2009

Insulin based inhibitors of human islet amyloid polypeptide (hIAPP) and their effect on hIAPP-mediated membrane damage in type 2 diabetes mellitus

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Insulin based Inhibitors of Human Islet Amyloid Polypeptide (hIAPP) and their effect on hIAPP- mediated membrane damage in Type 2 Diabetes Mellitus

by

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Thesis

Submitted to the Department of Chemistry

Eastern Michigan University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Chemistry

Dr. Deborah Heyl-Clegg, PhD, Chair

December, 2009

Ypsilanti, Michigan.

DEDICATION

This research is completely dedicated to my parents, who gave me much support and encouragement during the course of my study. My life could never have been possible without their love, affection, and blessings. I deeply express my love to my mother, Mrs. Jayalakshmi, for her unending support and motivation during tough times. I am indebted to my father, Mr. Mallikarjun, for his strong belief and hope in me and providing me the necessary assistance. I would also thank my friends and colleagues (Shyam, Ratheesh, and Srikanth) who encourage me during my stay at Eastern.

ACKNOWLEDGEMENTS

I deeply express my sincere gratitude to my research advisor, Dr. Deborah Heyl-Clegg, for her endless support, guidance, and confidence throughout my Master's program at Eastern.

I would like to thank my graduate coordinator, Dr. Timothy Brewer, for his invaluable guidance during the course selection process and providing me the financial support throughout my program.

I sincerely thank my committee members, Dr. Timothy Brewer and Dr. Hedeel Guy Evans, for being in my thesis committee and giving their valuable suggestions during the review of my thesis.

I would like to thank our Head of the Department, Dr. Ross Nord, for providing financial support throughout the program and also for his valuable time to review my thesis.

I would like to thank my earlier graduate coordinator, Dr. Krishna Swamy Rengan, for his advice and support during the odd times.

Also, I would like to thank the teaching and non-teaching staff of Chemistry Department, especially Carol Orlowski and Brain Samuels, for their timely help during my learning experience at Eastern.

I would like to thank EMU Graduate School for providing me academic and financial support.

Abstract

Amylin (Islet Amyloid Polypeptide, IAPP) is a 37 amino acid polypeptide, co-secreted with insulin from pancreatic beta cells, that plays a role in the damage of cell membranes by forming amyloid fibrils in Type 2 diabetes. Insulin has been found to inhibit hIAPP (Human Islet Amyloid Polypeptide) aggregation. The HLVEALYLVC amino acid region of insulin contacts hIAPP near the N-terminus. Truncated and modified analogs of insulin containing the binding region (VEALYLV, VEALFLV and EALYLV) were synthesized and purified, and their actions were studied on model lipid membranes in the presence of hIAPP 1-19 and hIAPP 1-37.

Results indicate that the presence of the aromatic hydroxyl group on tyrosine is not a requirement for activity and may be detrimental to the interaction with hIAPP. The analogs were found to be ineffective against the actions of hIAPP 1-37 and were found to show similar actions to insulin itself. In addition, at high molar ratios of inhibitor to hIAPP 1-37, they were found to promote membrane damage. However, VEALYLV was found to be effective in reducing the dye leakage caused by hIAPP 1-19 at higher molar ratios. This implies that the small inhibitors may block initial damage caused by the N-terminus of hIAPP but are unable to stop fiber formation mediated by the C-terminal region.

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1. INTRODUCTION

1.1 What is Diabetes?

Diabetes is also known as diabetes mellitus (DM) and is characterized by high blood glucose levels (hyperglycemia), which is due to reduced insulin secretion or reduced insulin action. Insulin is responsible for a number of physiological effects, among them allowing the movement of glucose from the blood into the cell. The symptoms of DM include polyuria, polydypsia, and increased hunger. DM has several types.[1] They are:

1. Type 1 diabetes mellitus (T1DM)
2. Type 2 diabetes mellitus (T2DM)
3. Diabetes due to other specific mechanisms/diseases
4. Gestational diabetes mellitus

Type1 diabetes mellitus

T1DM is also called insulin dependent diabetes mellitus (IDDM) or juvenile onset diabetes mellitus, which occurs due to loss of insulin-producing pancreatic beta cells. This will lead to deficiency of insulin. Children are the most common victims of T1DM, but it may be found in adults also. The treatment for T1DM includes injection of insulin hormone.

Type 2 diabetes mellitus

T2DM is called non-insulin dependent diabetes mellitus (NIDDM) or adult onset diabetes mellitus (AODM), which occurs due to insulin resistance and further leads to

decreased production of insulin.[2] Insulin resistance is defined as the incapability of muscle, liver, and fat cells to utilize the available insulin. This leads to high levels of insulin (as the beta cells secrete more in response to the insensitivity) and glucose in blood, causing T2DM.

Diabetes due to other mechanisms

This form includes mutations in the specific genes responsible for beta cell function and other pathological diseases. Some of the genes responsible for beta cell function are HNF 1α, HNF 4α, HNF 4β, and the gene for enzyme glucokinase. Mutations in these genes lead to the development of DM.

The pathological diseases responsible for DM are pancreatic diseases, liver diseases (chronic hepatitis, liver cirrhosis), endocrine diseases (Pheochromocytoma, Cushing syndrome, Hyperthyroidism), and drug and chemical induced diseases (Glucocorticoids).[1]

Gestational diabetes

This specific type of diabetes occurs during pregnancy. This is due to changes in levels of hormones. Once the delivery occurs, this diabetes will no longer exist.[2]

1.1.1 Current statistics

According to the American Heart Association, in 2005, 17 million people in the United States were diagnosed with diabetes mellitus. Every year, 1.6 million new cases are diagnosed. Sixty-five percent of the U.S. population suffering from diabetes mellitus will die due to heart-related disorders.[3]

According to the National Diabetes Information Clearing House (NDIC), in 2007, 23.6 million people in the United States, that is 7.8% of the U.S. population, had diabetes. This is a large increase in two years.[4] In 2007, the estimated cost for the treatment of diabetes mellitus was $174 billion.

1.2 Peptides

Peptides are polymers of amino acids. In this combination, the carboxyl group of one amino acid reacts with the amino group of the other, leading to formation of an amide bond or peptide bond with the elimination of a water molecule. These peptides are classified into different types, based on their size. They are dipeptides (formed by combination of 2 amino acids), tripeptides (formed by combination of 3 amino acids), oligopeptides (formed by more than 3 and less than 8 amino acids), and polypeptides (more than 8 amino acids). If the peptide sequence consists of more than 100 amino acids, it is called a protein.[5]

1.3 Amylin or Islet Amyloid Polypeptide (IAPP) and its role in Diabetes Mellitus

Amylin (also called Islet Amyloid Polypeptide or IAPP) is a 37 amino acid polypeptide belonging to the family of calcitonin, which is co-secreted along with insulin in the islets of Langerhans of pancreatic beta cells. The human form of IAPP, known as hIAPP, is responsible for formation of amyloid deposits. These amyloid deposits are known to cause beta cell membrane damage and, further, beta cell death, which leads to reduced production of insulin and the development of type 2 diabetes mellitus.[6, 7]

Studies reveal that amylin is amyloidogenic (forms amyloid plaques) in species like humans, monkeys, and cats, whereas it is non-amyloidogenic in the case of mice and rats. The amyloidogenic species is toxic to beta cells and is responsible for development of type 2 diabetes. [6]

1.3.1 Amyloid

Amyloid is a fibrillar structure formed due to aggregation and self-assembly of proteins, peptides, and polypeptides. Amyloid formation leads to degenerative processes such as cell death.

A reduction in beta cell mass can be seen due to the islet amyloid formation. These amyloid deposits are found post mortem in the pancreatic beta cells of more than 90% of type 2 diabetic patients. Apart from diabetes, amyloid deposits can be seen in pathologic conditions like Alzheimer's disease, Parkinson's disease, Medullary thyroid carcinoma and Insulinomas.

In vitro studies from Lorenzo et al. (1994) and Hiddinga and Eberhardt (1999) found that the synthetic human form of IAPP is toxic to beta cells (by forming fibrils), whereas the rodent IAPP is not toxic to beta cells (doesn't form fibrils). This indicates that only the aggregated or fibrillar form of IAPP is cytotoxic, whereas the soluble form is not cytotoxic.[7, 8]

1.3.2 Amyloid formation

Highly ordered amyloid fibrils are formed from self-association of proteins by a process known as amyloidogenesis. Several studies suggest that the process of amyloid

formation is mainly via nucleation dependent fibril formation and nucleation-independent fibril formation.

Nucleation-dependent fibril formation involves two steps, namely a lag phase and an elongation phase. In the lag phase, addition of protein monomers occurs, to form a highly ordered and stable nucleus. The monomer addition continues to form aggregates of the nucleus to produce long fibers known as amyloid fibrils. This is called the elongation phase.[9]

Nucleation-independent fibril formation involves addition of monomers into large molecular mass structures known as oligomers. The formation of oligomers is by a nucleation-independent process. In this process, the mechanism of amyloid fibril formation is unclear as to whether the addition of monomers or aggregation of existing oligomers leads to formation of amyloid fibrils.[10]

1.3.3 Structure of hIAPP

Previous studies about the three-dimensional structure of soluble hIAPP have shown that the hIAPP molecule contains a random coil with an α-helix. Also, studies involving x-ray diffraction, electron microscopy, and electron diffraction methods have shown that the 3D structure of the hIAPP fibril contains a cross-beta structure, natural for amyloid fibrils.

That means that in the process of fibril formation, the hIAPP random coil/helix transforms into a structure which consists of mostly β-sheet.[11, 12, 22] The 37 amino acid sequence of the human form of amylin is shown below.

KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY

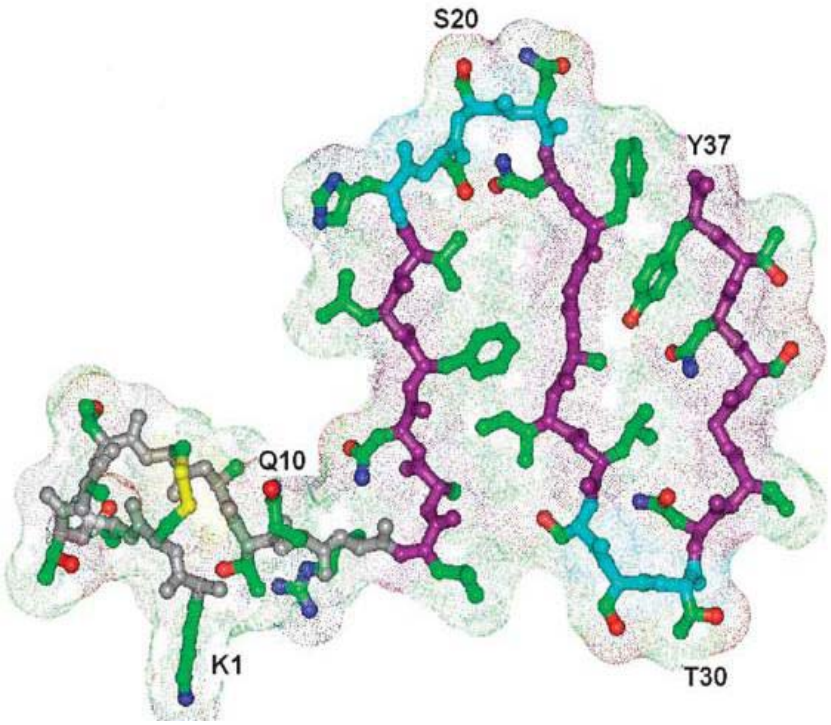

Figure 1. The beta-structure for amyloid fibrils of hIAPP 1-37. The beta strands can be seen in purple, and the atoms carbon, oxygen, nitrogen, and sulfur are represented by green, red, blue, and yellow balls, respectively. Figure was taken from reference 12.

Recent studies suggest that the N-terminal region of hIAPP (hIAPP 1-19) is responsible for the initial interactions with the membrane rather than the central amyloidogenic region of hIAPP (hIAPP 20-29). The hIAPP 20-29 region has been found to play a role in formation of amyloid fibrils.[11]

1.3.4 Cell membrane toxicity by hIAPP

The exact mechanism of beta cell membrane disruption by hIAPP is not well understood. Some researchers claim that the hIAPP oligomers are responsible for membrane damage and beta cell death, [13] whereas some claim that the fibrils that are grown at the cell membrane are responsible for the membrane damage and cell death.

According to the theory of beta cell membrane disruption by hIAPP oligomers, these oligomers form small pores on the cell membrane that allow the cell contents to pass out, causing cell membrane destabilization.[7] The process of oligomer formation is unclear, that is whether the preformed hIAPP oligomers bind to the membrane causing cell membrane disruption, or whether interaction of monomers with the membrane, followed by formation of oligomers at the membrane, causes the membrane disruption.

The mechanism of membrane damage by fibril growth at the membrane involves attachment of hIAPP monomers or oligomers to the surface of the cell membrane, followed by interactions between membrane bound hIAPP monomers or oligomers, leading to fibril growth at the membrane and causing membrane disruption and distortion. After the membrane distortion, the hIAPP fibrils detach from the cell membrane.[11]

Figure 2 represents the three different models for membrane disruption by hIAPP.

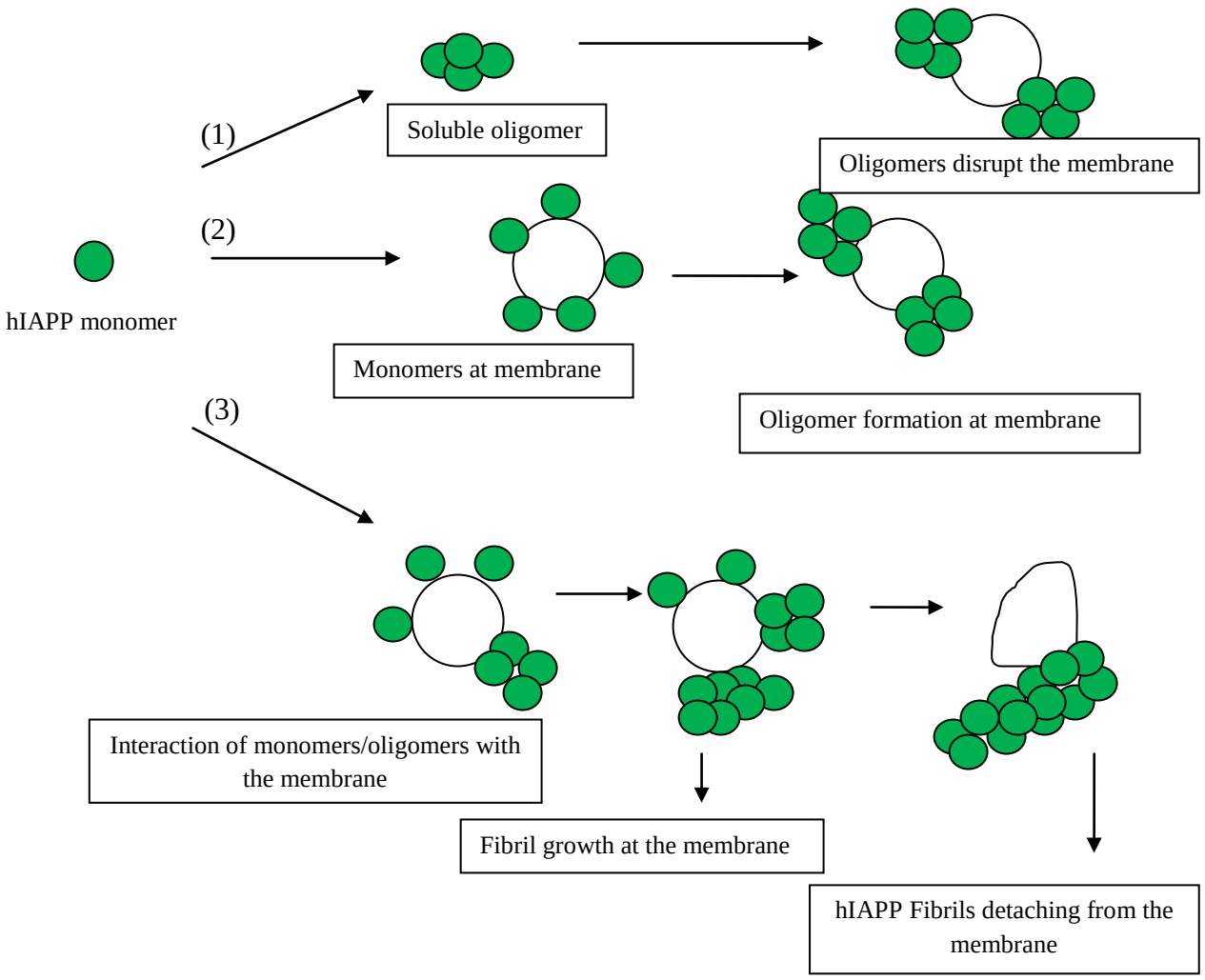

Figure 2. Different models of hIAPP-membrane interaction with respect to membrane damage and hIAPP toxicity. The small green colored circles represent hIAPP monomers, and clusters of 4 or more circles represent hIAPP oligomers and hIAPP fibrils, respectively. The large black circles indicate the cell membrane, and arrows indicate the processes. Model 1 represents the formation of soluble hIAPP oligomers in the initial step, followed by

interaction of toxic oligomers with the membrane, leading to cell death. Model 2 represents 2 steps: (i) interaction of hIAPP monomers at the membrane, and (ii) formation of toxic hIAPP oligomers at the membrane. Model 3 represents 3 steps: (i) interaction of hIAPP monomers or oligomers with the membrane, (ii) growth of hIAPP fibrils at the membrane, causing membrane disruption and damage, and (iii) finally the detachment of mature fibrils from the disrupted membrane.[11]

1.4 Insulin

Insulin is a peptide hormone, secreted in the islets of Langerhans by pancreatic beta cells. Insulin plays a major role in the regulation of blood glucose levels. Insulin is responsible for the uptake of glucose from the blood by body cells, which include muscle, liver, and fat tissues. The glucose is then stored as glycogen in the tissues as an energy source.

Insulin is made up of two polypeptide chains, namely the A-chain and B-chain. The A-chain is composed of 21 amino acids and the B-chain is composed of 30 amino acids. These two chains are connected by two disulphide bonds. The disulphide bonds are formed between cysteine residues of the A and B chains. One is between A7 and B7, and the other is between A20 and B19. Apart from these two disulphide bonds, the A chain itself possesses an additional disulphide bond between A6 and A11 cysteine residues.[14]

1.4.1 Role of insulin in prevention of hIAPP induced membrane damage

Insulin, which is co-secreted with amylin in the islets of Langerhans, acts as a potent inhibitor of hIAPP fibril formation. The region of insulin responsible for binding and interaction with hIAPP is HLVE**ALYLV**C. This decapeptide, present in the B-chain of insulin, inhibits the transition of random-coiled IAPP to the fibril-forming beta sheet conformation. This segment of insulin interacts with IAPP at the sequence of QRL**ANFLV**HS. The most important region of the above decapeptide is ALYLV. This pentapeptide core has sequence homology with the internal segment (ANFLV) of IAPP and is responsible for contact with IAPP.

The concentration ratio of hIAPP to insulin plays an important role in the prevention of hIAPP-induced membrane leakage. Insulin, at substoichiometric concentrations, acts as an inhibitor of IAPP fibril formation.[9, 23]

Research studies by Wei Cui et al. (2009) based on a light scattering assay and a Thioflavin T assay have revealed that insulin acts as a kinetic inhibitor of amylin aggregation (inhibition for a limited time period), but it has been found to enhance amylin fibril formation after a certain time period. This enhanced fibril formation of amylin is due to copolymerization of insulin and amylin. These effects were found to be concentration dependent (the higher the insulin/amylin ratio, the greater the effect).[15]

Transmission electron microscopy (TEM) images were found to show inhibitory properties of insulin at low concentrations (10 μM insulin incubated with 10 μM amylin for 6 hours). It was also found that amylin, incubated with insulin for 6 hours, showed reduced

fibril formation compared to amylin itself.[15] In contrast, the action of insulin was no longer inhibitory after 12 hours of incubation with amylin, but rather was found to enhance the aggregation of amylin in a concentration dependent manner. These results also indicate that the effects of insulin are due to interaction of insulin with amylin monomers or oligomers but not with preformed fibrils.

