific bio-molecules or foreign molecules to interact and bind to this region.

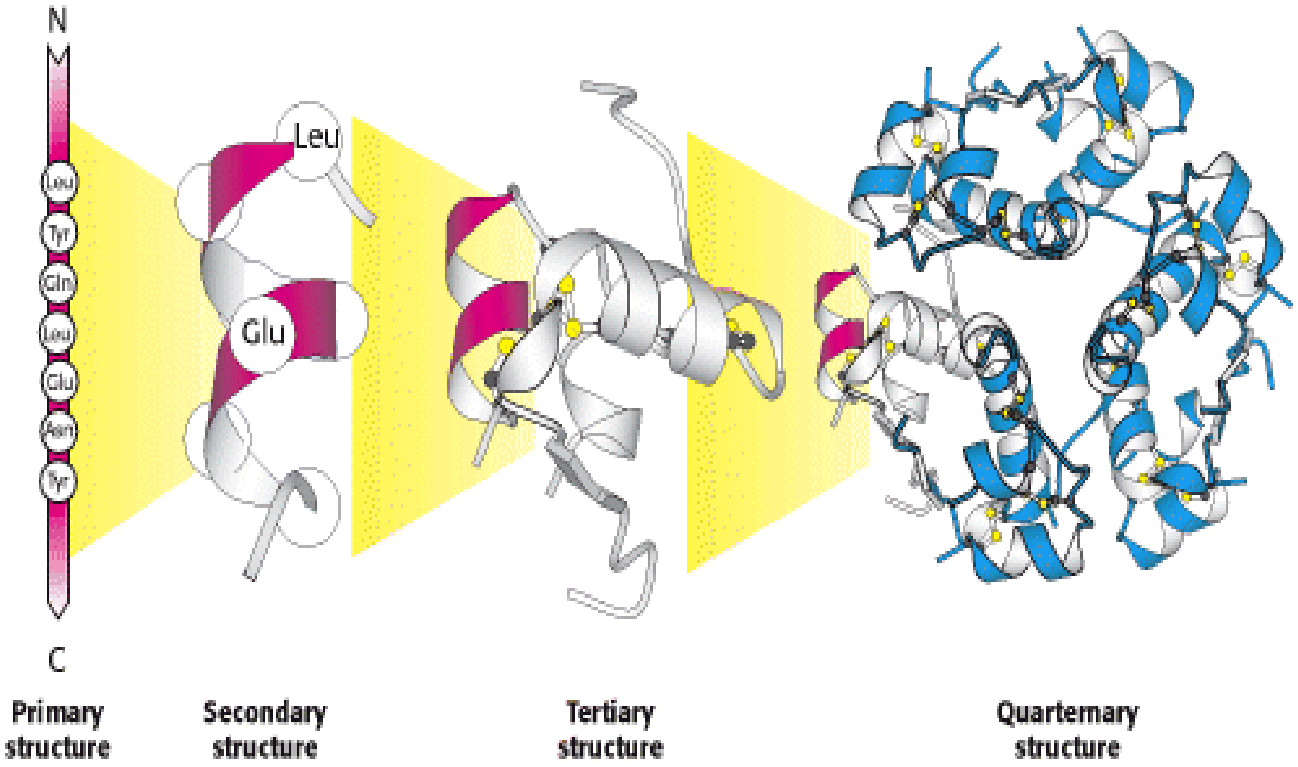


Figure 2. Schematic representation of protein structure[2].

If proteins adopt their regular conformation, they can perform normal physiological functions. But if there is any mis-folding in the protein, then the protein generally becomes insoluble and forms aggregates and damages the cells around them to causes diseases. One of the proteins that is implicated in the development of disease is known as amylin or human islet amyloid polypeptide.

1.3 Diabetes

The term *diabetes* is defined as a disease state associated with elevated blood sugar levels due to improper use of blood glucose. As we ingest food, it needs to be assimilated, processed,

and digested in our body with the help of a hormone called insulin. Insulin is secreted from the pancreas, in the islets of Langerhans. Diabetes is of two types[2]: Type I diabetes and Type II diabetes.

Type I diabetes is diagnosed generally in people aged less than 15 years and is believed to be due to hereditary causes, the passage of genes from one generation to another. This type contributes only ten percent of the world diabetes population.

Type II diabetes is generally observed in older people, usually above 45 years, and is caused by a metabolic disorder. Type II diabetes is also called non-insulin dependent diabetes mellitus (NIDDM). Type II diabetes affects roughly 150 million people around the globe[3] and is a chronic and progressive disease. Type II diabetes usually is associated with obesity, high blood pressure, and hypercholesterolemia. This type contributes about ninety percent of the world diabetes population, so immediate attention is required to help the people suffering from this dangerous disease.

1.4 IAPP

Islet Amyloid Polypeptide, also known as amylin, is of particular importance in Type II diabetes because of the way it mis-folds, causing amyloid fibril formation, which, in turn, can cause many diseases. The pathology of Type II diabetes (T2DM) is the cytotoxic deposits of amyloid fibrils in the islets of Langerhans, and the main component of these deposits is the Islet Amyloid Polypeptide (IAPP) [4]. Eighteen percent of Americans over the age of 60 develop Type II diabetes, and the estimated cost for the treatment of diabetes is around $132 billion, which is

10% of geriatric health care costs[3]. As the population gets older, the impact of this disease is expected to grow dramatically, making the study of this disease even more important.

1.5 hIAPP

Human IAPP (hIAPP) or amylin is a 37-residue polypeptide that is co-secreted along with insulin from pancreatic β-cells. Amylin protein is generally involved in appetite suppression. In 1900, the appearance of IAPP in the islets of Langerhans by hyalinization was first noted by Eugene Opie in diabetic patients. This protein is thought to behave as a hormone in normal individuals, and this was shown by Cooper et al in 1988.[5]

Later Friedrich and Kekule demonstrated the presence of amylin protein in amyloid masses, and today amyloid refers to fibrillar structures that are found to be a major component of Alzheimer's disease, Prion disease, Type II diabetes mellitus and Parkinson's disease. This misfolded protein is closely related to disease progression and is also found to contribute to cell death.[6]

The aggregates of misfolded islet amyloid polypeptide found in Type II diabetes patients are composed of β sheet amyloid fibers. Such amyloid deposits have been found in the pancreas of more than 90% of diabetic patients upon autopsy. When amino acid sequences of unrelated amyloid forming proteins are compared, it is seen that aromatic residues are conserved among these proteins.[7,8]

1.5 .1 hIAPP Functions:

hIAPP is also called amylin, IAPP or DAP (diabetes associated protein).[5] Its synthesis and secretion have been well studied but in contrast, the exact physiological function of the polypeptide remains unclear. Some of the functions of hIAPP are suppression of insulin-mediated glucose uptake in skeletal muscle and inhibition of glucose-stimulated insulin secretion (Swiss Prot Id P10997).[9] It also suppresses glucagon release from isolated islets[10] and regulates food intake and controls body weight.[10] The peptide also regulates renal filtration, with IAPP binding sites which are localized to the kidney, in addition to calcium homeostasis and vasodilation.

1.5.2 hIAPP Gene structure and location

The total size of gene hIAPP is 6599 base pairs (Entrez gene id: 3375). The precursor protein contains 89 amino acids (Refseq: NP_000406). Its mRNA is 1462 base pairs long and contain 3 exons (NM_000415). It is located on chromosome 12 (NC_000012.10) of the human genome, gene locus is 12p12.3-p12.1.

The ensemble data base shows the protein and its components clearly in Figures 3 and 4.[11]

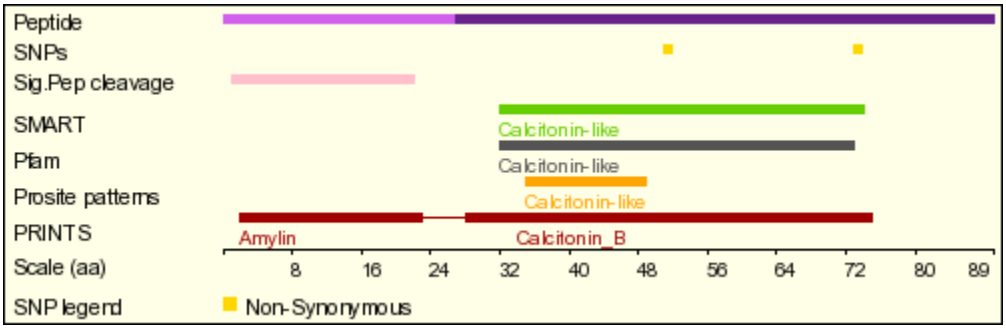


Figure 3. Scale (aa) shows total length of the protein, here different parts of the protein are clearly distinguished. There are two snp (single nucleotide polymorphism) sites at the 53rd (SNP ID: rs 1800203)[12] and 75th positions (SNP ID: rs11558904).[12] The average residual weight of the protein is 110.184, the charge of the peptide is 7.5, and isoelectric point is at pH of 10.3921(Ensembl peptide id is ENSP00000240652).[12]

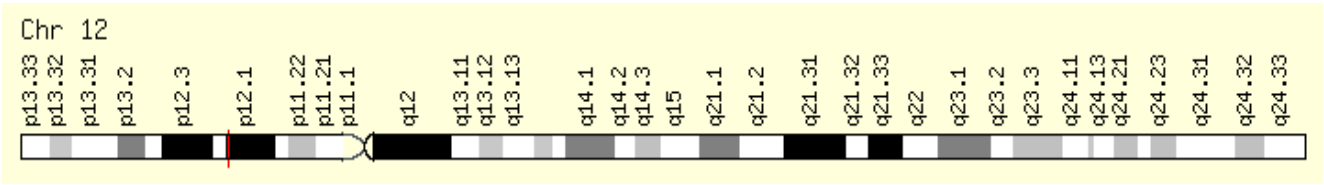
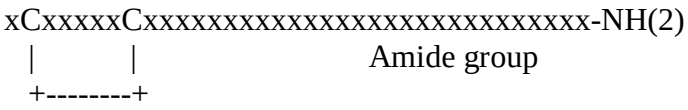


Figure 4. Location of hIAPP in humans according to gene cards[13] on chromosome12.

