Role of aromatic pi-stacking on the aggregation of human islet amyloid polypeptide (hIAPP)

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Role of Aromatic Pi-stacking on the Aggregation of Human Islet Amyloid Polypeptide (hIAPP)

by

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To

My loving mother
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ABSTRACT

Human islet amyloid polypeptide (hIAPP) is secreted in the β-cells of the pancreas, which also secretes insulin. In type 2 diabetes mellitus, hIAPP undergoes self-aggregation, forming fibrils. This self-aggregation is cytotoxic and is thought to be linked to type 2 diabetes mellitus by causing β-cell membrane destruction. The N-terminus of hIAPP (1-19) contains a binding site (residues 14-18) for self-aggregation. Aggregation is thought to be mediated by pi-stacking interactions between phenylalanine residues of hIAPP. In this study, the hIAPP 1-19 sequence was modified by replacing phenylalanine with alanine and naphthylalanine, to study if the modification of the aromatic side chain alters aggregation and membrane destructive activity. The activity of the modified hIAPP sequences was tested against differently charged lipids, using a fluorescent dye leakage assay. Both modified hIAPP 1-19 sequences were found to be less active when compared to the original hIAPP 1-19 sequence. This might be due to lack of aromatic pi-stacking interactions at the 15th position or due to inappropriate structural conformation of the naphthylalanine analog.
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1. INTRODUCTION

1.1 Amyloid Proteins

The study of proteins is a constantly evolving field posing innumerable challenges and offering a great scope for future exploration. Researchers have deciphered a lot of links between different proteins and diseases, yet a large number of peptides and their significance in various diseases still remain unstudied. Amyloids are a class of proteins which are formed from amyloid precursor proteins. The amyloid proteins are known to form amyloid deposits, which have a common presence in the case of various diseases like Alzheimer’s disease, type II diabetes, and Parkinson’s disease. The plaques are found in different parts of the human body and are usually similar structurally, with slight distinctions depending upon the regions in which they are present. Therefore, the study of amyloid proteins carries a great importance due their involvement in various pathological occurrences.¹

1.2 Amyloid Deposits

Amyloid deposits are formed due to misfolding of different amyloid proteins. The misfolding can occur either in the prefibrillar or the fibrillization assembly of the oligomers. The appearances of the amyloid deposits differ somewhat between disease states. These deposits are predominantly observed in the case of neurodegenerative disorders like Alzheimer’s disease, Down’s syndrome, and in pancreatic disorders like type 2 diabetes. During fibrillization of the amyloid proteins, different types of assembly states are observed, which exhibit a different spectrum of toxicities. Although the amyloid deposits contain proteins with different sequences, the basic structural characteristics of the deposits are similar.²
1.3 hIAPP

Islet amyloid polypeptide (IAPP) is a 37-amino acid peptide which is secreted by the β-cells of the mammalian pancreas. Human islet amyloid polypeptide (hIAPP) is the human version of IAPP. Insulin and hIAPP are co-secreted from the pancreas at varying ratios. The role of hIAPP is to maintain the homeostasis of glucose in the body. Previous studies have indicated strong evidence for the presence of hIAPP aggregates in the pancreas of people suffering with type 2 diabetes. The in vitro mechanism of the hIAPP peptide aggregation has been extensively studied, but very little is known about the in vivo mechanism. The amyloid fibrils which are formed due to aggregation of the hIAPP peptide have been found to be cytotoxic to the β-cells of the pancreas, resulting in reduction of insulin production. The imbalance of the insulin level in the body affects the glucose metabolism in the body and subsequently leads to type 2 diabetes mellitus.

1.4 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus is a rapidly growing disease which is affecting a population of approximately 140 million throughout the world. Type 1 diabetes mellitus is an autoimmune disorder which leads to apoptosis of the pancreatic β-cells, leading to a lack of insulin production in the body, which further leads to abnormal glucose levels in the body. In the case of type 2 diabetes, a resistance is developed by the cells towards insulin, leading to a subsequent increase in the insulin levels followed by a decrease in the β-cell mass. This in turn leads to the abnormal regulation of glucose levels in the body. The insulin resistance of the cells triggers the islets to produce more insulin. As the insulin production is increased, the IAPP levels also increase.
proportionally. Increased production of IAPP leads to the formation of amyloid fibrils which cause β-cell death, leading to type 2 diabetes.\textsuperscript{5}