Therefore, insulin was found to act as a natural inhibitor of amylin, as well as an enhancer for amylin aggregation over time, in type 2 diabetic patients. This variability in activity between different studies and in different time frames presents an interesting problem to study to gain a better understanding of the process.

1.5 Solid phase peptide synthesis

Solid phase peptide synthesis (SPPS) was first developed by Robert Bruce Merrifield.[16, 17] The methodology involved in solid phase peptide synthesis is an initial reaction of polystyrene resin with the carboxy terminal of an amino acid, where the amino group is protected by an Fmoc (Fluorenyloxymethylcarbonyl) protecting group. There are two different kinds of protecting groups available, namely Fmoc and Boc (tert-butyloxycarbonyl). Currently, Fmoc protected amino acids are used for most of peptide syntheses because the Boc strategy requires a strong cleaving agent like hydrogen fluoride. Further, the Fmoc protecting group is removed using a basic deprotecting agent, namely 20% piperidine (v/v) in dimethylformamide (DMF). Then, the free N-terminus of an amino acid that is attached to the solid resin support will react with another amino acid that has a free C-terminus and an Fmoc protected N-terminus. Several cycles of deprotection, washing, and coupling will finally lead to the synthesis of the required sequence. Then, the peptide is

subjected to cleavage from the resin, which also removes any side chain protecting groups. Normally, the cleaving agent used is trifluoroacetic acid (TFA).

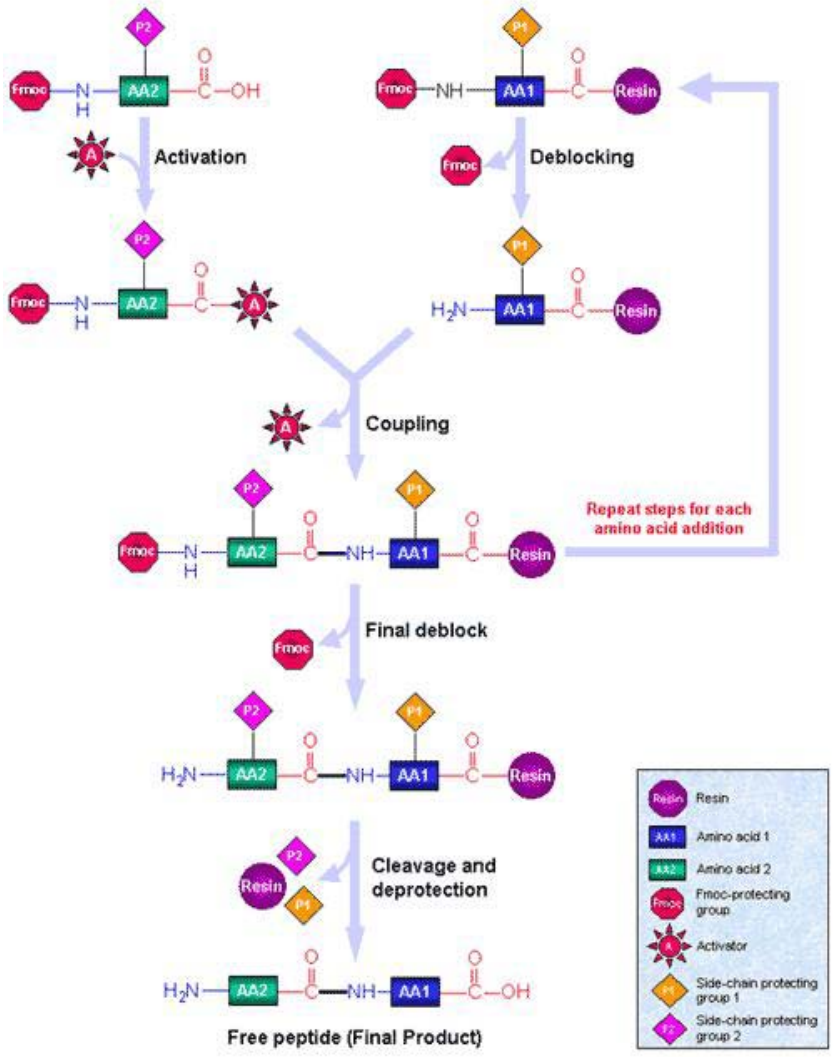

Figure 3. Scheme of Solid Phase Peptide Synthesis.[18]

1.6 Dye Leakage Analysis

Fluorescent dye leakage analysis was employed to determine the activity of the peptides on model lipid membranes. This method is based on the measurement of fluorescence produced as a result of membrane damage by peptides. Later, the percent fluorescence was plotted against time to calculate the percent membrane damage.

1.6.1 Lipids used to test the activity of peptides

The following lipids were used (manufactured by Avanti Polar Lipids, Inc), to test the activity of peptides. They are POPG (1-palmitoyl-2-olenyl-sn-glycero-3-phosphoglycerol), and a mixture of DOPC (1, 2-dioleoyl-sn-glycero-3-phosphocholine): DOPS (1, 2-dioleoyl-sn-glycero-3-(phospho-L-serine) in the ratio of 7:3, shown in Figures 4-6. The 7: 3 mixture of DOPC: DOPS lipid mimics the actual beta cell membrane composition and can be used as a model for pancreatic beta cells.

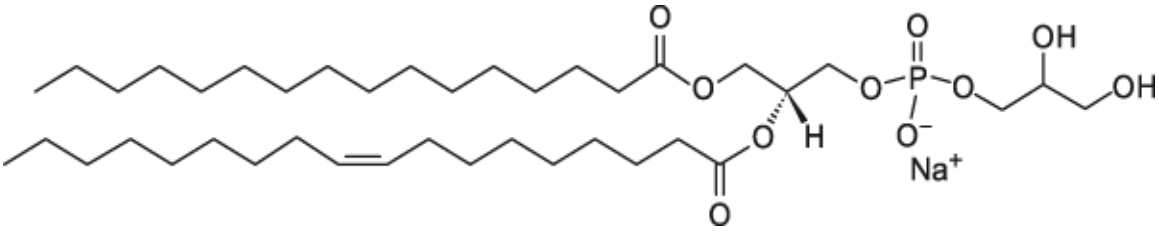

Figure 4. Structure of POPG[19] (a negatively charged lipid)

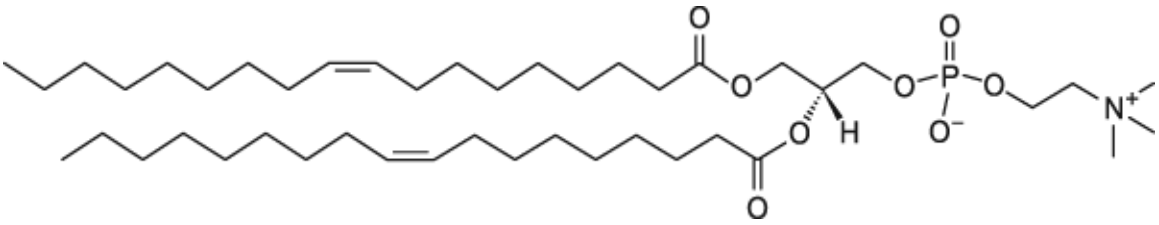

Figure 5. Structure of DOPC[20] (a neutral lipid)

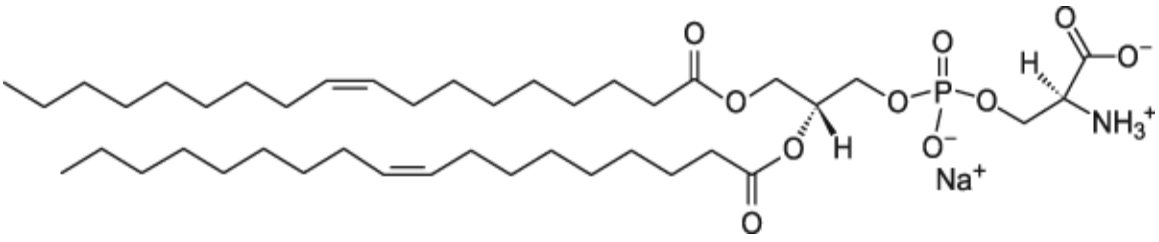

Figure 6. Structure of DOPS [21](a negatively charged lipid)

2. RESEARCH GOAL

Our research goal was to synthesize insulin based inhibitors of human amylin (hIAPP) with a minimal sequence that can prevent the aggregation of hIAPP itself and inhibit beta cell membrane damage. This was done by performing a truncation study of the shorter amylin-binding sequence of insulin while retaining the core sequence ALYLV. In this process, our first step was to synthesize and purify the required peptides, and the next step was to determine the activity of the insulin based inhibitors on lipid membranes in the presence of hIAPP 1-19 and hIAPP 1-37.

The following peptides were synthesized and purified:

- hIAPP 1-19
- Insulin based analog VEALYLV (analog 1)
- Insulin based analog VEALFLV (analog 2), where F was substituted for Y to test for the necessity of the para-hydroxyl group.
- Insulin based analog EALYLV (analog 3)

The activity of the above mentioned insulin based inhibitors was determined on different lipid membranes, POPG and 7:3 DOPC: DOPS.

- VEALYLV and VEALFLV were tested in POPG in the presence of hIAPP 1-19, and the activities of these two inhibitors were compared with each other.

- VEALYLV and VEALFLV were tested in 7:3 DOPC: DOPS in the presence of hIAPP 1-37, and the activities of these two inhibitors were compared with each other.

- VEALYLV and EALYLV were tested in 7:3 DOPC: DOPS in the presence of hIAPP 1-37, and the activities of these two inhibitors were compared with each other.

- The activity of VEALYLV in 7:3 DOPC: DOPS against hIAPP 1-19 was compared with that of VEALYLV against hIAPP 1-37.