The structure of hIAPP is closely related to calcitonin gene-related peptide, CGRP (PF00214).[14] "Two cysteine residuals are conserved from the N-terminal of these peptides and are known to be involved in a disulphide bond."[14] (Figure 5).



'C': conserved cysteine involved in a disulphide bond.

Figure 5. Two cysteine residues are involved in disulphide bonding.[14]

1.5.3 Role of hIAPP in Type II diabetes

Although definite functions have yet to be clearly ascribed to IAPP, its fundamental pathogenic role is the formation of the islet amyloid in the pancreas of individuals with Type II diabetes. hIAPP plays an important role along with insulin in healthy individuals to maintain glucose homeostasis.[15] In beta cells of the pancreas, hIAPP is present in very small quantities, roughly about one percent when compared to that of insulin in the beta cells. Because it is present in lower quantities, the clearance rate is also low. This makes the hIAPP highly concentrated during the fasting state when compared to insulin, at 10-15% of insulin levels.[16]

As mentioned previously, hIAPP is implicated as the main component in amyloid fibril deposits, which have been found by post-mortem examination in more than 95% of Type II diabetic patients. There seems to be a natural mechanism whereby these fibrils of hIAPP are not found in healthy individuals, where hIAPP is soluble and functional.

When hIAPP forms fibrils, they are dense, insoluble, and long beta sheet structures. These fibrils allow the entry of calcium ions into the pancreatic beta cells, due to the formation of small pores or holes, or complete disruption of the membrane. Due to this membrane

disruption, integrity of the cell is lost, thereby creating extra work for mitochondria to maintain the ion-balance, and this extra pressure leads to oxidative stress and eventually cell death (apoptosis). A lot of research still needs to be done to determine whether cells develop pores or the membrane is completely disrupted; it's still a million dollar question to the scientists working in the field of membrane disruption and hIAPP.

Figure 6 illustrates some of the possible mechanisms that researchers believe may lead to membrane disruption. The method that causes disruption is dependent on the environmental conditions. At low concentrations in solution, peptides are seen as monomers as shown in Figure 6. As the concentration of peptide is increased, monomers start to self-assemble.[17] As the concentrations are increased even further, three mechanisms are possible: barrel-stave, detergent like, and torodial-wormhole. Each of these three mechanisms is completely different in how it causes membrane disruption. In the barrel-stave model, peptides form transmembrane pores by aggregating. In the detergent model, the membranes are pulled apart leading to complete disruption. In the torodial-wormhole model, both the lipids and peptides act as a membrane lining, and this lining is destabilized, creating a curvature strain.[18]

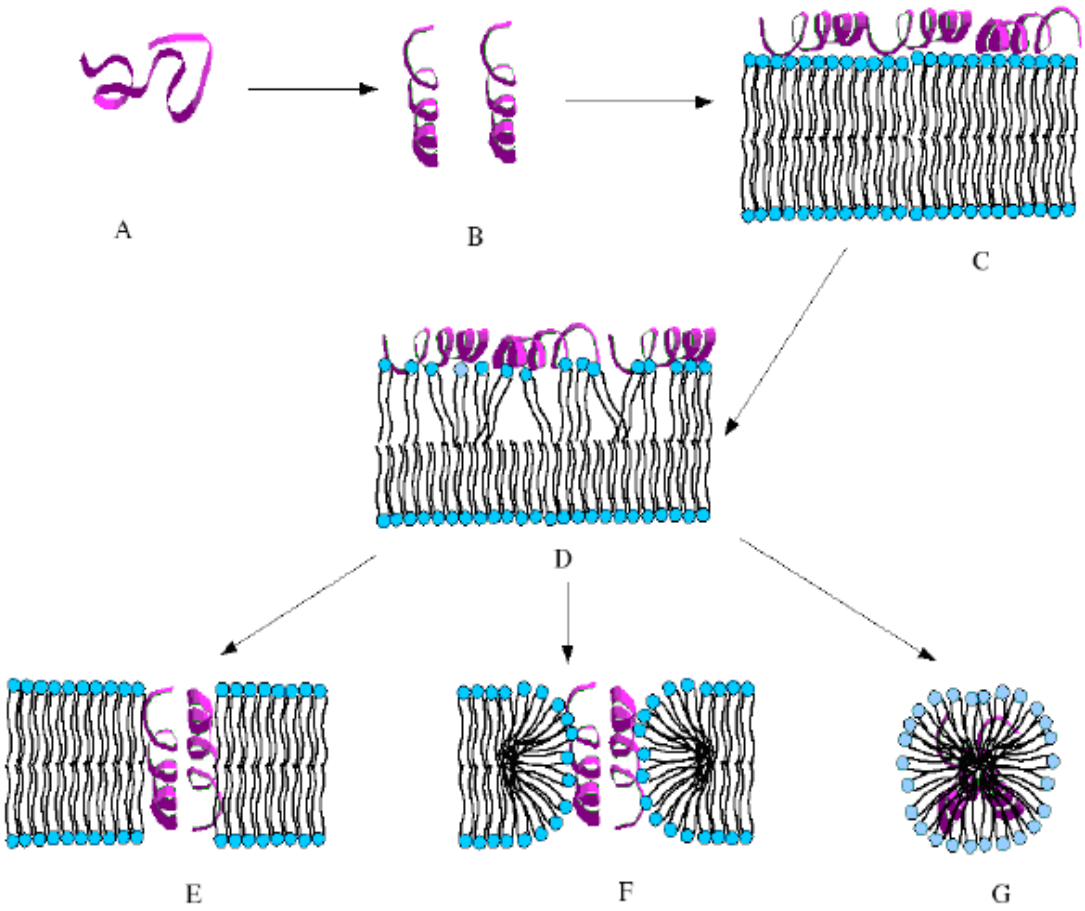


Figure 6. Six different states of how peptides are involved in membrane disruption.[17]

A: Peptides in solution, usually unstructured. B: Peptide acts as monomer. C: Peptides self assembled on a surface. D: Barrel-stave model. E: Torodial wormhole model. F: Detergent model.

Interestingly, the mature fibrils of hIAPP in solution are not cytotoxic to beta cells, implying that development of the Type II disease is not solely caused by the physical appearance

of fibrils but that the fibril forming process may be cytotoxic.[19] hIAPP fibrilization occurs *in vitro* at almost 100 fold less concentration than that *in vivo* within beta cells where both hIAPP and insulin are stored. A reasonable explanation for this would be that stabilizing agents are present *in vivo,* which help the hIAPP not to form fibrils.[20] Insulin has been proven to inhibit fibril formation *in vitro*.[21] Fibril formation of hIAPP in the absence and presence of insulin has been monitored and sub-stoichiometric amounts of insulin increase the conversion time to fibers by eight-fold. In addition, the half time for conversion increases in a linear fashion with insulin concentration.[4]

After many years of research with insulin and hIAPP, the recognition sequence between them was identified in the year 2006.[20] It was shown that the B-chain of insulin inhibits the intermediately formed coiled of hIAPP, which eventually forms a fibril forming beta-sheet conformation. Based on the results obtained from previous studies, several decapeptides based on insulin were synthesized and tested for interaction with hIAPP, and the domain corresponding to 10-19 (HLVEALYLVC) was found to bind strongly to hIAPP. This region is thought to interact with hIAPP 10-19 (QRRANFLVHS). It was proposed that this region was the recognition interface of hIAPP and insulin. In addition to this, there are some homologous regions in both hIAPP (ANFLV) 13-17, and insulin (ALYLV) 14-18, which may represent core recognition sites between the two sequences, which can prevent fibril formation. In fact, interestingly the aromatic tyrosine found in the center of the insulin sequence aligns exactly at the aromatic phenylalanine of the hIAPP sequence, making it possible for π interactions that may disrupt those between hIAPP molecules at the beginning stages of amyloid formation.[8] In this project, the recognition site of the native inhibitor insulin was employed in designing smaller peptides in an attempt to prevent or slow down the process of fibril formation and membrane

damage in model liposomes. As previously stated, insulin is a natural inhibitor of hIAPP aggregation and fibril formation. The peptide analogs were designed based on the insulin sequence reported to make contact with hIAPP. The initial sequence HLVEALYLVC was then truncated one amino acid at a time to determine the shortest insulin sequence that prevents fibril formation. The shorter sequence was designed with the aim of making it economically advantageous and easy to synthesize and may also serve as a basis for peptidomimetic drug inhibitors to cure fibril formation and the cytotoxity of hIAPP.