1.5 Amyloid Fibril Formation

A substantial number of previous studies have proposed a common mechanism by which the hIAPP forms aggregates. The predicted mechanism involves a pathway leading to polymerization of the peptide, which is driven by nucleation.\textsuperscript{6} The mechanism is subdivided into two stages. In the first step, oligomeric seeds are formed, initiated by oligomeric nuclei. This stage is called the lag phase, which initiates the monomeric nuclei to form soluble oligomers and subsequently leads to formation of a chain of oligomers, also called protofibrils.\textsuperscript{7} The protofibrils combine together to form mature amyloid fibrils. Previous studies have indicated that the soluble oligomers exhibit higher cytotoxicity than the protofibrils and mature fibrils. Despite numerous attempts to understand the fibril formation, the actual mechanism is still not known.\textsuperscript{8} Figure 1 shows the transformation of the peptide from monomers to amyloid deposits.
Figure 1: Formation of amyloid deposits-A and B depict the peptide in the monomeric form, gradually forming aggregates (C) leading to formation of protofibrils (D), transforming to mature fibrils (E) and finally forming amyloid deposits (F). 

1.6 Active sites of hIAPP

Different fragments of the hIAPP peptide exhibit distinct roles in forming aggregates. A significant amount of research done on the 20-29 region revealed that it plays a major role in the fibrillization of hIAPP. The 20-29 region is suspected to form anti-parallel conformations of beta sheets with other regions of the hIAPP, leading to aggregation. The 11-19 and 30-37 regions of hIAPP are thought to be interacting with the 20-29 region, causing anti-parallel conformations that almost look like an “S” shape. These monomers then interact to form the oligomers and fibrils. When compared to hIAPP, the 20-29 region of rat IAPP (rIAPP) does not form the anti-parallel beta sheet complex, which in turn restricts the formation of the pleated beta sheets and fibers. This phenomenon in rats is thought to be due to the presence of proline residues which prevent the aggregation of the 20-29 region. 

Most recently, the 1-19 region of the hIAPP is being widely studied due to its similarity in activity to that of the full length hIAPP despite its inability to form fibers by itself. This indicates that damaging membrane effects may be mediated by more than fiber formation.
1.7 hIAPP 1-19

In recent studies, the hIAPP 1-19 region showed similar activity to that of the full length hIAPP in causing membrane disruption. The 1-19 sequence of hIAPP was found to show a stable conformation rendering an alpha-helical form, unlike the unstable beta-helical conformation of the full length hIAPP. Some studies indicate that the N-terminal region of the hIAPP 1-19 sequence is involved in inducing the membrane disruption in addition to the 20-29 region. The membrane disruptive activity of the hIAPP 1-19 sequence was studied by comparing it with the rat version, which showed that the hIAPP 1-19 sequence showed a higher intensity of membrane disruption than that of the rIAPP 1-19. Also, hIAPP 1-19 contains the 14-18 region which is thought to be a mediating site for the self-aggregation of the hIAPP sequence. Due to the smaller fragment size of the hIAPP 1-19 sequence and the similarity in activity to the full length sequence, it is easy to synthesize a relevant model to study hIAPP. The aromatic phenylalanine at the 15th position also allows study of aromatic pi-stacking interactions which are suspected to mediate the aggregation. Studies have also indicated that at higher concentrations, hIAPP 1-19 is actually more active than the full length hIAPP in causing membrane damage.

1.8 Role of Aromatic Pi-stacking Interactions

Recent studies have proposed that aromatic pi-stacking interactions play a role in the aggregation of the hIAPP peptide. The interactions between aromatic rings play a major role in the self-assembly of various biological molecules. These are nonbonding interactions which are found due to the alignment of the aromatic rings of different amino acid residues in the same parallel plane. This is a phenomenon in the self-assembly of proteins into their specific
conformations. These pi-stacking interactions play a major role in the self-assembly of the double helical structure of DNA, as well as forming a stable tertiary structure in the case of proteins. The favorable pi-stacking energy produced by these interactions was found to be entropy driven, causing the release of water molecules which are bound to the aromatic rings. Four possible pi-stacking interactions have been suggested, namely parallel displaced, T-shaped, parallel staggered and herringbone. Out of the four different models, the parallel displaced mechanism is highly prevalent among proteins. In the case of the parallel displaced pi-stacking mechanism, the aromatic residues are stacked in an anti-parallel direction upon each other in the same plane. In the case of hIAPP, this leads to the production of entropy-driven pi-stacking energy, which is suspected to cause the misfolding of the peptide and form aggregates. Figure 2 shows the possible pi-stacking mechanisms that are commonly observed during the self assembly of various biological molecules.\textsuperscript{12} The hIAPP 1-19 fragment contains the aromatic phenylalanine at the 15\textsuperscript{th} position, which might be studied for its role in aromatic pi-stacking interactions with other regions of hIAPP as well as insulin.
Figure 2: Aromatic pi-stacking mechanisms-(a) Parallel displaced mechanism, which is more prevalent in the case of proteins, (b) T-shaped mechanism, (c) Parallel staggered, and (d) Herringbone mechanism.$^{12}$