- Also, the activities of insulin against hIAPP 1-19 in both POPG and 7: 3 DOPC: DOPS, and insulin against hIAPP 1-37 in 7:3 DOPC: DOPS, were determined for comparison.

The purpose of the above comparisons was to determine the most effective inhibitor of hIAPP 1-19 and hIAPP 1-37. To obtain this information, initially, we tested some of our inhibitors (VEALYLV and VEALFLV) against hIAPP 1-19 in POPG. Later, some researchers found that the mixture of DOPC and DOPS in 7:3 ratio was the best mimic of the actual beta cell membrane and a model for pancreatic beta cells. From that point on, we carried out our tests in the 7: 3 mixture of DOPC: DOPS.

3. EXPERIMENTAL

3.1 Peptide synthesis

All peptides (hIAPP 1-19 and insulin based inhibitors) were synthesized using an automated Rainin PS3 peptide synthesizer on a 0.1 mmole scale basis. The required amount of Fmoc protected amino acids (a 4- fold excess) were weighed and transferred into small plastic vials. To those vials, 0.4 mmoles (0.152g) of HBTU (Hydroxy-O-(Benzotriazol-1yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate), a coupling agent, was added. This coupling agent was activated by 0.4M N, N- diisopropylethylamine in DMF. A solution of 20 % piperidine (v/v) in N, N-dimethylformamide was used as the deprotecting agent, and methyl benzhydrylamine (MBHA) resin was the solid support. Peptides were synthesized after several steps of deprotecting, washing and coupling, which proceeds in a C-terminal to N-terminal fashion on 0.1 mmole of resin. The synthesized sequences of peptides are shown in Table 1.

Table 1. Sequences of Synthesized Peptides.

S.no	Peptide	Sequence
1.	hIAPP 1-19	KCNTATCATQRLANFLVHS
2.	Insulin analog 1	VEALYLV
3.	Insulin analog 2	VEALFLV
4.	Insulin analog 3	EALYLV

3.2 Peptide Cleavage

The synthesized peptides were cleaved from the resin using trifluoroacetic acid (TFA), which also removes any side chain protecting groups from the amino acids. A small amount of DMF was poured into the reaction vessel containing peptide-resin, and the solution was filtered through a 15 mL fritted disc Buchner funnel (Baxter F7700-9). The excess DMF was drawn off with the help of vacuum suction and the resin was washed with ethanol, followed by 2-3 rinses with methylene chloride. The contents on the funnel were dried under vacuum for 30 min to 1 hour. Then, the peptide-bound resin was transferred to a 50 mL beaker with a stirring bar and placed in an ice bath. A cocktail was prepared by adding 9.0 mL of TFA, 0.5 mL of distilled water, 0.5 mL of phenol, and 200 microliters of triisopropylsilane (TIS) in a 10 mL graduated cylinder. Then, the cocktail mixture was added to the beaker containing peptide-bound resin and stirred for 5-10 min. The beaker was then taken out of the ice bath, covered with parafilm, and stirred at room temperature for 1.5 to 2.5 hours. The cleavage mixture was then filtered using a side arm flask and coarse fritted funnel, and the peptide solution was collected in the side arm flask. To that peptide solution, 50 mL of cold diethyl ether was added slowly, which caused the peptide to precipitate. Since this is an exothermic process, the flask was kept in an ice bath to maintain the temperature and to prevent any side reactions that may occur. Then the precipitate was poured into a fine fritted disc Buchner funnel in a 250 mL side arm flask, and the ether was removed using a vacuum line (water aspirator). Last, the dried peptide was transferred to a lyophilization flask and dissolved in a small amount of 70% acetonitrile/water and diluted with an equal volume of distilled water, followed by shell freezing in a dry ice/acetone bath and lyophilized overnight.

3.3 Peptide Purification

The peptides were separated and purified by using reversed-phase high performance liquid chromatography (RP-HPLC) on a Waters instrument. For the above separation and purification processes a UV detector (Waters 484) was used. In the initial step, peptides were separated from impurities by preparative RP-HPLC using a Phenomenex Jupiter column (C18, 10µm, 250 x 21.20 mm, 300Å). The mobile phase consisted of 0.1% TFA in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B). A gradient type eluted at a flow rate of 10 mL/min. The peptide was injected in a minimal amount of TFA at 90%A and 10%B, and as time progressed the organic nature of the mobile phase increased to 50%. The peptides were eluted and detected at a wavelength of 254 nm. The pure peptide solutions were collected in test tubes, frozen, and lyophilized overnight.

3.4 Peptide analysis

Peptide purity was estimated by using analytical RP-HPLC (Waters dual pump system with gradient controller and UV detector) that consisted of a Phenomenex Jupiter column (C18, 5µm, 250 x 4.6 mm). The mobile phase used was 0.1% TFA in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B). A gradient type of elution was performed using 0 to 66% of organic component (solvent B) over 33 min at a flow rate of 1 mL/min. The purity of all peptides (hIAPP 1-19 and insulin analogs VEALFLV, VEALYLV and EALYLV) was found to be greater than 95%. The peptide mass in each case was confirmed by liquid chromatography coupled to electrospray mass spectrometry (LC-MS). The molecular weights of the various synthesized peptides are shown in Table 2.

Table 2. Molecular Weights of Various Peptides Confirmed by LC-MS.

Peptide	**Molecular weight**
hIAPP 1-19	2078
VEALFLV	789.3
VEALYLV	805.5
EALYLV	706.2

3.5 Fluorescent dye leakage assay

The principle involved in the fluorescent dye leakage assay is the measurement of fluorescence from carboxyfluorescein dye that leaks out from inside lipid vesicles due to cell membrane damage caused by hIAPP. These dye leakage assays can be performed in various types of lipids. Lipids used for these assays include Palmitoylolenylphospoglycerol (POPG), Palmitoylolenylglycerophosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS).

3.5.1 Preparation of lipid vesicles

The vesicles required for the dye leakage assay were prepared in our laboratory by weighing 5 mg of the required lipid (Ex: POPG, DOPC, DOPS) and dissolving it in a test tube, then adding 2 mL of chloroform. After the lipid was dissolved in chloroform, nitrogen was used to evaporate excess chloroform, leaving a thin film of lipid on the test tube wall.

Then the test tubes containing lipid were dried under vacuum by putting them on a lyophilizer overnight.

Dye encapsulated vesicles were prepared in the lab by adding 5.64 mg of carboxyfluorescein dye to 0.5 mL of sodium phosphate buffer (pH 7.5). Then 0.5 mL of dye solution was added to the test tube containing dried lipid. The test tube was vortexed to ensure proper mixing, and it was frozen using liquid nitrogen and thawed five times. These freeze and thaw cycles cause the lipid to encapsulate dye molecules and form vesicles. At this point, the test tubes were labeled and stored at -20°C.

3.5.2 Extrusion of dye encapsulated lipid vesicles

At the time of the assay, the lipid test tubes were removed from the freezer and thawed. After thawing, the lipid solution was passed through a lipid extruder purchased from Avanti Polar Lipids for 21 times. This consists of a 0.1 micron polycarbonate membrane and two Hamilton syringes. Extrusion is necessary to get uniform and desired size vesicles. The dye-encapsulated vesicles were then purified to remove free dye using a gel exclusion column packed with G-50 gel beads. The dyed vesicle solution was poured on the top of the gel exclusion column with a Pasteur pipette and allowed to pass through the packed gel bed by adding sodium phosphate buffer on to the top of column. The larger dyed vesicles (a yellow colored fraction) separated quickly by size as the solution passed through the gel bed, and they were collected into a microvial. Then the column was flushed with buffer solution.

In the same way, an undyed vesicle solution was prepared, except that no carboxyfluorescein was added and it was not passed through the column. For the assay, a solution of diluted dye-containing vesicles was prepared by adding 10% by volume of dyed vesicles to undyed vesicles and mixing by test tube inversion. After the solution had been prepared, the lipid extruder and syringes were washed with water followed by ethanol.

3.6 Preparation of samples for dye leakage assay

3.6.1 Preparation of detergent (100% leakage) solution

This detergent solution was prepared by using Triton-X 100 detergent. 40 µL of this detergent was added to 250 µL of sodium phosphate buffer. This detergent solution causes breakage of the liposome, which leads to 100% leakage of dye from vesicles.

3.6.2 Preparation of control (0% leakage) solution

Control solution was prepared by adding 50 µL of dimethyl sulfoxide (DMSO) and 20 µL of lipid vesicles to 1430 µL of sodium phosphate buffer. This control should not cause leakage to the membrane as it has no peptide or detergent.

The volume of components in detergent and control tubes are shown in Table 3.

Table 3. Volume of Components in Detergent (100% leakage) and Control (0% leakage) Sample Tubes.

Trial	**Amylin (µL)**	**Insulin (µL)**	**Vesicles (µL)**	**Buffer (µL)**	**DMSO (µL)**	**Detergent (µL)**
Detergent	0	0	20	1390	50	40
Control	0	0	20	1430	50	0

3.6.3 Preparation of peptide samples

Amylin/amylin fragment and insulin/insulin analog samples were prepared by weighing out a calculated mass of peptide based on its molecular weight and dissolving in the required amount of DMSO to produce a stock solution of 0.72 M concentration of peptides.

To prepare an amylin (hIAPP) solution, 1.0 mg of amylin (MW is 3905 g/mol) was weighed and dissolved in 350 µL of dimethylsulfoxide (DMSO) into a vial to produce a stock solution of concentration 0.72 M.

The amylin fragment hIAPP 1-19 was prepared by weighing out 3.1 mg of the peptide and dissolving in 1.1 mL of DMSO to give a stock solution of 0.72 M.

The insulin sample was prepared by weighing out 3.0 mg of insulin (MW=5808 g/mol) and dissolving in 715 µL of DMSO to give a stock solution concentration of 0.72 M.

Based on their molecular weight, insulin analogs were weighed out and dissolved in the required quantity (usually 375 µL) of DMSO to produce a stock concentration of 0.72 M. To study the effect of amylin or insulin itself, the sample test tubes were made up as in Table 4.

Table 4. Volume of Components in Sample Tubes of Amylin/Insulin.