1.6 Peptide synthesis overview

There are several types of peptide synthesis available, but the most convenient and effective small scale production of peptides for research labs is solid phase peptide synthesis. Solid phase peptide synthesis (SPPS) was introduced by Merrifield.[22, 23] In solid phase peptide synthesis, one can easily control the peptide length as well as modify the peptide by adding any desired unnatural or D-amino acids. The important mechanism behind this process is repeated coupling and deprotection of amino acids at each and every step by various solvents. Once the solid support resin is placed in the reaction vessel, it is deprotected and the C-terminal amino acid (with amine protection) is added to attach to it. Then the N-terminus of the peptide is deprotected to allow the next coupling to occur. This process is repeated and the peptide can be obtained with ease, since it stays attached to the solid support while soluble impurities are washed away at each step. If done properly, a yield of more than 90% can be achieved. A scheme is in Figure 7.[24]

There are two types of solid phase synthesis available. One is Fmoc and the other is tBoc. These are classified on the basis of the amine protecting groups at the end of the amino acids

where further addition takes place. Fmoc, Fluorenyl-methoxy-carbonyl, is the advanced and widely used protectant group on solid phase peptide synthesis. The tBoc method was the first to be used but is less utilized today due to the requirement to use dangerous hydrofluoric acid to remove the peptide from the resin. In the Fmoc method to remove the protective group, 20% piperdine in DMF is regularly used. Last, the peptide is cleaved from the resin using trifluoroacetic acid.

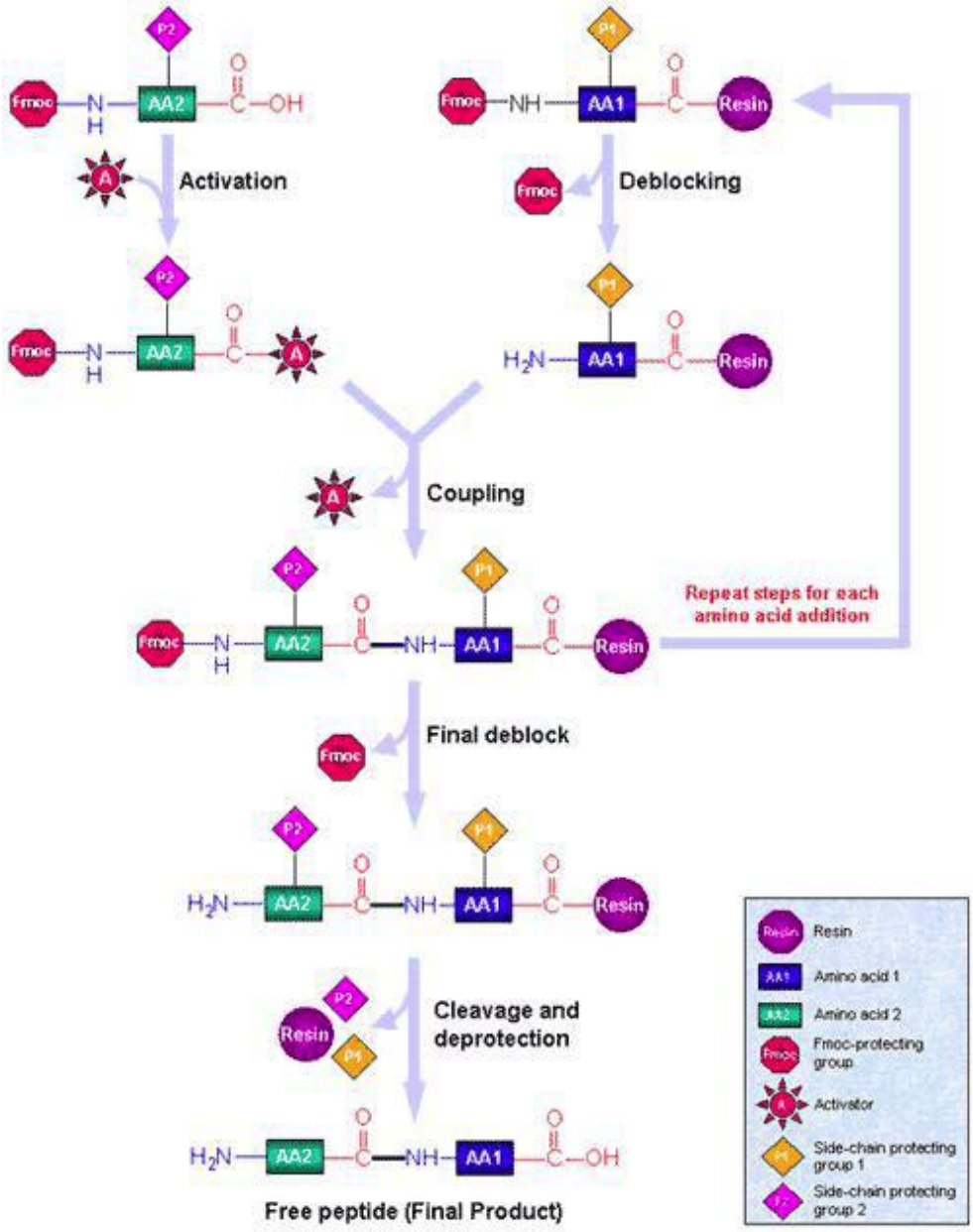


Figure 7. Schematic diagram of solid phase peptide synthesis.[24]

1.7 Dye Leakage Studies

Dye leakage studies are the best technique to study membrane damage models. Dye leakage assay studies use the property of fluorescence to determine the leakage caused by IAPP of encapsulated dye from lipid membranes. The fluorescence studies use a dye called carboxyfluorescein. First the lipid vesicles are created, allowing incorporation of carboxyfluorescein dye into the vesicles. When the membrane gets damaged, the dye is released into the media. The concentration of the leaked dye can be measured using a scanning spectrofluorometer. This can be correlated to the extent of membrane damage. Later, the percent of dye leaked can be plotted against time to determine time dependent effects.

The lipid membranes which are commonly used in dye leakage studies are Palmitoylolenylphospoglycerol (POPG), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS). The structures and relative charges of POPG, DOPC and DOPS are shown in Figure 8.[26, 27, 28]

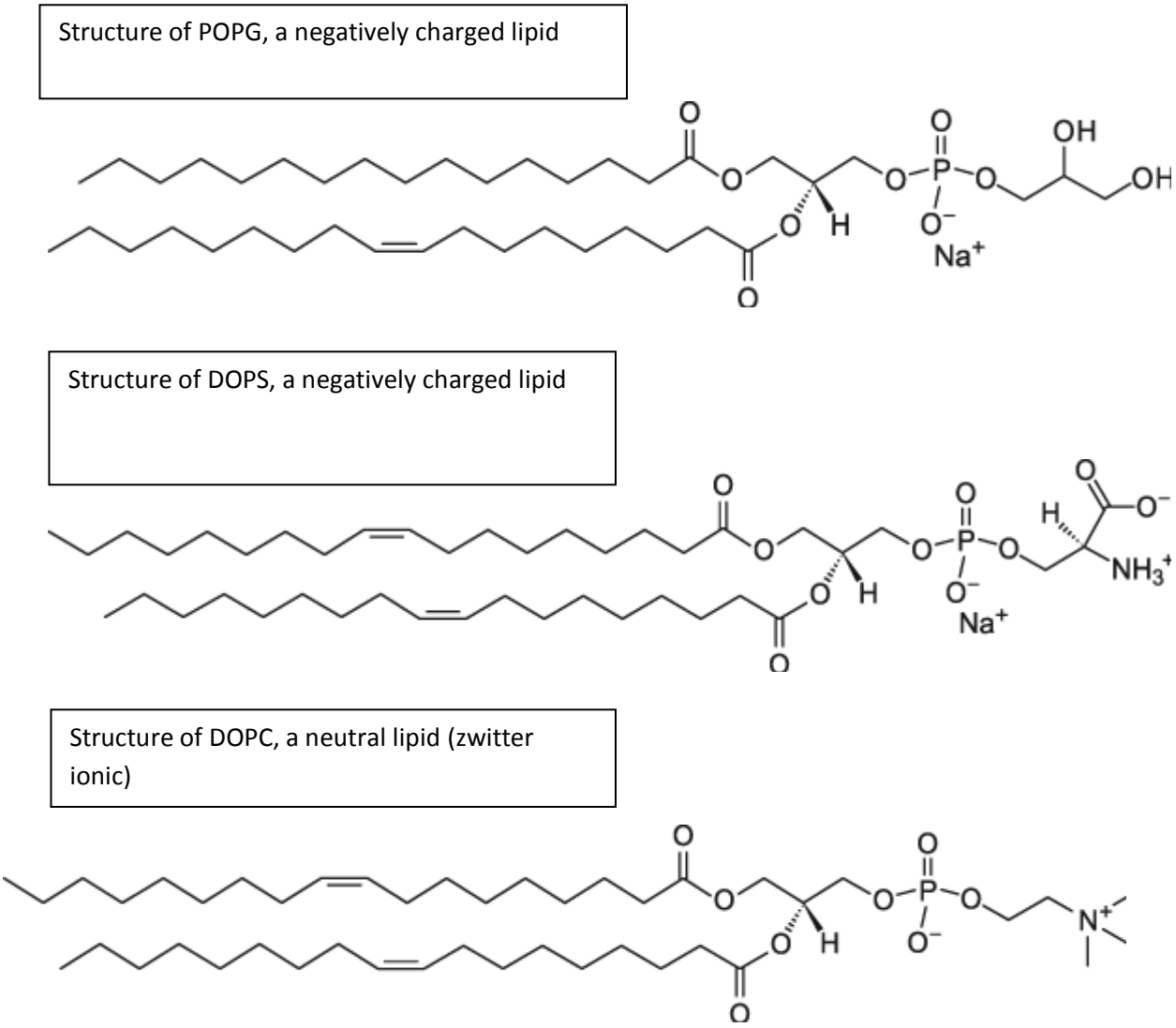


Figure 8. Structural representations of lipids POPG, DOPS and DOPC.[25, 26, 27]

CHAPTER 2

Research goals and objectives

2.1 Hypothesis

The main goal of this project was to synthesize varying length insulin-based inhibitors of hIAPP to prevent aggregation and eventually prevent cell death. Two peptides of length 9 and 10 amino acids were synthesized using solid peptide synthesis, and their action was observed in the presence of models resembling lipid membranes. The main focus of this study was to truncate a sequence of insulin that was known to make contact with hIAPP and see its action in preventing the aggregation process. In order to see its effects, first two different peptides were synthesized, LVEALYLVC and HLVEALYLVC, which were purified and confirmed with analytical HPLC and LC-MS. Then the activity of the peptides was tested in the presence of hIAPP 1-19 (the N-terminal region also known to cause damage) and hIAPP 1-37 in model membranes.