1.9 Membrane Disruption Mechanism of IAPP

Several mechanisms have been suggested to explain the membrane disruptive activity of IAPP. The peptides are usually monomers at lower concentrations, and as the concentrations increase, they tend to assemble into an aggregate.$^{13}$ At higher concentrations, the peptides are hypothesized to cause membrane disruption by three different mechanisms. The first mechanism is the barrel-stave mechanism, where the peptides aggregate resulting in the formation of pores within the membrane. The membrane disruption is predominantly caused by mature fibrils, and some studies indicate that the permeability of the membrane ends after complete fibrillization.
The second mechanism is the detergent-like mechanism, which involves rupturing the membrane by the peptide by forming high density micelles on the surface of the membrane. The third model is the toroidal wormhole, which works by lining the peptide within the lipid membrane, producing a curvature strain and subsequently leading to membrane disruption. The actual mechanism of the membrane disruption is still not understood completely. Based on the current evidence, it is suggested that all three types of membrane disruption mechanisms occur randomly. Figure 3 shows the different types of possible membrane disruption mechanisms.
Figure 3: A schematic for the different mechanisms of membrane disruption. A- Peptide in the solution, B- Monomeric form of the peptide, C- Assembly of the peptide on the surface of the membrane, D- Barrel-stave mechanism, E- Toroidal wormhole mechanism and F- Detergent like action.
1.10 Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) is one of the widely used techniques for peptide synthesis. The method is well known for its option of choosing the protecting groups for the required peptide sequence and also offers flexibility in introducing synthetic amino acids. Both single and double coupling of the amino acids can be automatically performed by this method. The synthesis is initiated by a coupling and subsequent deprotection of amino acids in repeated cycles. The synthesizer is equipped with a reaction vessel which holds the resin required for the binding of the peptide. The resin is deprotected first, followed by the coupling of the carboxy terminus of the incoming amino acid, followed by deprotection of its N-terminus. This process is continued for all of the amino acids required for the peptide sequence. In the final step, acetic anhydride can be used to cap the N-terminus of the peptide if desired. SPPS is classified into two forms, based on the protecting groups of the amino acids. The first form is the Fmoc form, which is currently in wide usage. A 20% v/v piperidine solution in N, N-dimethylformamide (DMF) is utilized for removing the fluorenyl oxymethyl carbonyl (Fmoc) protecting group of the amino acid. The second form is the one containing the t-butyl oxy carbonyl (Boc) deprotecting group. This method requires usage of hydrofluoric acid for final cleavage, which is considered hazardous. Although the Fmoc method is most commonly used for its simplicity and lesser risk, the Boc method has some advantages over the Fmoc method as it can be used for more complex processes. Also, when compared to Boc, the deprotection of the Fmoc group is more time-consuming, because of the anionic nitrogen which is released as the end product of the synthesis.¹⁶
2. RESEARCH GOAL

Type 2 diabetes mellitus is a major disease which affects a vast population around the world. A lot of investigation is underway to understand the pathology and to design a cure for the rapidly spreading epidemic. A common marker in all the cases of type 2 diabetic patients is the presence of human islet amyloid polypeptide (hIAPP) aggregates in the pancreas. The hIAPP aggregates are found to be cytotoxic to the pancreatic β-cells causing type 2 diabetes. The hIAPP protein is a 37 amino acid sequence, and various fragments of this peptide show different activities with regard to the amyloid fibril formation, which is believed to be the major driving force for cytotoxicity. Different theories have been proposed and new ones are being explored to understand the aggregation mechanism of the hIAPP peptide.

An aromatic pi-stacking interaction between various aromatic amino acid residues is one suspected reason for the aggregation of hIAPP. This hypothesis is not completely proven and is attracting a lot of research. Recent studies have indicated that the hIAPP 1-19 region is similar in activity to the full length hIAPP sequence. Due to a shorter amino acid sequence, the hIAPP 1-19 fragment is economically feasible and easier to synthesize. Also it contains the 14-18 region of hIAPP which is linked to the self-aggregation process of the peptide.