Trial	**Sodium phosphate buffer (µL)**	**Amylin/Insulin (µL)**	**Lipid vesicles (µL)**	**Concentration of Amylin/Insulin (µM)**
1	1479	1	20	0.5
2	1477.5	2.5	20	1.2
3	1475	5	20	2.4
4	1470	10	20	4.8
5	1467.5	12.5	20	6
6	1455	25	20	12
7	1442.5	37.5	20	18
8	1430	50	20	24

To study the effects of variable amounts of insulin or insulin analogs with a constant amount of amylin (2.4 µM), the sample tubes were made as in Table 5.

Table 5. Volume of Components in Constant 2.4 µM Amylin plus Varying Insulin/Insulin Analog Assay.

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

3.7 Measurement of fluorescence values using FLx 800 Microplate Reader

Sample tubes consisting of sodium phosphate buffer, peptides, and vesicles were mixed properly by inversion. 300 µL of each test tube contents were transferred to a 96 well plate. Tips of the pipette were changed for each sample. All trials were performed in triplicate, and average values were later calculated.

A Bio-tek FLx fluorimeter and computer was used for this study. The excitation wavelength was set to 485 nm and the emission wavelength was set to 528 nm. Depending upon the number of samples, the range of wells was input (for example, for 4 different samples with 9 trials each, the range was given as A1 to D9). When samples were ready for analysis, the 96 well plate was placed in the instrument and the lid was closed. The instrument collected the fluorescence values based on membrane leakage for a given period of time. For all of these assays, the run time was 3 hours and interval time was 59 seconds. The fraction of dye leaked from membrane was calculated by using the formula:

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

The percent fraction leaked (% fluorescence) was plotted against time for each concentration of each peptide to determine the percent membrane disruption.

4. RESULTS AND DISCUSSION

We have synthesized three different truncated analogs of insulin, namely VEALYLV (insulin analog 1), VEALFLV (insulin analog 2), and EALYLV (insulin analog 3). These analogs were synthesized based on the insulin decapeptide region (HLVEALYLVC), which is thought to be responsible for interaction with hIAPP and inhibition of its aggregation.

The activity of the three truncated insulin analogs on different lipid membranes (POPG and 7:3 mixture of DOPC: DOPS) in the presence of hIAPP 1-19 and hIAPP 1-37 was compared to that of insulin and also compared to each other, in order to find the optimal length analog of insulin that can prevent the membrane damage caused by hIAPP and therefore further the treatment of T2DM. In addition, analog 2 tests for the importance of the tyrosine hydroxyl by replacement of tyrosine with phenylalanine.

In this assay, the lipid vesicles that took up the carboxyfluorescein dye were treated with peptide, causing the dye to leak from vesicles as percent fluorescence was monitored. From the dye leakage assays, we were able to plot the percent fluorescence, which is directly proportional to the membrane damage, against time. These graphs indicate the extent of damage to the cell membrane caused by the peptides. All of the assays were run for 3 hours in triplicate, and average values were plotted.

4.1 VEALYLV versus VEALFLV in the presence of hIAPP 1-19 on POPG

Initially, the insulin analogs (analogs 1 and 2) were assayed in the presence of a constant concentration of hIAPP 1-19, in POPG, a negatively charged lipid. The activities of these analogs were compared to those of insulin in the presence of hIAPP 1-19.

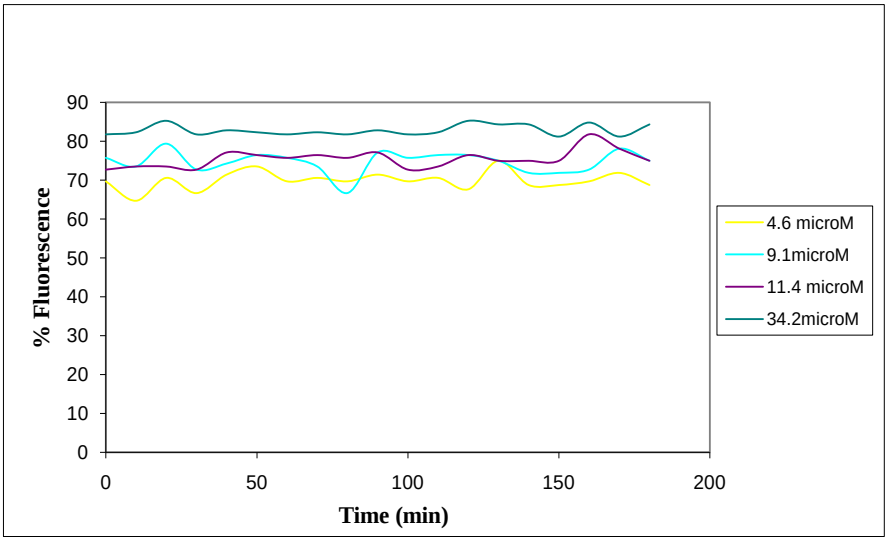

Figure 7. Activity of hIAPP 1-19 at varying concentrations on POPG.

The activity of varying concentrations of hIAPP 1-19 was assessed first so that an appropriate amount could be chosen for the inhibition assay. Figure 7 represents the activity of hIAPP 1-19 on POPG membrane at varying concentrations of 4.6 µM, 9.1 µM, 11.4 µM, and 34.2 µM, respectively. The damage caused by hIAPP 1-19 to the POPG lipid membrane was found to be concentration dependent. At a concentration of 4.6 µM, it caused 70% membrane damage, and at higher concentrations of 9.1 µM, 11.4 µM, and 34.2 µM, it caused membrane damage up to a greater extent of 75%, 75-80%,

and 80%, respectively. Therefore, the concentration of 4.6 µM was chosen for the inhibition study in order to conserve peptide, since the damage caused at this concentration was significant and measurable.

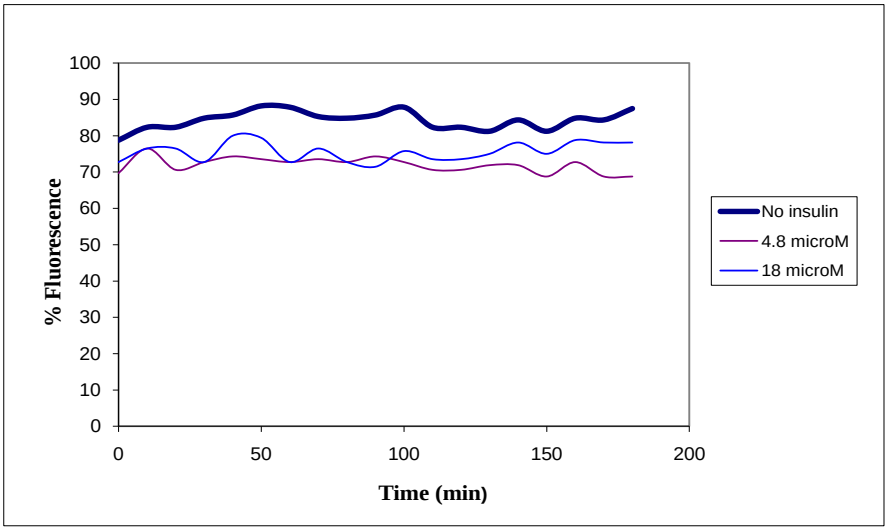

Figure 8. Activity of 4.6 µm hIAPP 1-19 on POPG in the presence of varying insulin.

Figure 8 represents the activity of 4.6 µM hIAPP 1-19 on POPG lipid in the presence of insulin at two different concentrations. Alone, hIAPP 1-19 at a concentration of 4.6 µM caused membrane damage in this assay to an extent greater than 80%. But in the presence of insulin at a 1:1 molar ratio (hIAPP 1-19: insulin), the activity of hIAPP 1-19 was reduced by about 10%. At a higher insulin concentration, a four-fold molar excess to hIAPP 1-19, the activity of hIAPP 1-19 was reduced by only 5%. Effectively, there was little

difference between the two concentrations, indicating that increasing the molar ratio of insulin to hIAPP 1-19 provides little benefit under these conditions.

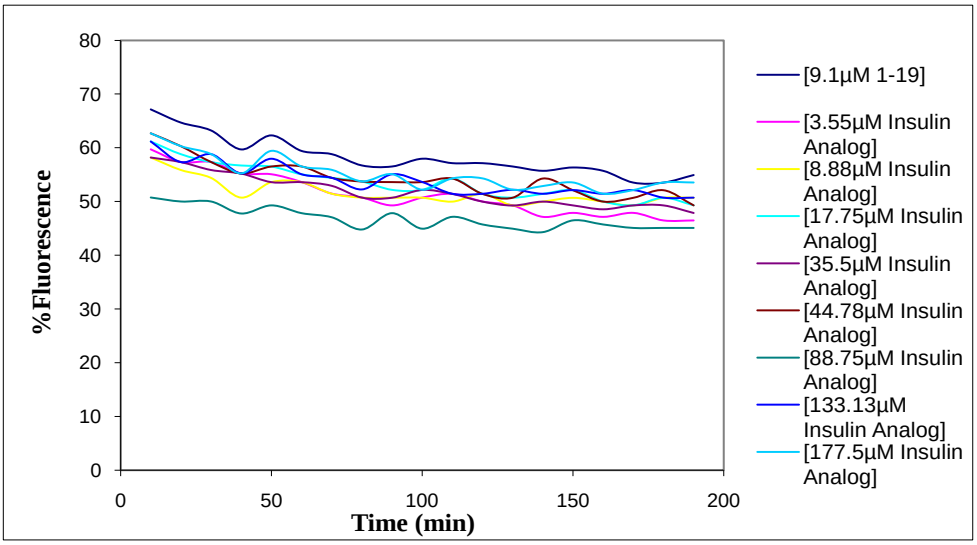

Figure 9. Activity of 9.1 µM hIAPP 1-19 on POPG lipid in the presence of insulin analog 1 (VEALYLV) at varying concentrations.

Figure 9 represents the membrane damage caused by 9.1 µM of hIAPP 1-19 by itself and in the presence of varying concentrations of analog 1, ranging from 3.55 µM to 177.5 µM. In this assay, 9.1 µM hIAPP 1-19 caused about 60% dye leakage by itself (with some decline overtime). Analog 1 was only minimally effective in preventing damage, by about 5-10%, even at high concentration and a large molar excess (20:1). The reduction in the membrane damage by this analog was not concentration dependent, and most of the concentrations were found to be clustered.