Initially we started testing insulin analog inhibitors in the presence of hIAPP 1-19 and hIAPP 1-37 in POPG. Later, based upon other studies, we confirmed the best mimicking membrane models were DOPC: DOPS in a 7:3 ratio. Then we continued performing experiments with the vesicles that best mimic the pancreatic cells in humans.

CHAPTER 3

Experimental Procedures

3.1 Peptide Synthesis

The insulin analog inhibitors LVEALYLVC and HLVEALYLVC were synthesized using standardized Fmoc methods using a Ranin PS3 peptide synthesizer on a 0.1 mmol scale basis. The required amino acids essential for the synthesis of peptides were weighed (0.4 mmoles each) in a vial. All vials consisted of a 1: 1 ratio of amino acid and coupling agent, HBTU (Hydroxy-O-(Benzotriazol-1yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate). The amino acid vials were arranged in sequence from C-terminus to N-terminus on the synthesizer. 0.4 M N, N- diisopropylethylamine in dimethylformamide (DMF) was used as the activator in peptide synthesis. The synthesis of peptides was done using a single coupling program. The solvent was N, N-dimethylformamide, and a 20% piperidine solution was used for deprotection. All of the amino acids used in the research project were bought from Midwest Biotech Inc. or Protein Technologies Inc., while solvents and other chemicals were purchased from Fischer Scientific. All of the amino acids, solvents, and chemicals were at least ACS standard grade.

3.2 Peptide Cleavage

After the peptide synthesis, the peptide bound to resin was transferred to a coarse fritted glass funnel with the help of methylene chloride and DMF. The resin in the coarse fritted glass funnel was washed with DMF, ethanol, and methylene chloride and dried under vacuum for 30 minutes to one hour. Then the peptide bound resin was transferred to a 50-ml beaker with a

stirring bar and placed in an ice bath. The cleavage cocktail was added, which contained 0.5 ml anisole, 10 ml TFA, 1 crystal of phenol, and 0.5 ml water. The reaction was stirred for two hours at room temperature. This enabled the resin to separate from the peptide, after which the peptide can be found in a dissolved state, whereas the resin is found in an insoluble state (remains as solid).

The resin was separated from the peptide through a filtration step, using a coarse fritted glass funnel. The filtrate was collected using a side arm flask, and repeated washing with TFA enabled the collection of all of the peptide through a filtration process. To the filtrate collected, 60 mL of cold diethyl ether was then added while stirring slowly, which precipitated the peptide from solution so that it could be separated and collected through filtration through a fine fritted glass funnel. This solid was vacuum dried for 30-45 minutes, and then it was transferred to a lyophilization flask containing 70% acetonitrile in water. An equal amount of distilled water was added and the flask and contents were frozen. The peptide was kept on a lyophilizer under vacuum overnight to remove any liquid, leaving behind solid peptide.

3.3 Peptide Purification and Analysis

Once the lyophilization was done, the peptide obtained was weighed on an analytical balance, and then the peptide was dissolved in the minimum amount of DMF and injected on to the reverse phase high performance liquid chromatography (RP-HPLC) column. The HPLC used was purchased from Waters, Inc. The absorbance used depended upon the type of amino acids present in the peptides. If the peptide contains phenylalanine the characteristic absorbance is seen at 254 nm, and peptides containing tyrosine have a characteristic absorbance at 280 nm. Both of the peptides used in this project contained tyrosine, so 280 nm was used. The solutions used for

the mobile phase were 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (ACN, solvent B). Initially to equilibrate the column, the mobile phase solvents were run for 20 minutes with 90% solvent A and 10% solvent B. For separation, a gradient was selected with a flow rate of 10 ml /min, changing the composition of the mobile phase to 50% solvent B over the time period of two hours. Depending upon the absorbance of the peaks, the elutant was manually separated in test tubes. Typically the larger peak was the desired peptide. The collected solutions of pure peptide were combined, frozen, and lyophilized overnight.

After the purification, the desired peptide identity and purity were confirmed in two ways. One was running the peptide solution through an analytical column by HPLC, and the other was confirming its mass by liquid chromatography combined with electrospray mass spectrometry (LC-MS). Using the first method, RP-HPLC, the peptide solution that was previously purified was run on the analytical column to make sure no other contaminants were present. The peptide purity was calculated by integration of peak areas using analytical RP-HPLC on a Waters dual pump system that consisted of a gradient controller and UV detector. The column used was a Phenomenex Jupiter column. The mobile phase used was solvent A (0.1 % TFA in water) and solvent B (0.1 % TFA in acetonitrile). Initially the pumps were run on isocratic to equilibrate the column at 1 mL/min with 100% solvent A. Then a gradient was designed at the same flow rate but changing the composition of solvent B over 30 minutes from 0 to 66 percent. Once the column was equilibrated, a tiny amount of pure peptide was dissolved in a small amount of DMF and injected. There should be only one major peak, which confirms that the peptide is pure, or the previous purification step must to be done again. In this case, the target peptides were found to be greater than 99 percent pure, and their respective molecular weights were 1022.28 (LVEALYLVC) and 1159.42 (HLVEALYLVC). The pure peptide was

then weighed, labeled, and stored in the refrigerator until the dye leakage assays were ready to be commenced. Table 1 below shows the description and details of both the preparative and analytical HPLC and the columns used in the research project.

Table 1. Specifications of HPLC used in the project

Name and Function	Specifications
Preparative HPLC	Water 600S controller Waters 616 pump Water 484 tunable Abs. detector Fisher Recordall Series 5000
Preparative column	Phenomenex Jupiter 10µm, C18 250 * 21.2 mm and 300 A^0
Analytical HPLC	Waters automated gradient controller Waters 515 HPLC pump Waters 490 E programmable multi-wavelength detector Shimadzu CR901 chromatopac integrator
Analytical column	Phenomenex Jupiter 5µm, C18, 250 *4.6mm and 300 A^0

3.4 Dye leakage assay experiments

The main principle used in this experiment was fluorescence, and the fluorescent dye carboxyfluorescein was used. When this dye escapes or leaks from the vesicles due to membrane damage caused by hIAPP, the fluorescence measured is proportional to the amount of dye

released. For this experiment we used two different types of lipids: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS).

3.4.1 Vesicle creation

For the type of vesicles being created, 5mg total of DOPC and DOPS in a 7:3 ratio were weighed in a test tube and dissolved in 2 mL of chloroform. Excess chloroform was blown off with nitrogen after the lipid was dissolved. Once the excess chloroform was blown off, it left a thin film of lipid behind, which was dried under vacuum by lyophilizer overnight. Then the dye was encapsulated in the lipids by dissolving 5.64 mg of carboxyfluorescein in 0.5 mL of sodium phosphate buffer at pH 7.5. Once the dye solution was ready, 0.5 mL was taken and added to the dried lipid test tube, which was then vortexed and subjected to freeze-thaw cycles five times using liquid nitrogen or dry ice/acetone. This enabled the dye encapsulation inside the lipid vesicles as they formed. The test tubes were labeled and stored at -20^{o}C for later use.

When it was time for the assay, the dye encapsulated lipid test tubes were removed from the freezer and thawed, and the extruder was assembled. Then the lipid solution was passed through the extruder 21 times. The extruder was purchased from Avanti Polar Lipids and consisted of seven pieces, including two plastic syringe insert pieces, a plastic washer, two exterior steel pieces, and two rubber o-rings that fit into groves of the plastic insert pieces. Glass syringes were inserted here. In between the two exterior steel pieces was placed a 0.1 micron polycarbonate membrane and two filter supports, which were immersed in buffer before carefully placing them between the syringe insert pieces. The extrusion was done by passing the solution through the poly-carbon membrane 21 times, which required some pressure. This made

the vesicles the desired size and uniform throughout the solution. Once the extrusion was complete, it was time to dissemble the extruder and save the vesicle solution in a test tube.

The vesicle solution was placed on the top of a gel exclusion column with a Pasteur pipette, and allowed to run through the gel bed. This technique separates small dye molecules from the large vesicles. The large vesicles reach the bottom first since they are excluded from the gel pores, and the smaller molecules move more slowly. As the vesicles passed through the column, different color bands started to appear. The first yellow-colored fraction that came off the column was collected in a microvial. Then the column was flushed with buffer. The gel used was Sephadex G-50 from Sigma. In a similar fashion, vesicles without dye were also prepared, created in the same way but without carboxyfluorescein. The same steps of extrusion were done, but the gel exclusion was not necessary since there was no free dye to separate from the vesicles.

3.4.2 Preparation of detergent, control, and peptide solutions

For the preparation of the detergent solution, Triton X 100 detergent was used. 40µL of detergent was added to 250µL of sodium phosphate buffer at pH 7.5. This detergent causes damage to all of the lipid membranes prepared, leading to 100% of the dye leaking from the vesicles. This provides a positive control (100% leakage) when 50µL of this solution and 20µL vesicle solution were added to 1430µL buffer.

