Aggregation of Amylin in type II diabetes:
Molecular recognition elements in islet amyloid assembly
and functional inhibition

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SUBMITTED BY
Yair Porat

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This work was carried out under the supervision of

Dr. Ehud Gazit
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Abstract

The formation of amyloid fibrils is associated with major human diseases including Alzheimer’s disease, Parkinson's disease, prion diseases, and type 2 diabetes. Methods for efficient inhibition of amyloid fibril formation are hence of high clinical importance. Although various amyloidogenic proteins do not share any simple sequence homology, all amyloid structures share similar ultrastructural and physicochemical properties, and understanding the molecular interactions which facilitate fibril assembly is a big challenge.

One of the most common amyloidogenic diseases is type 2 diabetes. Pancreatic amyloid deposits composed of the human islet amyloid polypeptide (hIAPP) are found in more than 90% of type 2 diabetes patients, and their toxicity is assumed to be an important factor in pancreatic β-cell failure.

Previous results have demonstrated the key role of a phenylalanine residue in the minimal amyloidogenic fragment of hIAPP (NFGAILSS). Based on this observation, and the remarkable occurrence of aromatic residues in other short amyloid-related peptides, we speculated that aromatic-stacking interactions might play a key role in the acceleration of amyloid fibril formation. To further understand the role of the phenylalanine residue in hIAPP fibril formation, we studied the molecular recognition between hIAPP and peptide array, composed of the core amyloidogenic peptide fragment of hIAPP, with substitutions of phenylalanine residue to all other natural amino acids except cysteine. The peptide array results clearly demonstrated that molecular recognition between hIAPP and its core amyloidogenic fragment is mediated by aromatic rather than hydrophobic interactions.

Substitution of phenylalanine with tyrosine in the context of NFGAILSS core peptide (NYGAILSS) resulted in substantial halt of fibril formation but did not affect molecular recognition to hIAPP. Addition of the same peptide analogue inhibited fibril formation by
hIAPP. The inhibition was significantly higher than the one achieved using a β-sheet breaker conjugated peptide NFGAILPP.

Based on the molecular arrangement of tyrosine-phenylalanine interaction, we suggest that the inhibition stems from the geometrical constrains of the hetero-aromatic benzene-phenol interaction. In line with this notion, we demonstrate remarkable inhibition of hIAPP fibril formation and cytotoxicity toward pancreatic β-cells by small polyphenol molecule, the non-toxic phenol red compound. Phenol red is shown to inhibit hIAPP fibril formation in vitro in a concentration dependent manner and IC50 of ~1 µM. Furthermore it is shown that addition of phenol red to growth media of beta cells that were grown with hIAPP for 36 hours increased cell viability from 50% to 80% (P<0.05).

Understanding the molecular pathway which leads to hIAPP cytotoxicity is crucial for future therapeutic approach. We specifically studied the membrane interaction activity of soluble and intermediate hIAPP assemblies at high temporal resolution. A colorimetric analysis using lipid/polydiacetylene bio-mimetic vesicles clearly demonstrated the transient formation of prefibrillar assemblies that strongly interact with the lipid vesicles. The transient nature of the membrane-active assemblies was independently confirmed by a fluorescence-quenching assay. Ultrastructural analysis using transmission electron microscopy also supported the transient existence of membrane permeating soluble species, by showing the transient membrane destructive effect of these intermediates. The non membrane-active effect of mature fibrils was also observed.

Taken together, our results provide experimental evidence that hIAPP forms transient soluble prefibrillar assemblies which are highly membrane-active. Our results also provide further experimental support for the potential role of aromatic interactions in amyloid formation and establish a novel approach for its inhibition.
1. Introduction:

1.1 Protein misfolding and amyloidogenic diseases:

The phenomenon of protein misfolding is characterized by conformational changes that are coupled to the aggregation of misfolded proteins inside or outside the cells (Cohen and Kelly, 2003). This process is linked to disease due to self assembly of cellular proteins into amyloid fibrils and amyloidogenic deposits and poses a key medical importance. A partial list of more than twenty amyloid-related diseases includes Alzheimer's disease, Parkinson's disease, Huntington's disease, prion diseases, familial amyloidosis and Type II diabetes, (Cohen, 1999; Dobson, 1999; Sadana and Vo-Dinh, 2001; Soto, 2001; Muchowski, 2002; Thompson and Barrow, 2002; Dobson, 2003; Gregersen et al., 2003; Soto, 2003; Uversky, 2003; Agorogiannis et al., 2004; Ross and Poirier, 2004)