The goal of this research is to understand the role of aromatic pi-stacking interactions in the aggregation of the hIAPP 1-19 sequence. Since the sequence contains an aromatic phenylalanine residue at the 15th position, it seems important to study whether the phenylalanine is involved in any aromatic pi-stacking interactions that might contribute to membrane damage. By replacing the aromatic phenylalanine with alanine, it might be possible to see a reduced activity on the membrane due to lack of an aromatic moiety and therefore no pi-stacking
interactions. Also, by increasing the aromatic nature by replacing the alanine with a 1-naphthylalanine, which has an extra aromatic ring relative to the phenylalanine, we may observe an increase in aromatic pi-stacking interactions and subsequently aggregation and membrane damage. The activities of the modified hIAPP 1-19 analogs can then be compared to that of the unmodified hIAPP 1-19 sequence and also to the full length sequence to yield reasonable conclusions.
3. EXPERIMENTAL PROCEDURES

3.1 Synthesis of Modified hIAPP 1-19 Peptide Sequences

The solid phase peptide synthesis (SPPS) technique was employed to make the desired peptides. The necessary amino acids were weighed accurately and transferred into their respective vials. A constant amount of 0.4 mmol O-benzotriazole-N, N’, N’-tetramethyluronium hexafluorophosphate (HBTU), a coupling agent, was added to all the vials. Each vial thus contained 0.4 mmol of the amino acid and 0.4 mmol HBTU. The amino acids required for the specific peptide were arranged in the PS3 peptide synthesizer, from C-terminus through N-terminus, and 0.1 mmol of 4-methylbenzylhydrylamine hydrochloride (MBHA) resin was added to the reaction vessel. N, N-dimethylformamide (DMF) was used as the primary solvent and 20% (v/v) piperidine solution in DMF was used as a deprotecting agent. The coupling process was activated by using a 0.4 M N, N-diisopropylethylamine in DMF. The peptide synthesis was performed using standard Fmoc procedures. The peptide sequences synthesized are listed in Table 1.

Table 1. The modified hIAPP 1-19 sequences that were synthesized.

<table>
<thead>
<tr>
<th>Modified hIAPP sequences</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hIAPP 1-19 (Phe → Ala)</td>
<td>KCNTATCATQRLANALVHS</td>
</tr>
<tr>
<td>hIAPP 1-19 (Phe → Naphthylalanine)</td>
<td>KCNTATCATQRLAN(Nal)LVHS</td>
</tr>
</tbody>
</table>
All of the ACS grade chemicals used in the synthesis were purchased from Fisher Scientific, and all of the amino acids used were purchased from Midwest Biotech Inc. Figure 4 shows the various amino acids that were replaced for the structural modification.

Figure 4: Modified amino acids- (A) is phenylalanine, which is replaced by naphthylalanine (B) to increase the aromatic nature and replaced by alanine (C) to decrease the aromatic nature of the hIAPP 1-19 sequence. \textsuperscript{17}
3.2 Peptide Cleavage:

After the completion of synthesis, the peptides were bound to the MBHA resin which was present in the reaction vessel. DMF was added into the reaction vessel to transfer the peptide-resin amalgam from the reaction vessel into a coarsely fritted glass funnel. The peptide-resin mixture was rinsed using methylene chloride and dried under vacuum for 30 minutes. A solution comprising 0.5 mL water, 0.5 mL anisole, 10 ml trifluoroacetic acid, and a single crystal of phenol was prepared for the cleavage of the peptides. To cleave the peptides from the resin, the dried peptide-resin mixture was added to the solution prepared for cleavage and was constantly stirred for two hours at room temperature. The resin was separated from the mixture by filtration since it remained solid, whereas the peptide dissolved in the solution. The solution containing the peptide and solid resin was transferred into a coarsely fritted glass funnel, filtered under vacuum, and the filtrate was collected in a sidearm flask by constantly washing with TFA to collect the peptide completely.