The control solution was prepared by dissolving 50µL of DMSO and 20µL of lipid vesicles in 1430µL of sodium phosphate buffer. The control will not cause any damage to the lipid membranes, causing 0% leakage. The control solution does not contain detergent or peptide to cause damage to lipid membranes.

Preparation of peptide solutions: Both amylin and insulin fragment stock solutions were prepared to 0.72 M concentrations of peptides. Both insulin and amylin analogs were weighed and dissolved in DMSO to make the concentration 0.72M.

For amylin samples, 1.0 mg of amylin (molecular weight =3905 g/mol) was weighed and dissolved in 385µL of DMSO, giving the 0.72M stock solution.

For insulin samples, 3.0 mg of insulin (molecular weight = 5808 g/mol) was weighed and dissolved in 715µL of DMSO in a vial, giving a 0.72 M stock solution. Based on their individual molecular weights, insulin analogs were weighed out and dissolved in the required amount of DMSO, usually 375µL of DMSO, which gave rise to the final concentrations of 0.72 M. For LVEALYVC (MW 1022.28), 0.28mg was dissolved in 375µL DMSO. For HLVEALYVC (MW 1159.42), 0.31mg was dissolved in 375µL DMSO. In order to test the effects of insulin and amylin themselves, the samples vials were made up as shown in Table 2.

Table 2. Sample tubes of Amylin/Insulin in varying volumes

Tube	Amylin/Insulin (µL)	Lipid vesicles (µL)	Buffer (sodium phosphate pH 7.5) (µL)	Concentrations of Amylin/Insulin (µM)
1	1	20	1479	0.5
2	2.5	20	1477.5	1.2
3	5	20	1475	2.4
4	10	20	1470	4.8
5	12.5	20	1467.5	6
6	25	20	1455	12
7	37.5	20	1442.5	18
8	50	20	1430	24

To study the effects of variable amounts of the synthesized insulin analogs with a constant concentration of amylin (2.4 µM), the sample tubes were made up as presented in Table 3.

Table 3. Samples of constant amylin plus varying concentrations of synthesized insulin analog inhibitors in varying volume

Tube	Amylin (µL)	Insulin Analog (µL)	Lipid Vesicles (µL)	Insulin Analog Conc. (µM)	Buffer (µL)	Ratio of insulin analog/amylin
0	5	0	20	0	1475	N/A
1	5	1	20	0.5	1474	1:5
2	5	2.5	20	1.2	1472.5	1:2
3	5	5	20	2.4	1470	1:1
4	5	10	20	4.8	1465	2:1
5	5	12.5	20	6	1462.5	2.5:1
6	5	25	20	12	1450	5:1
7	5	37.5	20	18	1437.5	7.5:1
8	5	50	20	24	1425	10:1

3.5 Measurements of fluorescence values using Flx 800 microplate reader

All of the test tubes containing buffer, peptides, and vesicles were mixed well, and 300 µL of each solution from the test tubes was transferred to a 96 well plate. Tips were changed for each sample. All of the trials were performed in triplicate, and average values were calculated.

A Biotek FLx Fluorimeter was used for this study. The excitation and emission wavelengths for the instrument were 485 nm and 528 nm, respectively. When samples were transferred and ready to go, the instrument lid was closed; then the instrument measured the fluorescence values depending upon the dye leaked through the vesicles. All assays in this study were run for three hours, and the interval time for reading was 59 seconds. The formula used to calculate fraction of dye leakage from the lipid membrane was:

Percent Fraction Leaked = [(Value-Control)/(Detergent-Control)] *100

Finally, the percent leakage versus time was plotted for each concentration of each peptide and compared against each other. Higher percent leakage implies higher damage to the membrane. From the graphs plotted, time dependence effects could also be observed.

4. RESULTS AND DISCUSSION

Using the solid phase peptide synthesis technique, two analogs of insulin were synthesized, LVEALYLVC and HLVEALYLYC. These two insulin analog inhibitors were designed to interact with hIAPP and prevent the aggregation process, thereby protecting the membranes from damage.

Dye leakage studies were done to see the activity of these inhibitors in different lipid membranes that mimic pancreatic cells. In this dye leakage assay, the lipid vesicles are treated with peptides (insulin analog inhibitors and hIAPP). The dye escapes or leaks from the vesicles, which enables the monitoring of fluorescence. Percent fluorescence is directly proportional to the amount of membrane damage in a given amount of time. These results indicate the extent of damage to the lipid membranes caused by the hIAPP in the absence and presence of synthesized peptides. All of these dye leakage studies were run for 3 hours in triplicate, and average values were taken to plot the graphs. It should be noted that the results obtained from each individual dye leakage assay may not be comparable to other assays, due to variability in conditions such as temperature and vesicle preparation quality, and unavoidable experimental errors. Results are considered valid within individual trials, however, since each trial has its own control tubes.

The lipid model membranes used to mimic the somatic body cells are 7:3 DOPC/DOPS. This ratio and lipids are commonly used in liposome models of membrane systems. DOPC is zwitterionic, and DOPS is a negatively charged lipid. This ratio with 30% negatively charged lipid membrane serves as the best model for pancreatic beta cells to study membrane disruption.

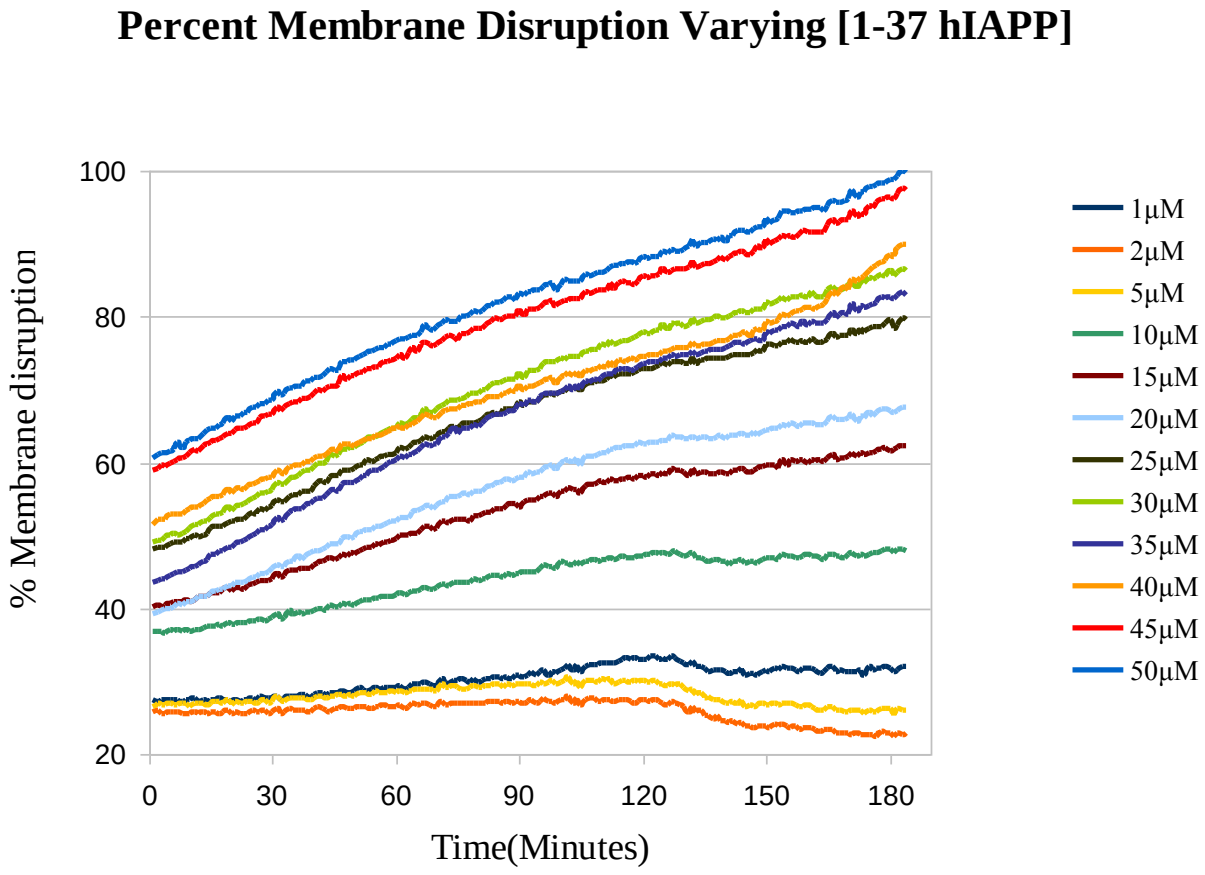


Figure 9. Percent membrane disruption of varying concentrations of hIAPP over time in 7:3 DOPC: DOPS.

From the dye leakage assay studies, the interaction between the pancreatic beta cell-mimicking model membranes and twelve concentrations of hIAPP are displayed in Figure 9. The fluorescence values were collected every minute for three hours. The graphs were made by using the formula described earlier, plotted against time for each concentration. From Figure 9 it can be seen that membrane leakage increased over time, implying time dependence and the possibility of aggregate formation. Concentration-dependent damage can also be seen. As the

concentration is increased, the damage increases proportionally, and the highest concentration tested, 50μM, yielded 100% leakage after 3 hours.