In spite of the fact that various amyloid-related proteins and polypeptides do not reveal any simple sequence homology, all amyloid structures share similar ultrastructural and physicochemical properties. All amyloid structures have characteristic elongated fibrillar morphology with 5-15 nanometers in diameter. Amyloidogenic proteins are rich with β-sheet structures as observed by circular dichroism and FTIR analysis (Cascio et al., 1989; Bouchard et al., 2000; Uversky and Fink, 2004). X-ray fiber diffraction studies of six different amyloid fibrils have shown a repeating pattern of 4.6-4.8 Å, which is considered to be β strand repeats that forms a complete β sheet whose strands run perpendicular to the fibril axis (Blake et al., 1996; Sunde et al., 1997). Another well-known characteristic of all amyloid fibrils is specific optical behavior such as birefringence following staining with the congo-red dye (Khurana et al., 2001; Roterman et al., 2001).
Experiments *in vitro* indicate that amyloid formation is generally characterized by a lag phase, followed by a period of rapid growth (Bitan et al., 2003; Caughey and Lansbury, 2003; Green et al., 2004). Such behavior is typical of nucleation processes such as crystallization, and the lag phase can be eliminated by the addition of preformed aggregates to fresh solutions, a process known as seeding (Jarrett and Lansbury, 1992; Lomakin et al., 1996; Padrick and Miranker, 2002). Furthermore, amyloidogenesis is a high-order kinetic process and therefore highly influenced by modest decrease in the protein concentration (Cohen and Kelly, 2003).

### 1.2 Interaction which facilitate amyloid fibril formation:

The ability of polypeptide chains to form amyloid structures is not restricted to the relatively small number of proteins, associated with recognized clinical disorders, but it now appears to be a generic feature of polypeptide chains (Bucciantini et al., 2002). The core structure of the fibrils seems to be stabilized primarily by interactions, particularly hydrogen bonds, involving the polypeptide main chain. Because the same chemical structure of the main chain is common to all polypeptides, this observation explains why fibrils formed from polypeptides of very different sequence seem to be so similar (Dobson, 1999). Even though the ability to form amyloid fibrils seems to be generic, the propensity to do so under given conditions can vary markedly between different sequences. The relative aggregation rates for a wide range of peptides and proteins correlates with the physicochemical features of the molecules such as charge, secondary-structure propensities, hydrophobicity and aromatic interactions (Gazit, 2002; Dobson, 2003).
1.3 Aromatic interactions and amyloid fibril formation:

The initial hypothesis on the role of aromatic interactions in amyloid fibril formation was based on the remarkable occurrence of aromatic residues in many amyloid-related proteins and short peptide fragments (Gazit, 2002; Gazit, 2002), and the well-known role of aromatic stacking in processes of self-assembly in chemistry and biochemistry (Burley and Petsko, 1985; Burley and Petsko, 1986; Aggeli et al., 1997; Claessens and Stoddart, 1997; Tartaglia et al., 2004). This hypothesis suggests that stacking of aromatic residues may play a role in the acceleration of the assembly process in many cases of amyloid fibril formation. Stacking interactions may provide an energetic contribution as well as directionality and orientation that are facilitated by the restricted geometry of planar aromatic rings stacking (Azriel and Gazit, 2001; Gazit, 2002; Naito et al., 2004). This is in line with the observations made by Burley and Petsko (Burley and Petsko, 1985) revealing that about 60% of aromatic side chains in proteins are involved in aromatic pairs. This theory was further demonstrated by showing that short aromatic dipeptides contain all the molecular information to self-assemble into well-ordered nanostructures that are structurally related to amyloid fibrils (Reches and Gazit, 2003).

Although the molecular mechanism of amyloid fibril formation and the direct correlation to disease in vivo is not fully understood, the appearance of disease-related fibril aggregates has been correlated with potent nonspecific cytotoxicity (Bucciantini et al., 2004). Furthermore, assemblies of non-disease-related amyloid fibril structures were also shown to induce significant cytotoxic effects (Bucciantini et al., 2002). In that regard, membrane permeation was proposed as a primary mechanism mediating amyloid fibril cytotoxicity, which might explain the generic and non-receptor-specific
activities of such assemblies (Mirzabekov et al., 1996; Lin et al., 1999; Zhu et al., 2000; Anguiano et al., 2002; Volles and Lansbury, 2002; Green et al., 2004).

1.4 Therapeutic approaches towards amyloidogenic diseases:

Several therapeutic approaches were suggested so far towards amyloidogenic diseases. These approaches include reduction in the production of amyloidogenic form of proteins, increasing the clearance rate of misfolded or aggregated proteins, increasing amyloidogenic proteins native state stability, and direct inhibition of the self assembly process (Cohen and Kelly, 2003). Some of the examples for these approaches include inhibition of BACE1 β-secretase which cleaves APP to its amyloidogenic form (Potter and Dressler, 2000), chemical chaperons (Petaja-Repo et al., 2002), the use of antibodies to increase the protein amyloidogenic form clearance (Spooner et al., 2002) or direct inhibition with specific antibodies (Lambert et al., 2001; Frenkel and Solomon, 2002; Kayed et al., 2003), the application of peptide or peptidomimetics based on short peptides which are highly similar to the native protein with some modification, and small molecule inhibitors (Cohen and Kelly, 2003).