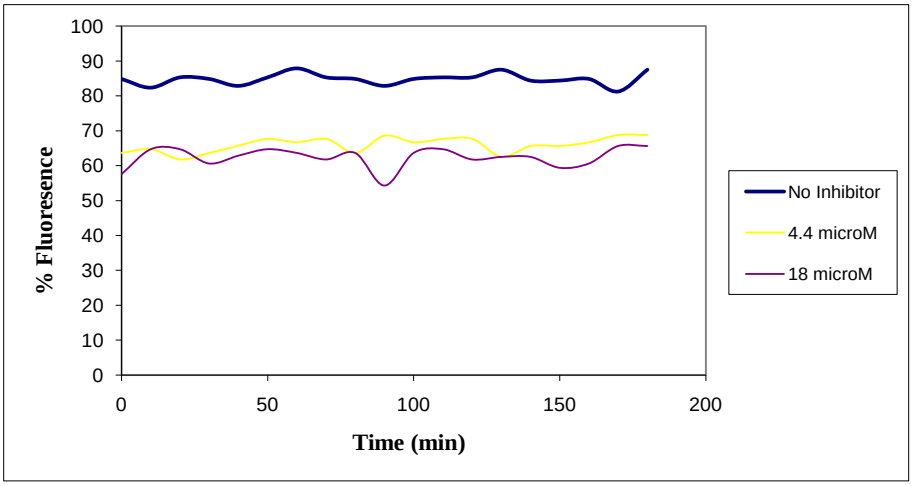

Figure 10. Activity of 4.6 µM hIAPP 1-19 on POPG in the presence of insulin analog 2 (VEALFLV) at varying concentrations.

Figure 10 represents the activity of 4.6 µM hIAPP 1-19 in the presence of VEALFLV (insulin analog 2) at two different concentrations, 4.4 µM, and 18 µM, respectively. As stated earlier, 4.6 µM hIAPP 1-19 by itself on POPG membrane caused dye leakage at an extent of 70%. hIAPP 1-19, upon treatment with insulin analog 2 at a concentration ratio of 1:1, showed a 20% reduction in membrane damage as measured by fluorescent dye leakage. Similar to the observation with insulin, increasing the inhibitor concentration four-fold relative to that of hIAPP 1-19 did not have much effect. Overall, the short analog was slightly more effective than insulin in reducing membrane damage at the same concentration.

Based on the above results (from Figures 8-10), the actions of the two different insulin analogs (analogs 1 and 2) can be summarized as follows:

- Full length insulin was able to reduce dye leakage caused by hIAPP 1-19 in the liposomes by 5-10% at a 1:1 molar ratio. No benefit was observed by increasing the insulin: hIAPP 1-19 ratio to 4:1.
- Insulin analog 1 (VEALYLV) at a concentration ratio of 10:1 with hIAPP 1-19 was found to reduce the membrane damage by only 10%. The activities of other concentrations of insulin analog 1 were found to be clustered in the same range. It therefore had similar activity to insulin at low concentration with no advantage observed by drastically increasing the molar ratio.
- Insulin analog 2 (VEALFLV) at a concentration ratio of 1:1 with hIAPP 1-19 was found to reduce the membrane damage by 20%, thus making it a more effective inhibitor against hIAPP 1-19 in POPG. This indicates that the aromatic hydroxyl on tyrosine in analog 1 is not important and may in fact be detrimental to the interaction of the inhibitor analog with hIAPP 1-19. Again, increasing the ratio to 4:1 provided little to no benefit.
- Insulin analogs 1 and 2, while much shorter in sequence than insulin, displayed very similar activity in POPG against the N-terminal region of hIAPP.

4.2 VEALYLV versus VEALFLV in the presence of hIAPP 1-37 on 7:3 DOPC: DOPS

The activity of insulin analogs 1 and 2 was tested on 7:3 mixture of DOPC: DOPS membrane (neutrally and negatively charged lipids, respectively, which mimics the composition of the pancreatic beta cell membrane).

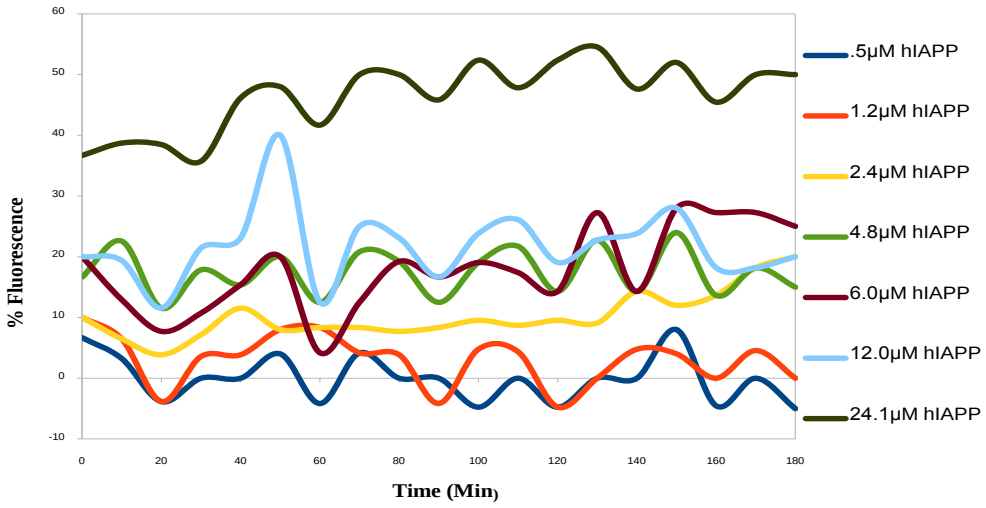

Figure 11. Activity of hIAPP 1-37 on 7:3 DOPC: DOPS at varying concentrations.

The activity of full length hIAPP on 7:3 DOPC: DOPS was found to be a maximum of 50-55% at a concentration of 24.1 μM (the highest tested) (Figure 11). The activity was roughly concentration dependent. Activity in this lipid was lower than that observed for POPG due to the varying charges of the lipid vesicles (POPG is negative, while this mixture is effectively only 30% negative). hIAPP is positively charged, so it has a greater effect on negatively charged lipids.

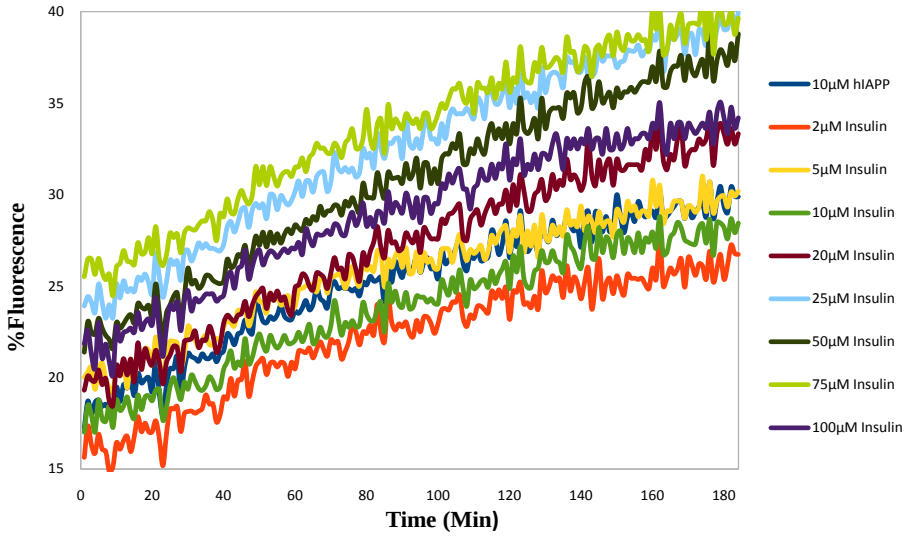

Figure 12. Activity of hIAPP 1-37 on 7:3 DOPC: DOPS in the presence of insulin at varying concentrations.

From Figure 12, it is clear that 10 μM hIAPP 1-37 itself caused membrane damage of approximately 30% after 3 hours. This concentration was chosen for the inhibition assay because damage was measurable, but the expensive peptide could be conserved as much as possible. As shown in Figure 12, when hIAPP 1-37 was treated with insulin at varying concentrations of 2 μM, 5 μM, 10 μM, 20 μM, 25 μM, 50 μM, 75 μM, and 100 μM, respectively, insulin generally was found to increase membrane damage by about 5-10% relative to hIAPP 1-37 itself, except at the lower concentrations of insulin (namely 2 μM, 5 μM, and 10 μM). These concentrations of insulin reduced membrane damage, but only by 5%. Effectively, these results overall show little influence of insulin on the activity of hIAPP 1-37 in this type of vesicle in our assay, but the variable direction of the effect at different concentrations is interesting. It is possible that higher concentrations

than 1:1 molar excess are actually aiding fiber formation, or even being incorporated into the fibers, while molar ratios less than 1:1 are slightly inhibitory.

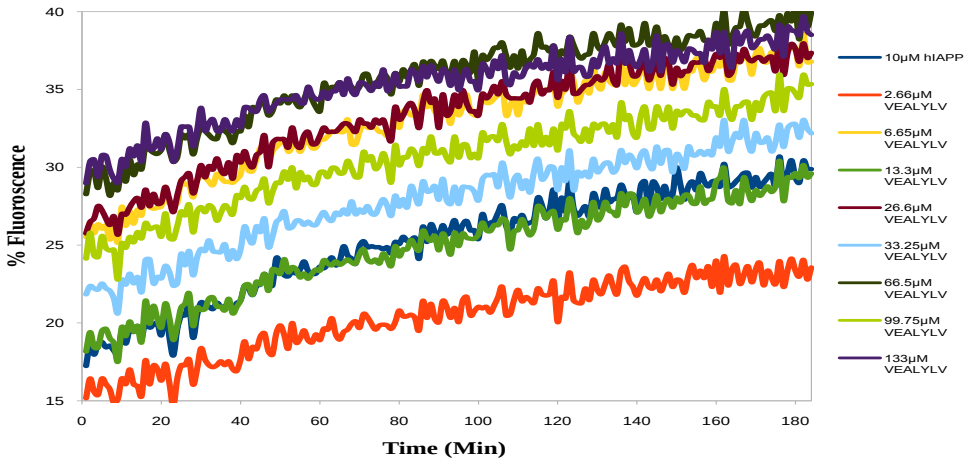

Figure 13. Activity of insulin analog 1 (VEALYLV) at varying concentrations on 7:3 DOPC: DOPS in the presence of constant 10 μM hIAPP 1-37.