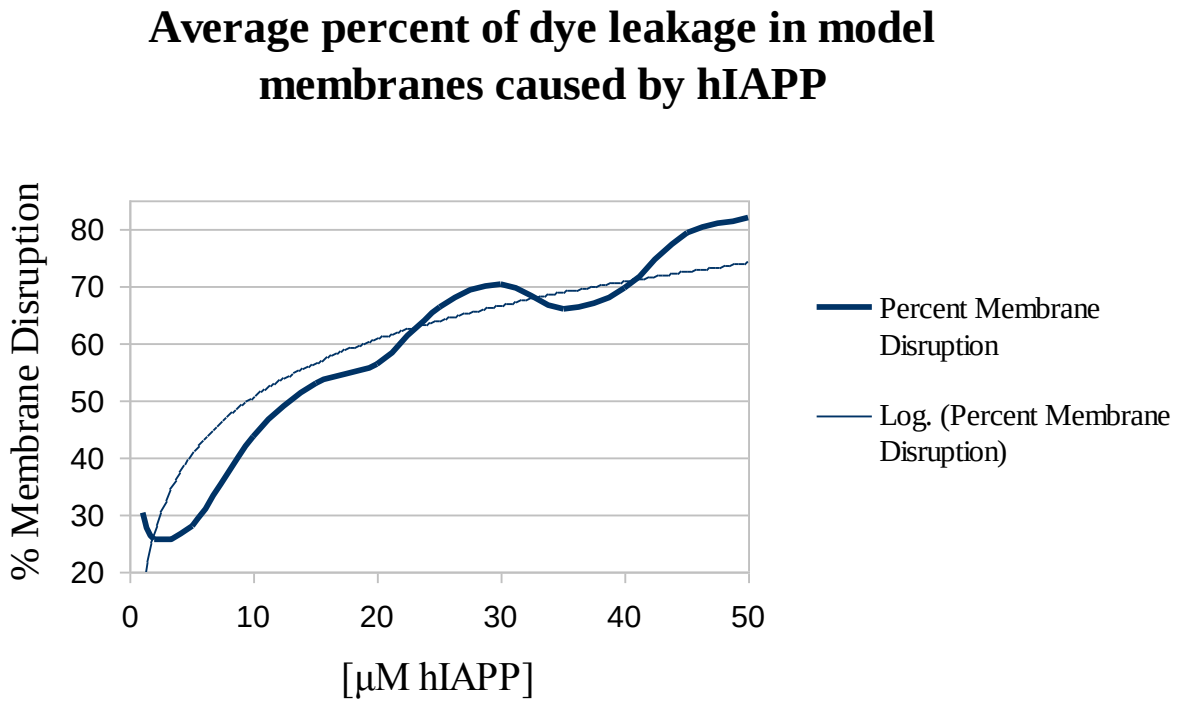


Figure 10. Average values of percent dye leakage against varying concentration of hIAPP in 7:3 DOPC/DOPS.

In order to show the concentration dependent damage of hIAPP, the average values were taken at each time point and plotted against concentration values of hIAPP. In Figure 10, the time-averaged percent membrane disruption was plotted against varying concentrations of hIAPP, and a clear concentration dependent damage can be seen. In this graph, 50μM hIAPP

produce over 80% membrane damage. A logarithm regression was also done on the graph with a correlation coefficient of 0.87.

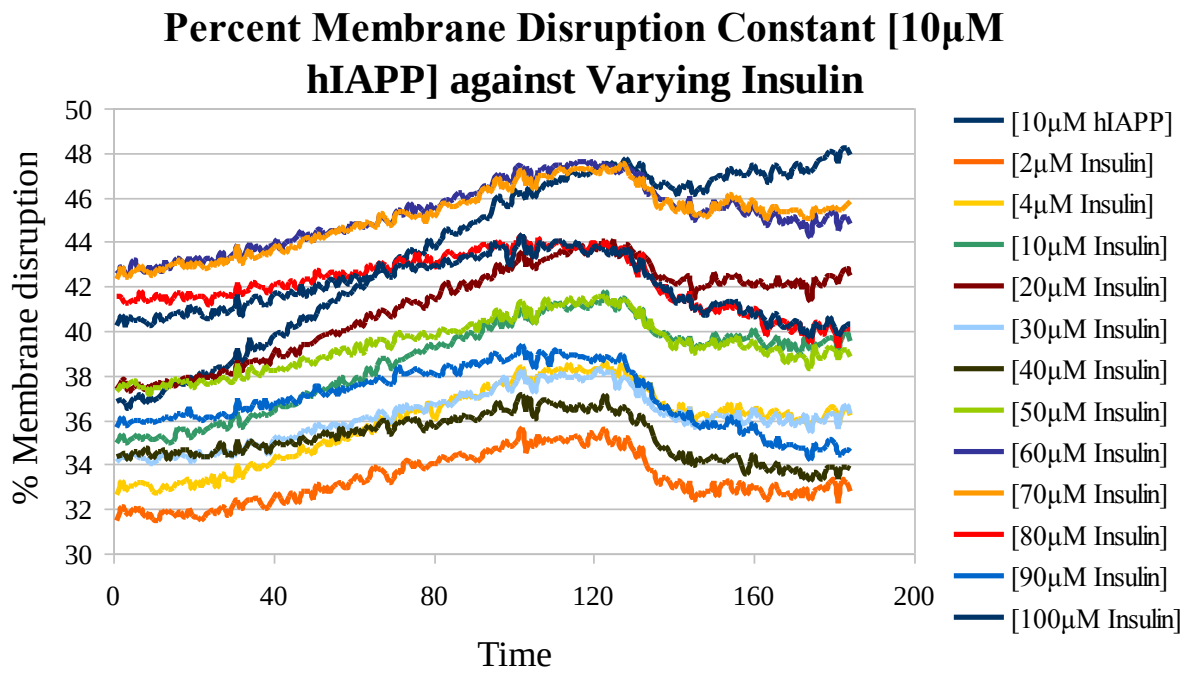


Figure 11. Percent membrane disruption observed with 10µM constant concentration of hIAPP and varying concentrations of insulin.

In order to observe both increases and decreases in membrane damage brought about by insulin, an optimal concentration of hIAPP was selected. A 10µM concentration of hIAPP was used and twelve varying concentrations of insulin, from 0µM to 100µM, were run against the constant hIAPP. Figure 11 shows a mixed effect of insulin with no clear evidence of concentration dependence. However, it is clear that hIAPP at 10µM concentration without insulin showed much more damage as the time increased. Addition of insulin does appear to

reduce membrane damage over time, implying a possible role in slowing the fiber formation.

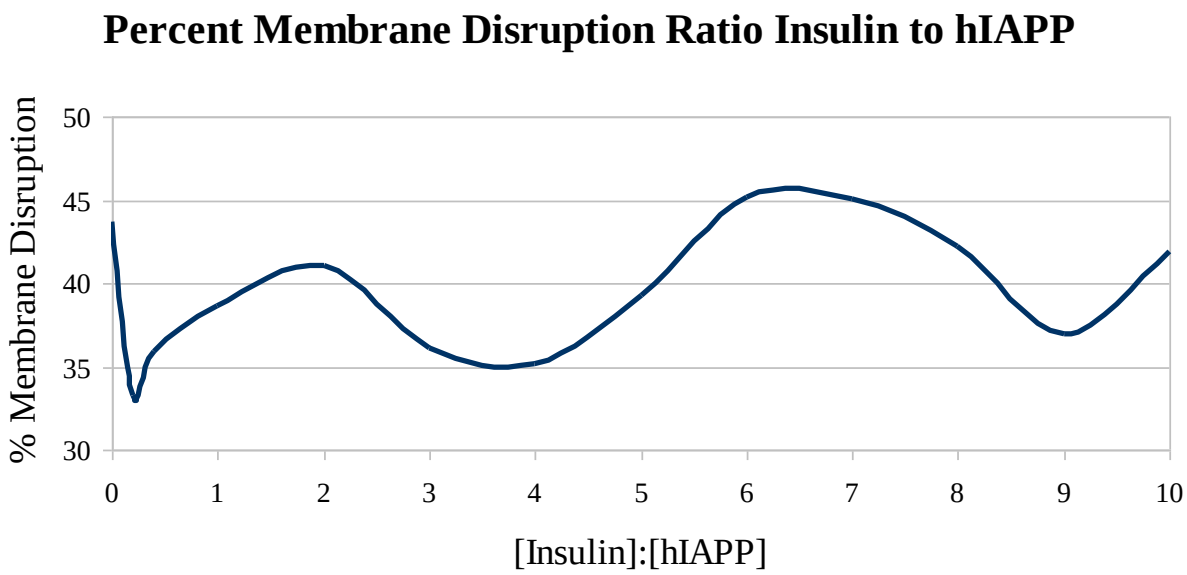


Figure 12. Average values of percent dye leakage for constant 10μM concentration of hIAPP against varying ratios of insulin to hIAPP.

The graph in Figure 12 was obtained by taking the average percent membrane disruption over time versus the ratio of insulin to hIAPP. It can be seen from the graph that membrane disruption is generally higher without insulin, confirming a possible role of insulin in preventing membrane damage. At a 0.2:1 ratio of (insulin)/ (hIAPP) there is a sharp decrease in the percent membrane damage, indicating a 13% reduction. Beyond this ratio of (insulin)/ (hIAPP), the membrane damage reduction is variable and less notable. One hypothesis for this observation is that hIAPP forms a five-subunit complex and binds to one subunit of insulin forming a six-unit complex (hexamer). This hexamer may not interact with membranes as the monomers do. This optimal ratio of 0.2:1 (insulin)/ (hIAPP) was seen in many trials and is interesting because

insulin is stored in secretory granules of the pancreas as a hexamer. Still, the reduction in membrane damage observed was minimal at 13%.