The peptide was precipitated from the filtrate by slowly adding 60 mL of cold diethyl ether with constant stirring. The solid peptide was separated from the solution by filtering it using a finely fritted glass funnel under vacuum for 25 minutes. The peptide was dissolved in a solution containing 70% acetonitrile/ H₂O solution and an equal aliquot of distilled water. This solution was transferred into a lyophilization flask and frozen at -20°C for approximately 10 hours. The flask containing the frozen solution was mounted on the lyophilizer overnight to completely dry out any trace of moisture from the peptide. The dry peptide was collected from the lyophilizer for further analysis.
3.3 Purification and Analysis of the Peptides

The crude peptide which was obtained after lyophilization was collected and weighed accurately using an analytical balance. Reverse phase – high performance liquid chromatography (RP-HPLC) was employed to separate the impurities from the crude peptide and to determine the purity of the peptide. A Waters HPLC composed of a Waters 616 pump, a Waters 484 tunable absorbance UV detector, and a gradient controller was used for the preparative separation of the peptide from impurities. Trifluoroacetic acid (TFA) was used to dissolve the peptides and to facilitate injection into the HPLC. Solvent A was composed of 0.1% TFA in water and solvent B was made of 0.1% TFA in acetonitrile. The column was equilibrated at 90% A: 10% B for 20 minutes after which the peptide was injected. A Phenomenex Jupiter column (250 x 21.2 mm, C18, 10 µm and 300 Å) was used for the preparative separation process. After the injection of the peptide, the gradient was run, which increased the percentage of solvent B to 70% over a time of 2 hours. The pure peptide was manually collected into specific test tubes at a maximum wavelength of 280 nm. The test tubes supposedly containing the pure peptide were transferred into a lyophilization flask, frozen, and subsequently lyophilized to obtain the desired pure peptide.

Analytical RP-HPLC was used to further determine the purity of the peptide obtained after preparative cleanup. A Waters HPLC containing two Waters 515 HPLC pumps, a Waters 490E programmable multi-wavelength detector, a gradient controller, and a Phenomenex Jupiter analytical column (5 µm, C18, 250x4.6 mm and 300 Å) were used. Solvent A and solvent B were the same as those of the preparative run. A mass (1 mg) of the peptide was dissolved in 1 mL TFA for injection. A gradient was run starting at 90% A and 10% B, which resulted in a solvent peak after approximately 2 minutes, followed by a single peptide peak after 30 minutes at
30% A and 70% B. For the hIAPP 1-19 sequence which was modified with alanine, the peptide peak was observed at a wavelength of 214 nm, and the hIAPP 1-19 peptide modified with 1-napthylalanine resulted in a peak at 254 nm. Peak integration revealed that the peptides were 99% pure. The purity of the peptides was further confirmed by electrospray ionization-mass spectrometry, which showed that the peptides had the correct molecular weight.

### 3.4 Fluorescent Dye Leakage Assay

In order to study the activity of the peptides on model lipid membrane systems, a fluorescent dye leakage assay was utilized. The assay was designed based on a similar method developed by researchers at the University of Michigan. The initial stage of the assay involved preparation of lipid vesicles of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC): 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (sodium salt) (DOPS) (7:3). During the process, 5 mg of the lipid was dissolved in 2 mL of chloroform, followed by evaporation of chloroform using a low flow of nitrogen gas. A thin layer of lipid was formed on the walls of the test tube due to evaporation of the solvent, which was desiccated overnight under vacuum. The resulting lipid was dyed by adding 500 µL of 30 mM carboxyfluorescein dye, dissolved in sodium phosphate buffer at pH 7.5. The lipid and the dye solution were thoroughly mixed, followed by freeze-thaw cycles using liquid nitrogen. In order to prepare uniform vesicles for the assay, the solution was further processed 21 times using a mini-extruder manufactured by Avanti Polar Lipids, Inc. The dye-encapsulated vesicles were separated from the free dye by using a Sephadex G50 size exclusion column. The peptide solution was prepared by dissolving the purified peptide in DMSO at a concentration of 1
mg/mL, from which different aliquots were taken to analyze the activity. A solution of Triton X detergent was prepared for addition in the 100 % leakage control. Figure 5 shows the structures of the different kinds of lipid membranes used in the experiment.
Figure 5: Structures of the lipid vesicles used in this study. (A) 1-palmitoyl-2-oleoyl-\textit{sn}-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG) (B) 1,2-dioleoyl-\textit{sn}-glycero-3-phosphocholine (DOPC), (C) 1-palmitoyl-2-oleoyl-\textit{sn}-glycero-3-phosphocholine (POPC), (D) 1,2-dioleoyl-\textit{sn}-glycero-3-[phospho-L-serine] (sodium salt) (DOPS).
Different aliquots of the peptide stock solution along with the required buffer solution and vesicles were mixed and analyzed using a Jasco FP-6300 spectrofluorometer. The excitation and emission wavelengths were set at 489 nm and 513 nm, respectively. The cuvette containing the required amount of the sample and vesicles was analyzed over the course of 300 s. A blank sample which contained DMSO, buffer solution, and vesicles was run initially, followed by a sample which contained the detergent as 100% leakage (positive control) since the detergent completely breaks open the vesicles, releasing the dye. This process was repeated for each concentration of the peptide. The data obtained from the fluorescence spectrum was analyzed using Microsoft Excel. A Bio-tek FLx-800 microplate fluorescence reader was also utilized to run some of the samples due to malfunction of the FT-spectrofluorometer. The concentrations of the samples to be analyzed were altered for the new spectrofluorometer, because the installed 96-well plate reader could accommodate only 300 µL samples. However, the new method was advantageous as it facilitated running triplicates of the samples, providing consistency and reproducibility of the results. It also allowed monitoring over a longer time period. The average values of the triplicate results were compiled using Microsoft Excel. The percentage fluorescence is calculated using the formula:

\[
\text{% Fluorescence} = \frac{\text{Fluorescence of the sample} - \text{Control (DMSO only)}}{\text{Fluorescence of the sample and detergent} - \text{Control (DMSO only)}}
\]
4. RESULTS AND DISCUSSION

4.1 Structural Modifications to hIAPP 1-19 Sequences

The hIAPP 1-19 sequence contains an aromatic phenylalanine residue at the 15\textsuperscript{th} position, which was suspected to play a possible role in the aromatic pi-stacking which results in the formation of aggregates. The attempt was to confirm the role of aromatic pi-stacking in forming aggregates and causing membrane disruption by synthesizing two modified analogs of hIAPP 1-19 peptide. In the case of the first analog, an attempt was made to reduce the aromatic nature of the peptide by replacing the phenylalanine with alanine. The second modification attempt was based on increasing the aromatic nature of the peptide by replacing the phenylalanine with 1-naphthylalanine, which has an extra aromatic ring when compared to phenylalanine.

The purification of the first analog (15-ala) was unsuccessful the first time, because of improper solvent choice in dissolving the sample for the preparative HPLC. Initially, DMF was used to dissolve the peptide, which resulted in a gel-like solution, for unknown reasons. Subsequently, methanol was used to dissolve the peptide, which also did not properly dissolve the peptide, and not all of it could be recovered. The peptide was synthesized for a second time, and TFA was used to dissolve the peptide for injection into the HPLC, and the purification was successful. The synthesis and purification of the second hIAPP 1-19 analog (15-naphthylala) was successful and did not require further attempts. The percentage yield of the first analog of hIAPP was only 50%, which is partially due to slowdown of the HPLC pump, which resulted in reduced flow rate. However, the purified peptide was further tested using mass spectrometry and analytical HPLC, which confirmed that the peptide was pure. The percentage yield of the second
analog of hIAPP was 85%, and the peptide was found to be pure based on mass spectrometry and analytical HPLC.

4.2 Activity of the peptides on POPG

The activity of the peptides was first tested on POPG lipid membranes. POPG is a negatively charged lipid which was supposed to show optimal activity with the hIAPP 1-19 peptide and its analogs, due to the overall positive charges of the peptides. The hIAPP 1-19 sequence, which was modified with alanine, showed an increase in dye leakage from the vesicles, as the concentration of the peptide was increased. An increase in the percentage fluorescence was observed as the concentration of the peptide was increased, which indicated an increase in the membrane disruptive activity of the peptide. There was a huge increase in the percentage fluorescence between the peptide concentrations 1µM and 3 µM. This sudden change in the activity indicates that the peptide might be active at higher concentrations, or that there might be a threshold amount of the particular peptide to be effective on the membrane. Figure 6 shows the increasing activity of the hIAPP 1-19 peptide modified with alanine over time, with increase in the concentration of the peptide. At the maximum concentration tested (30 µM peptide), the percentage fluorescence was approximately 87%, which indicates a high membrane disruptive activity.
Figure 6: Percentage fluorescence due to dye leakage over time of the hIAPP 1-19 peptide modified with alanine on POPG.