Figure 13 shows the membrane damage of 30% caused by 10 μM hIAPP 1-37 itself after 3 hours. Insulin analog 1, at varying concentrations of 2.66 μM, 6.65 μM, 13.3 μM, 26.6 μM, 33.25 μM, 66.5 μM, 99.75 μM, and 133 μM in the presence of constant 10 μM hIAPP 1-37, was found to cause membrane damage up to an extent of 40% (10% higher than that of hIAPP 1-37 itself), except for the lowest concentration (2.66 μM). This concentration of insulin analog 1 was found to reduce the membrane damage by 7%, which is similar to the effect of 2 μM insulin. The 13.3 μM concentration had no effect. The actions of insulin analog 1 were therefore found to be similar to those of insulin in

influencing membrane damage, showing little effect within experimental error, but influencing the effect in different directions at low versus high concentration. In this case again, ratios of inhibitor to hIAPP close to 1:1 or lower (with the exception of 6.65 µM) were slightly inhibitory, while those higher were damage-enhancing.

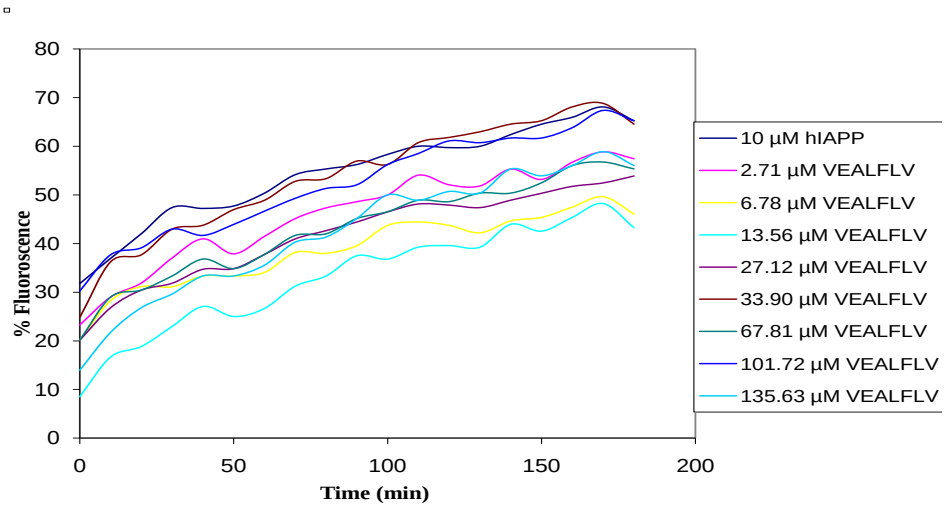

Figure 14. Activity of insulin analog 2 (VEALFLV) at varying concentrations on 7:3 DOPC: DOPS in the presence of hIAPP 1-37.

From Figure 14, the activity of 10 µM hIAPP 1-37 by itself was found to induce 70% dye leakage in this run, which is higher than previous runs (30% damage was seen in earlier studies). This is likely due to variability in the liposome preparation, but results are still comparable due to standards run within the assay. Insulin analog 2, at concentrations ranging from 2.7-135.6 µM, was found to show some inhibitory activity on 10 µM hIAPP 1-37, but it was not concentration dependent. The best inhibition was displayed at concentrations of 6.78 µM and 13.56 µM, both of which reduced membrane damage by

about 20%. Interestingly, this analog not only showed better inhibitory activity than both insulin and analog 1, but it also failed to exhibit the bidirectional effect. This may be due to its slightly different sequence, lacking the tyrosine hydroxyl group, which may influence its interaction with hIAPP or its conformation. Similar activity was noted in POPG.

Based on the above results (Figures 12, 13 and 14), the actions of insulin analogs 1 and 2 against hIAPP 1-37 on the 7:3 DOPS: DOPS lipid membrane can be summarized as follows:

- Insulin analog 1 was found to show similar activity to that of insulin in the presence of 10 μM hIAPP 1-37 on 7:3 DOPC: DOPS lipid. Both insulin and analog 1 were found to be minimally effective at low concentration, reducing the membrane damage to an extent of 5-7% at concentrations ranging from 2-3 μM (Here the ratio of insulin/insulin analog 1: hIAPP 1-37 was 1:1 to 0.2:1). However, higher concentrations (molar ratios greater than 1:1, up to 20:1 for analog 1) were ineffective to detrimental, actually increasing dye leakage.
- Insulin analog 2 (VEALFLV) at a concentration ratio of roughly 1:1 (hIAPP 1-37: insulin analog 2) was found to reduce membrane damage by 20%, making it more effective than VEALYLV (analog 1). However, no concentration dependence was observed, and higher molar ratios failed to increase the dye leakage as they did for insulin and analog 1.

4.3 VEALYLV versus EALYLV on 7:3 DOPC: DOPS in the presence of hIAPP 1-37

The 10 µM hIAPP 1-37 shown in Figure 15 caused between 33-40% damage to the membrane after three hours. Insulin analog 3 at varying concentrations in the presence of 10 µM hIAPP 1-37 was found to have minimal effect on this damage even at high concentrations. The best reduction in dye leakage was only 6%, at a concentration of 15 µM, and no direct concentration dependence was observed. Therefore, it can be summarized that both VEALYLV and the shorter EALYLV are ineffective against hIAPP in this assay.

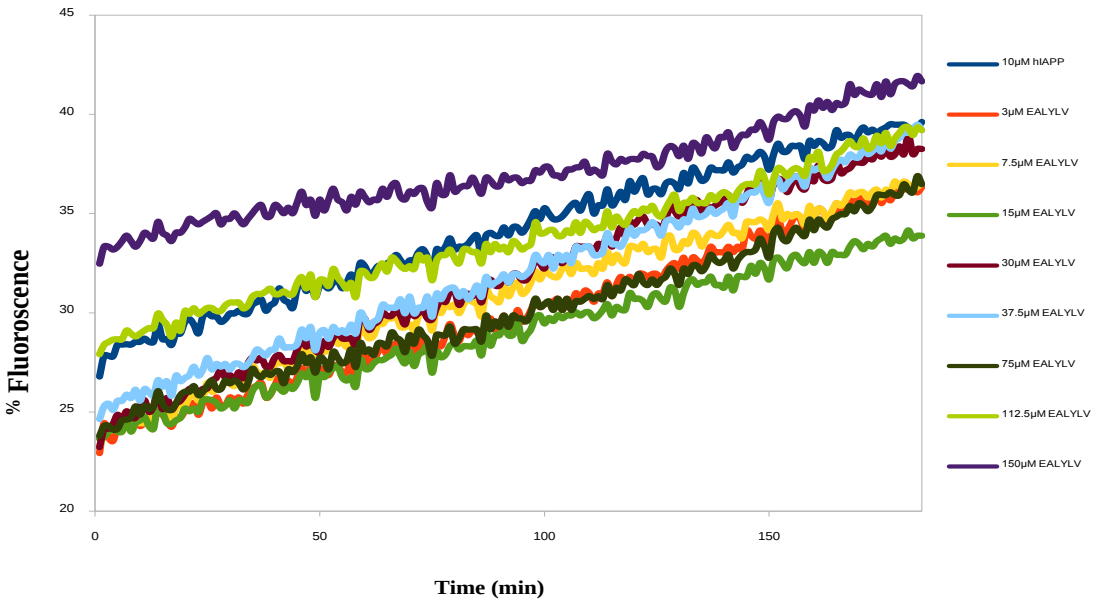

Figure 15. Activity of insulin analog 3 (EALYLV) at varying concentrations in the presence of 10 µM hIAPP 1-37.

Both analogs displayed less than 10% reduction in membrane damage when in approximately a 1:1 molar ratio with hIAPP. However, only the very highest concentration of analog 3 (150 µM, or 15:1 molar excess to hIAPP) caused an increase in dye leakage. Perhaps the shorter and less hydrophobic analog 3 is less able to enhance fiber formation at high concentration relative to analog 1.

4.4 VEALYLV against hIAPP 1-19 versus VEALYLV against hIAPP 1-37 on 7:3 DOPC: DOPS

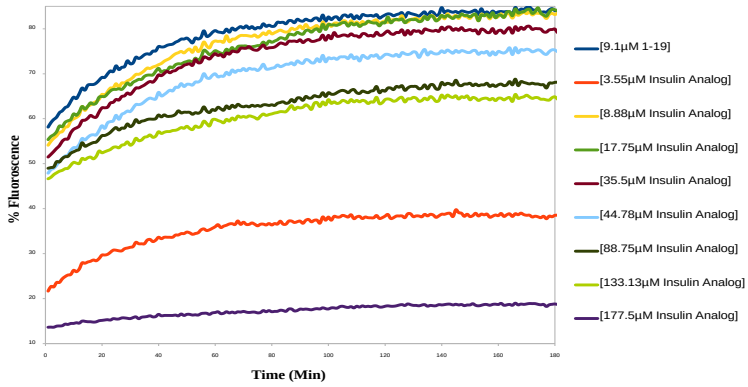

Figure 16. Activity of 9.1 µM hIAPP 1-19 on 7:3 DOPC: DOPS in the presence of insulin analog 1 (VEALYLV).

Figure 16 shows membrane damage of 80-85% by 9.1 µM hIAPP 1-19 itself after three hours in 7:3 DOPC: DOPS. Dye leakage increased as the time progressed. Interestingly, insulin analog 1 was found to be effective in reducing the membrane damage caused by 9.1 µM hIAPP 1-19 in a generally concentration dependent manner (with some exceptions like the high activity of the 3.55 µM sample). This concentration dependence can be seen clearly in Figure 17.