Figure 13 shows the effect of varying concentrations of the first inhibitor, HLVEALYLVC, in 7:3 DOPC/DOPS in the presence of 10μM hIAPP.

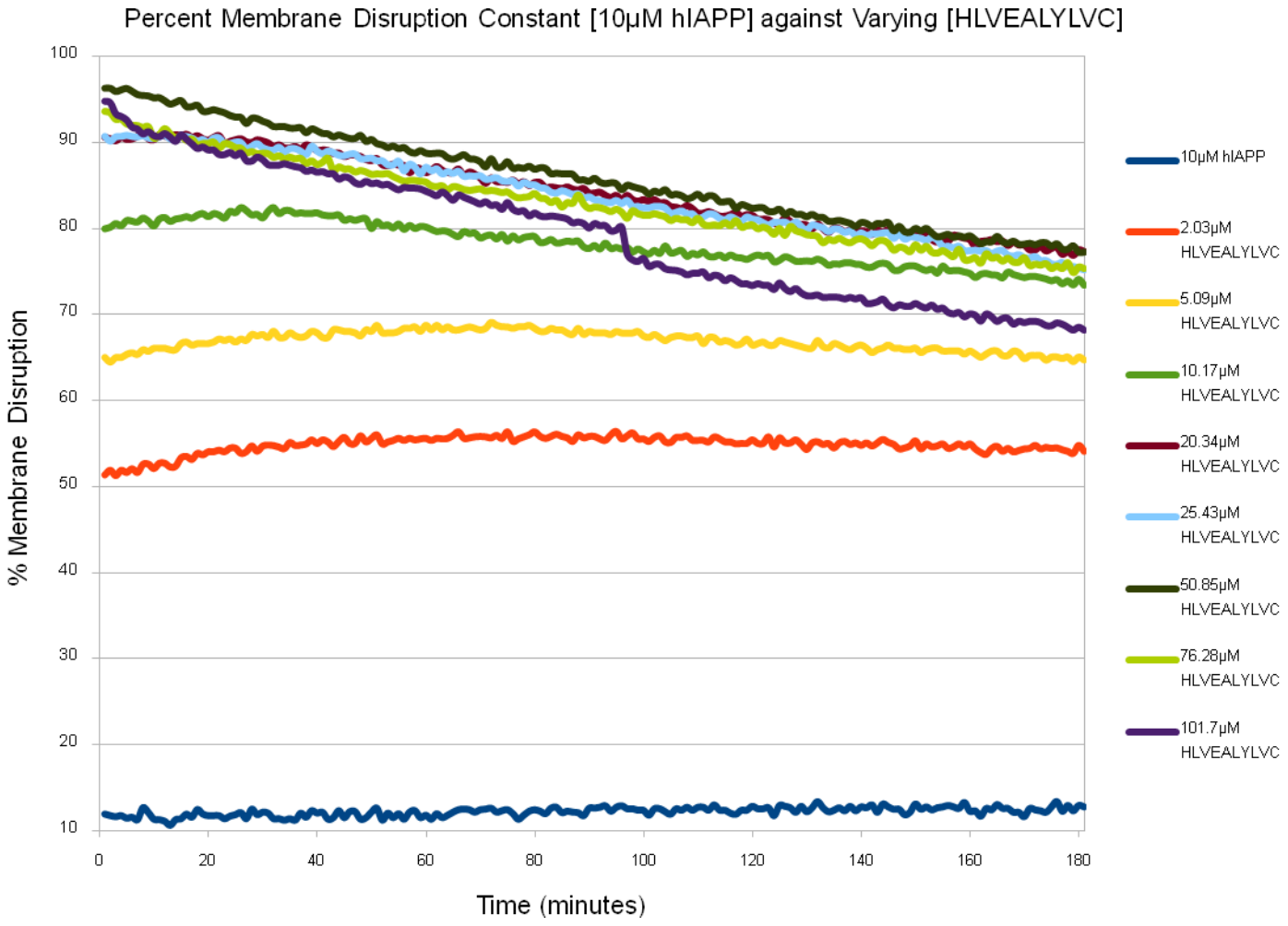


Figure 13. Percent membrane disruption of HLVEALYLVC against 10μM hIAPP 1-37.

The percent membrane disruption caused by hIAPP in the presence of HLVEALYLVC was observed by keeping the hIAPP concentration constant at 10μM. The insulin analog concentration was varied from 0μM to 101.7μM. As shown in Figure 13, as the concentration of analog is increased, the percent membrane disruption also increases significantly. Even at low concentrations, the insulin analog is apparently aiding in causing damage to the lipid mimicking membrane models. As the concentration is increased to 10μM inhibitor, the percent membrane disruption levels goes to about 80%. After that point, the damage levels off. This significant increase in membrane damage may be caused by the analog interacting with the hIAPP. Since the only difference in sequence between this analog and the other one (which showed a different effect, as will be discussed below) was the histidine at the N-terminus, it is also possible that the partial positive charge of this side chain gives the analog the ability to interact with the membrane directly rather than with hIAPP, making their effect additive.

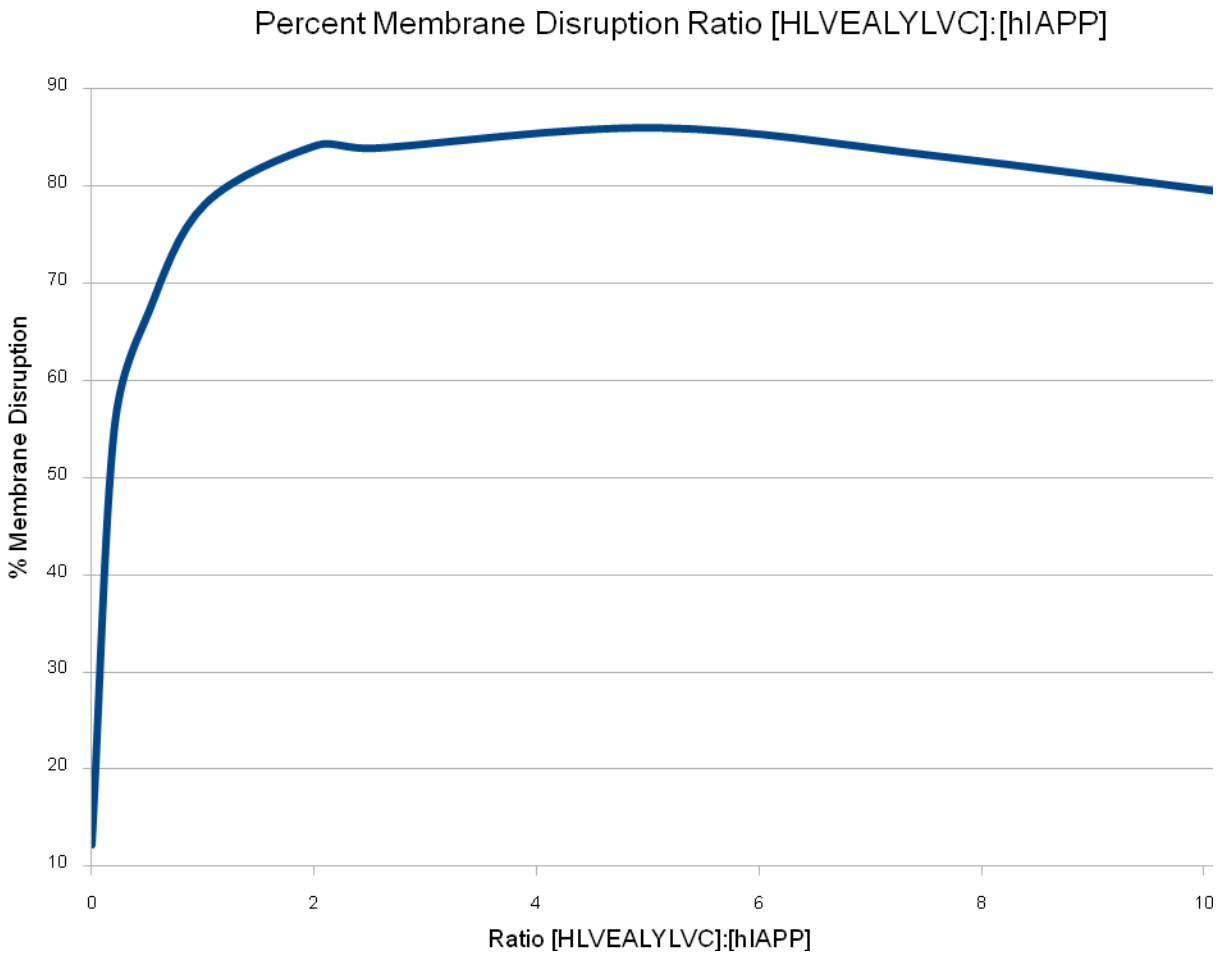


Figure 14. Ratio of insulin analog HLVEALYLVC to hIAPP versus percent membrane disruption.

Figure 14 shows time averaged disruption versus the ratio of inhibitor to hIAPP. It is clear from the graph that the insulin analog is not preventing membrane disruption at any ratio and is in fact contributing somehow to the membrane damage. However, once the 1:1 ratio is reached, damage is maximized and further increases in peptide have no significant effect.