The activity of the second analog of hIAPP 1-19, which was modified using naphthylalanine, was also tested on the POPG lipid. The naphthylalanine analog was expected to show increased activity relative to the non-aromatic analog of hIAPP 1-19, and unmodified hIAPP 1-19, because the increase in the aromaticity caused by naphthylalanine was supposed to induce the pi-stacking interactions which contribute to the increase in the membrane disruptive activity. The naphthylalanine containing analog was tested on POPG with increasing concentrations of the peptide, and concentration dependence was observed. The activity of the naphthylalanine modified hIAPP 1-19 analog was found to be similar to that of the analog modified using alanine. Figure 7 shows the activity of the naphthylalanine modified analog over time. The maximum percentage fluorescence observed at 30 µM was approximately 71%, which was slightly less than that of the alanine modified analog of hIAPP 1-19. The slight decrease in the percentage fluorescence of the naphthylalanine modified peptide was not clearly understood.
but could be due to variability in the assay. A similar threshold concentration of 3 µM was observed. The activity of the naphthylalanine modified hIAPP 1-19 analog on POPG was found to be higher than that of the activity on DOPC: DOPS (7:3) membrane, which showed a threshold activity of approximately 25% leakage.

Figure 7: Percentage fluorescence due to dye leakage over time of the hIAPP 1-19 peptide modified with naphthylalanine on POPG.
4.3 Activity of the peptides on POPC

The peptides were further tested on POPC lipid, which is zwitterionic in nature. The peptides were expected to show lower activity on POPC than on POPG, due to its zwitterionic rather than negative nature, and the overall positive charges of the peptides. Both of the modified hIAPP 1-19 analogs were tested on POPC and showed less activity than on POPG as expected. Figure 8 shows the activity of the hIAPP 1-19 analog modified with alanine on POPC. At the highest concentration of 30 µM, the peptide exhibited approximately 25% of the fluorescence of the detergent treated vesicles, and an increasing pattern of leakage was observed with time at each concentration, especially the higher ones.

![hIAPP 1-19 modified with Alanine on POPC](image)

Figure 8: Percentage fluorescence due to dye leakage over time of the hIAPP 1-19 peptide modified with alanine on POPC.
The activity of the hIAPP 1-19 analog modified with naphthylalanine showed similar activity on POPC. Figure 9 displays the activity of the hIAPP 1-19 analog modified with naphthylalanine on POPC. The maximum percentage fluorescence observed at 30 µM peptide concentration again was approximately 25%. However, the activity of the peptide at lower concentrations was less than that of the hIAPP 1-19 analog modified with alanine, which was evident from the lower percentage fluorescence values at lower concentrations of the peptide. A similar trend was also observed in the activity of the peptides on POPG.

Figure 9: Percentage fluorescence due to dye leakage over time of the hIAPP 1-19 peptide modified with naphthylalanine on POPC.
4.4 Activity of the peptides on DOPC: DOPS (7:3)

The modified hIAPP 1-19 analogs were tested on vesicles composed of DOPC: DOPS (7:3) lipids. This mixture is generally considered to be closest to that of the β-cell membrane. DOPC is zwitterionic, and DOPS is negatively charged. The analog altered with alanine showed a maximum percentage fluorescence value of 13% at 30 µM. The activity of this analog was expected to be low due to its non-aromatic nature. The percentage fluorescence values increased consistently with increasing concentration of the peptide as expected. The overall activity of this peptide on DOPC: DOPS (7:3) was found to be less than that on both POPG and POPC. In the case of POPG, this might be due to the more positive nature of DOPC: DOPS (7:3) compared to POPG, but the lower activity on this mixture than was observed on POPC was unexpected. Time dependence was not significantly observed during the assay. The pattern of the membrane disruptive activity of the hIAPP 1-19 modified with alanine is depicted in Figure 10.
Figure 10: Percentage fluorescence due to dye leakage over time of the hIAPP 1-19 peptide modified with alanine on DOPC: DOPS (7:3).

The activity of the analog modified with naphthylalanine (Figure 11) displayed a similar trend as that of the non-aromatic analog on DOPC: DOPS (7:3). At equal concentrations, the activity of the hIAPP 1-19 analog modified with naphthylalanine was higher than that of the analog modified with alanine by roughly 3-8 fold. The percentage fluorescence value at the maximum concentration (30 µM) was approximately 30%, which was between the values of the activities recorded on POPC and POPG. However, at lower concentrations, the naphthylalanine analog showed less activity in this case than it did on the other two lipids. The reason for this poor activity at lower concentration may be due to a required threshold for interaction with the membrane. Indeed, a similar pattern of much lower activity at lower concentrations of the peptide was also evident in the case of its activity on POPG and POPC.
Figure 11: Percentage fluorescence due to dye leakage over time of the hIAPP 1-19 peptide modified with naphthylalanine on DOPC: DOPS (7:3).