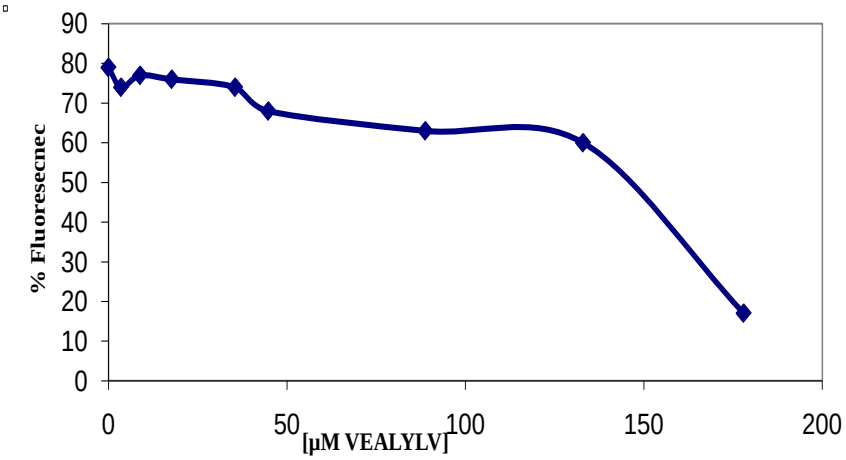

Figure 17. Activity of Insulin analog 1 (Average values) in the presence of 9.1 µM hIAP 1-19 on 7:3 DOPC:DOPS.

There was a reduction in membrane damage by 60% at a concentration of 177.5 µM of analog 1 (roughly 20:1 ratio of inhibitor to hIAPP 1-19). This analog was found to be very effective against the N-terminal fragment, hIAPP 1-19, in 7:3 DOPC: DOPS.

However, the analog was not effective in the same membrane composition against full length hIAPP (Figure 13), and in fact enhanced the damage at most concentrations.

This result seems reasonable since the insulin/inhibitor binding region lies in the N-terminal fragment (1-19) of hIAPP. It is possible that the inhibitor binds both peptides but that it is unable to influence the conformation of the full length analog as much as it does the shorter N-terminal 1-19 region. It is also possible that two separate events cause damage: binding of the N-terminus by both peptides, followed by fiber formation for the full length hIAPP only. In this case, it is possible that only the initial membrane binding is blocked by the inhibitor, but not the fiber formation. In fact, it is further possible that the inhibitors (especially analog 1) and insulin itself, which is known to bind to hIAPP, actually co-polymerize with hIAPP when in molar excess, aiding in fiber formation. Since hIAPP 1-19 does not fibrilize, the enhancement in dye leakage is not observed with this peptide upon treatment with insulin or the inhibitors. Last, it is possible that the full length hIAPP adopts a different conformation from the 1-19 region itself, leaving the interaction site with the inhibitor less accessible. Even insulin itself failed to show any significant inhibition against full length hIAPP in this assay.

It should be noted that the 7:3 DOPC: DOPS lipid composition is considered a better model of the pancreatic beta cell than POPG, which due to its negatively charged nature interacts more strongly with hIAPP (which may be why analog 1 was less effective against hIAPP 1-19 in that lipid).

4.5 VEALYLV against hIAPP 1-19 versus Insulin against hIAPP 1-19 on 7:3 DOPC: DOPS

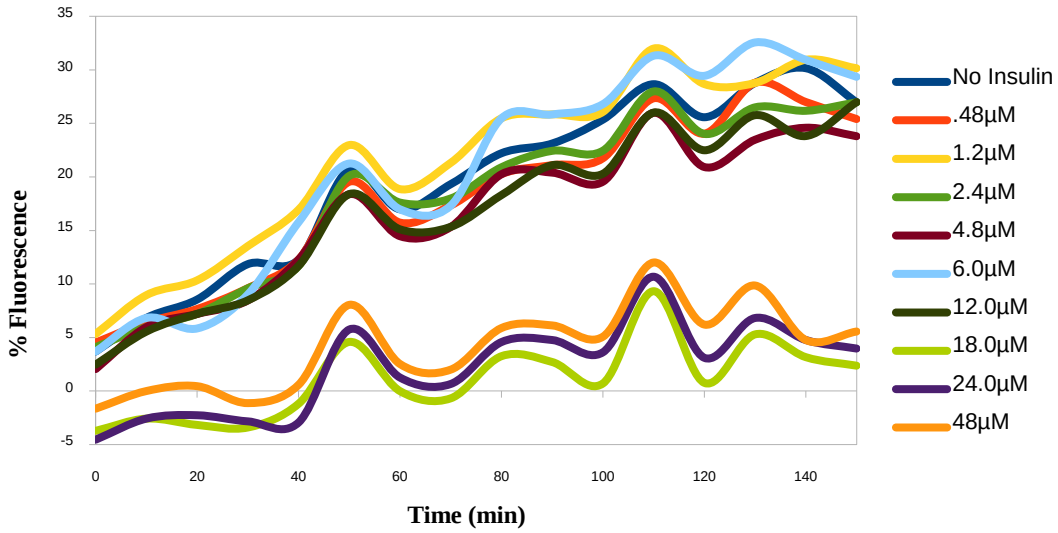

Figure 18. Activity of 9.1 μM hIAPP 1-19 on 7:3 DOPC: DOPS in the presence of varying concentrations of insulin.

Figure 18 represents the dye leakage caused by 9.1 μM hIAPP 1-19 in the presence of insulin on 7:3 DOPC: DOPS membrane. There were some negative fluorescence values due to internal error in background fluorescence. Insulin was found to decrease the membrane damage due to hIAPP1-19 by 17% at a concentration close to 48 μM (5:1 molar excess).

The effect at lower insulin concentrations was less significant. The effect of insulin analog 1 was found to be similar (13% reduction in membrane damage, Figure 16) to insulin against hIAPP1-19 on 7:3 DOPC: DOPS at a concentration of 44.5 μM. Also, at the highest concentration tested (177.5 μM, a 20: 1 ratio to hIAPP 1-19), analog 1 reduced dye leakage by about 65%. This result is promising, although it should be noted that insulin itself was not tested at this high concentration, so a direct comparison cannot be drawn. However, it is promising that at low concentrations, the truncated analog 1 can mimic insulin in reducing membrane damage caused by hIAPP.

5. SUMMARY

In POPG lipid, insulin analog 1 was found to show similar activity to insulin against hIAPP 1-19 in reducing dye leakage from the vesicles at low concentrations. There was no advantage observed upon increasing the molar ratio of analog 1 to hIAPP 1-19. Analog 2, however, was found to be slightly more effective against hIAPP 1-19 at low molar ratios (1:1 ratio of analog 2 to hIAPP 1-19). Again, little or no benefit was observed upon increasing the molar ratio to 4:1. By comparison to the actions of insulin analog 1 and 2, it can be concluded that the presence of an aromatic hydroxyl group on tyrosine (as in analog 1) is not a requirement for activity and may actually be detrimental to interaction with hIAPP 1-19.

In 7:3 DOPC: DOPS lipid, insulin analog 1 was found to show activity similar to insulin in reducing the dye leakage caused by 10 μM hIAPP 1-37. Both insulin and analog 1 were found to be minimally effective at low concentrations, showing inhibition to an extent of 5-7%. This effect was seen at a ratio of 0.2:1 to 1:1 (insulin/insulin analog 1: hIAPP 1-37). However, molar ratios greater than 1:1 were found to be ineffective to harmful (increasing dye leakage in the presence of insulin/inhibitor). Interestingly, insulin analog 2 was found to show a reduction in dye leakage by 20% at a molar ratio of 1:1 (insulin analog 2: hIAPP 1-37), making it a better inhibitor than insulin and analog 1, with no increase in dye leakage upon increasing the molar ratio. Insulin analog 3 was found to show very little inhibition, to an extent of 6% at a concentration of 15 μM. In addition, it was also found to cause an increase in dye leakage at very high concentrations (15:1 ratio of analog 3 to hIAPP).

Insulin analog 1 in 7:3 DOPC: DOPS lipid was found to be very effective against hIAPP 1-19 in reducing the membrane damage, to an extent of 60%, at a molar ratio of 20:1 (analog 1: hIAPP 1-19). When analogous concentrations were examined, analog 1 was found to show a similar effect to that of insulin. Analog 1 reduced dye leakage to an extent of 13% at a concentration of 44.5 μM, whereas insulin showed a 17% inhibition in membrane damage at 48 μM. This indicates that analog 1 is effective against the N-terminal region of hIAPP 1-19 but not against full length hIAPP in this lipid.

Based on the above results, insulin analog 1 was found to show different actions against hIAPP 1-19 and hIAPP 1-37 in 7:3 DOPC: DOPS membrane. Analog 1 was found to show a significant reduction in membrane damage against hIAPP 1-19 at a high concentration ratio of 20:1, whereas, it actually enhanced membrane damage caused by hIAPP 1-37.

Since the inhibitor/hIAPP binding region lies in the N-terminus of hIAPP, it is possible that the short inhibitor analog is better able to interact with hIAPP 1-19 than the full length hIAPP. This N-terminal region of hIAPP is thought to bind to the membrane to induce transient damage without forming fibers. The short analogs are as effective as insulin in inhibiting this form of damage. However, the secondary damage, most likely caused by fiber formation of the full length analog, is apparently not inhibited by either insulin or the shorter analogs. In this case, the “inhibitors” actually seem to promote the damage at high concentration and may even be incorporated into the fibers.

6. FUTURE DIRECTIONS

Future work will include testing a larger range of these insulin-based analogs in different lipid membranes by choosing appropriate compositions of each component, so that the resulting lipid vesicles mimic the actual pancreatic beta cell environment at various stages of life (i.e., varying amounts of cholesterol and oxidized fatty acids). Inhibitors of different lengths and those with modified amino acids may be tested to optimize the effect. Last, new assays will be developed to assess fiber formation so that effects of the inhibitors on that process can be understood.

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