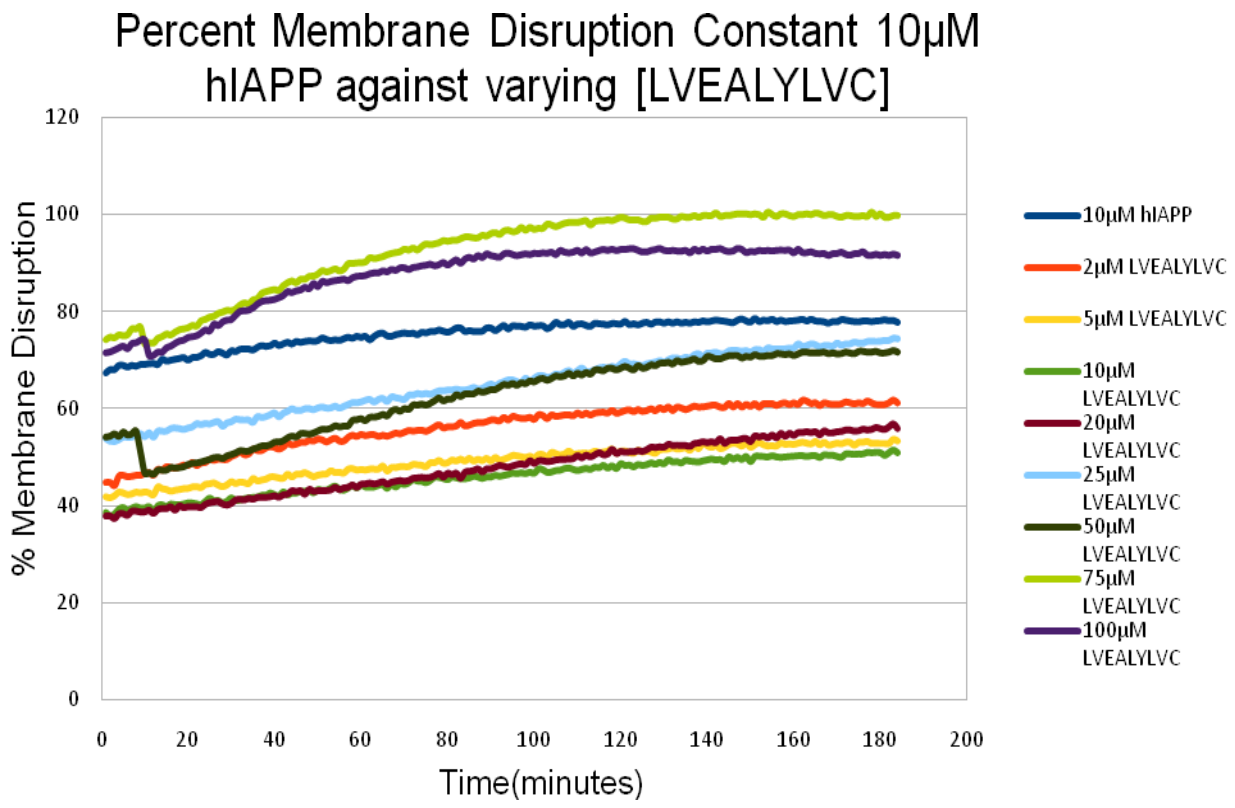


Figure 15. Percent of dye leakage from model 7:3 DOPC/DOPS liposomes in the presence of 10μM hIAPP and varying concentrations of LVEALYLVC over time.

In Figure 15, we can see the effect of insulin analog LVEALYLVC, keeping the hIAPP concentration constant at 10μM and running the assay over 180 minutes. The insulin analog concentrations ranged from 0μM to 100μM. From the graph the insulin analog LVEALYLVC is protecting the membrane from disruption caused by hIAPP at lower concentration; the membrane disruption is reduced at LVEALYLVC concentrations lower than 50μM. When the concentration of insulin analog LVEALYLVC is increased to more than seven times that of

hIAPP, it becomes damaging and disrupts the membrane above the levels of hIAPP itself.

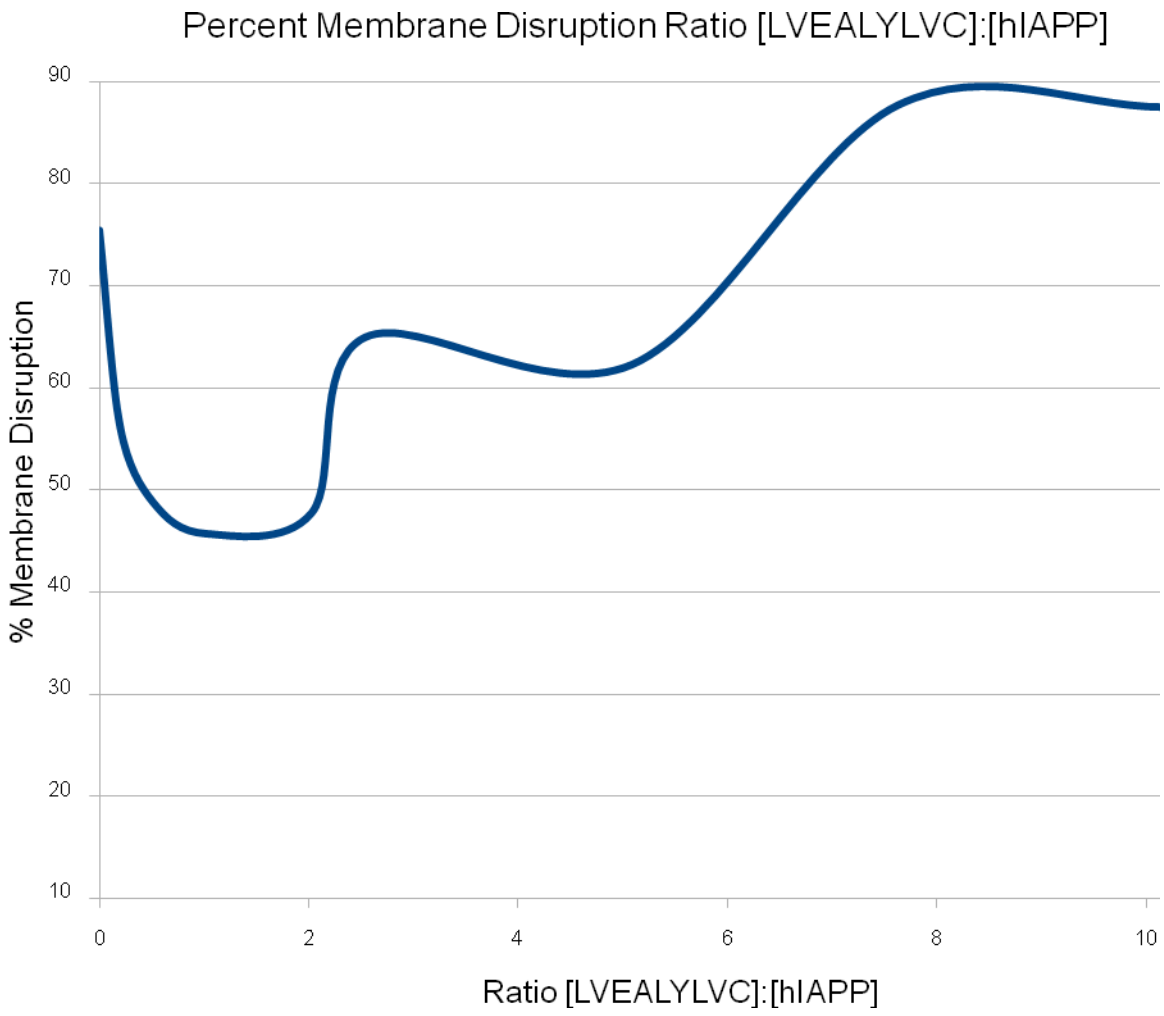


Figure 16. Average percent of dye leakage from model membranes in the presence of varying ratios of [LVEALYLVC]: [hIAPP].

Figure 16 shows percent membrane disruption versus the ratio of inhibitor LVEALYLC to hIAPP. It is clear from the graph that insulin analog is reducing membrane disruption at the 0.2: 1 ratio but is contributing somehow to the membrane damage as the ratio of inhibitor is

increased. The liposomes are disrupted to a 15% greater extent at 75μM peptide concentration. This result is interesting since the synthesized peptide shows the same approximate ratio range for optimal inhibition as does insulin itself (0.2:1 to 2:1), and its reduction in hIAPP induced damage (30%) is actually better than that of insulin (15%). However, the increased damage at high ratios was not observed with native insulin.

5. SUMMARY

Overall, the two synthesized peptides behaved differently at low concentration, with the shorter LVEALYLVC demonstrating a similar profile to that of insulin in reducing damage caused by hIAPP. These results are somewhat promising in that truncated analogs may be able to mimic the action of insulin in reducing cell damage caused by hIAPP, leading to potential peptidomimetic drugs. The longer sequence HLVEALYLVC is not protective at any concentration and is in fact an activator of membrane damage. At high concentrations, both peptides are destructive, unlike insulin itself. They may actually co-aggregate with hIAPP at high concentrations. Given their similar sequences to the internal sequence of hIAPP (ALYLV versus ANFLV in hIAPP), they may actually be acting as analogs of hIAPP rather than insulin.

6. FUTURE DIRECTIONS

Future work will include Thioflavin T assays that measure aggregation instead of membrane damage so that the cause of the membrane activity can be further elucidated. This will provide more information regarding the mechanism of damage and the aggregation process (i.e, determine if dye leakage and aggregation/fibril formation are correlated). Eventually more peptides will be tested and a shorter and/or more effective insulin analog may be identified. Instead of membrane mimicking lipids, the final goal is to do studies in pancreatic cells *in-vivo*. We also want to see if hIAPP competes with insulin and binds at the insulin receptor to impair glucose utilization. This may give more insight into understanding how Type II diabetes develops.

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