4.5 Activity of full length hIAPP and hIAPP 1-19 on DOPC: DOPS (7:3)

Previous studies have indicated that the activity of the hIAPP 1-19 sequence is similar to that of the full length hIAPP 1-37 sequence. Studies carried out by Anitha Jayaprakash and Joshua Osborne in our laboratory have indicated that the activity of the full length hIAPP is actually slightly lower than the truncated hIAPP 1-19 sequence in this assay, at similar concentrations. Both the peptides displayed concentration dependence and slight time dependence. Figures 12 and 13 show the activities of the full length hIAPP and hIAPP 1-19, respectively, on DOPC: DOPS (7:3) membrane. The hIAPP 1-19 sequence showed the highest percentage leakage of about 80% at 30 µM, whereas the full length sequence showed a leakage of approximately 55% at 24.1 µM concentration. Also, at 6 µM concentration, the activity of the full length hIAPP sequence showed a percentage leakage of approximately 25% which is low.
when compared to that of the unmodified hIAPP 1-19 sequence, which showed a percentage fluorescence of 40%.

Figure 12: Percentage fluorescence due to dye leakage over time of the full length hIAPP peptide on DOPC: DOPS (7:3).
The activities of both the modified hIAPP 1-19 analogs were found to be less than that of the unmodified hIAPP 1-19 sequence on the DOPC: DOPS (7:3) membrane. This might be due to improper folding of the peptides into a different conformation than the one required for causing membrane disruption. In the case of the 15-ala modified analog, the activity was expected to be low due to lack of aromatic phenylalanine residue. But this does not seem conclusive because the activity of the 15-naphthylala analog was also low, which was not expected. This indicates that disturbing the natural structure of the peptide might lead to unpredictable conformations. Also, it might be possible that the aromatic pi-stacking interactions do not play a significant role in the formation of aggregates in the case of hIAPP.
5. SUMMARY

The activity of the hIAPP 1-19 region was found to be slightly higher than the activity of full length hIAPP 1-37 in causing disruption of lipid vesicles in the DOPC: DOPS (7:3) model membrane system. The mechanism of membrane disruption may be more than just fibril formation, since the truncated peptide lacks the 20-29 region commonly thought to primarily mediate fibrilization. The results indicate that the hIAPP 1-19 region can be used as a model in drug target studies, in place of the expensive and synthetically challenging full length hIAPP.

The activity of both of the modified peptides was found to be lower than that of the original hIAPP 1-19 sequence on DOPC: DOPS (7:3), which is similar to the actual pancreatic cell membrane. This suggests that aromatic pi-stacking has no significant influence on the aggregation of the peptide or that the membrane disruption might be due to other mechanisms such as causing transient disorder of the acyl chains or head groups at the surface. It is also possible that pi-stacking is important, but that the naphthylananine analog is unable to adapt to the appropriate structural conformation for aggregation. It might also be possible that a different geometrical isomer of naphthylanaine or a different aromatic moiety may adapt to the required structural conformation. Any deviation from the native sequence/structure appears to be detrimental at this position. The aromatic pi-stacking interactions might exist at other aromatic residues, but not at the 15th position.
6. FUTURE WORK

The role of insulin in the aggregation of hIAPP is widely being studied. Therefore it might be useful to study the activity of the hIAPP sequence along with insulin in the future. Testing the interactions between various structurally modified hIAPP sequences along with insulin analogs on various lipid membranes may help us understand the role of insulin in the aromatic pi-stacking interactions, which might subsequently lead to aggregation of hIAPP. Since insulin has been shown to inhibit fiber formation under some conditions, insulin-based inhibitors of the process may be developed. It is also important to focus on developing new assays to understand the extent of fibril formation. The use of POPG must be avoided as much as possible due to inconsistent results. The artificially negatively charged lipid leads to too much interaction with the peptides. As the activity on the DOPC: DOPS (7:3) membrane is similar to the activity on the actual pancreatic membrane, it can be considered more reliable when compared to POPG and POPC. This significantly reduces the length of the experiment and also helps to yield better results. Also, using molecular modeling may provide a good platform to predict the different structural conformations of hIAPP.
7. REFERENCES