The peptide inhibitors line includes similar to native peptides such as KLVFF for Alzheimer disease (Tjernberg et al., 1996; Scrocchi et al., 2002; Watanabe et al., 2002), β-sheet breaker peptides with proline insertions (Soto et al., 1998; Thakur et al., 2004) or other β-sheet breakers (Cruz et al., 2004; Gilead and Gazit, 2004). Another approach is the use of retroinverso or all D-amino acid peptides (Tjernberg et al., 1997; Findeis and Molineaux, 1999; Cohen and Kelly, 2003). Although very promising the peptide approach suffers from low penetration, low stability, and fast clearance rates of the inhibitory peptides.
Small molecule inhibitors approach was initially based on the long known finding that molecules such as congo red and thioflavin T interact specifically with amyloid fibrils and inhibit their formation (Lorenzo et al., 1994; Lee, 2002; Poli et al., 2003). In the past few years there is an accumulation of reports describing small molecule inhibitors of amyloid fibril formation. A partial list of these reports which include more than 50 inhibitors suggests that aromatic rich small molecular inhibitors are efficient \textit{in vitro} to inhibit amyloid fibril formation and some of them were shown to dramatically inhibit cell death in cell culture assays (Lorenzo et al., 1994; Lorenzo and Yankner, 1994; Tomiyama et al., 1996; Hertel et al., 1997; Findeis, 2000; Kuner et al., 2000; Levites et al., 2001; Lashuel et al., 2002; Lee, 2002; LeVine, 2002; Ono et al., 2002; Ono et al., 2002; Yager et al., 2002; Aitken et al., 2003; Bartolini et al., 2003; Conte et al., 2003; Hartsel and Weiland, 2003; Kocisko et al., 2003; Nishimura et al., 2003; Ono et al., 2003; Poli et al., 2003; Sabate and Estelrich, 2003; Cordeiro et al., 2004; Hutter-Paier et al., 2004; Iuvone et al., 2004; Ono et al., 2004). So far only some green tea polyphenols such as epigallocatechin gallate were reported to have a generic inhibition features and were reported to inhibit α-synuclein, β-amyloid and Scrapie associated prion protein (Levites et al., 2001; Kocisko et al., 2003; Ono et al., 2003), respectively. The main drawback of these inhibitors is theirs lack of specificity (Cohen and Kelly, 2003).

Unfortunately none of these approaches resulted in an efficient drug that has passed phase II clinical trials so far.

1.5 Human islet amyloid polypeptide and type 2 diabetes:

One of the most common amyloidial diseases is Type 2 diabetes. According to the Center for Disease Control and Prevention (CDC), it is estimated that more than 15%
of the American population above the age of 65 suffer from Type II diabetes. In the postmortem of Type II diabetes patients more than 90% have amyloid deposits in their pancreas (Jaikaran and Clark, 2001; Hoppener et al., 2002; Clark and Nilsson, 2004; Hull et al., 2004). These deposits are composed of the human islet amyloid polypeptide (hIAPP), a 37-residue peptide hormone that is produced in the pancreatic β-cells and co-secreted with insulin. The early stage of type 2 diabetes is characterized by insulin resistance, followed by increased insulin and hIAPP secretion. This secretion initiates an increase in extracellular IAPP concentrations that may exceed 100-fold of the normal IAPP concentration (Hoppener et al., 1999; Kahn, 2003). The elevated concentration is probably a key issue in amyloid formation as fibril assembly is nucleation-dependent, and the lag time needed for the nucleation of amyloid fibrils growth is strongly correlated with protein concentration (Jarrett and Lansbury, 1992; Kayed et al., 1999; Gazit, 2002; Padrick and Miranker, 2002).

Even though a direct correlation between hIAPP fibrillization and in vivo β-cell death has not yet been fully established, some evidence creates the linkage between hIAPP and type 2 diabetes. Early onset of type 2 diabetes in the Chinese and Japanese populations was found to be correlated to a single mutation (S20G) in hIAPP, which was later found to enhance hIAPP aggregation in vitro as well (Sakagashira et al., 1996; Sakagashira et al., 2000; Seino, 2001). Several animal rodent models have shown that over expression of human IAPP accompanied by knock out of rodent IAPP induced symptoms which resemble type 2 diabetes, especially in conjugation to high fat diet or obesity (van Hulst et al., 1997; Ahren et al., 1998; Soeller et al., 1998; Hoppener et al., 1999; Mulder et al., 2000; Westermark et al., 2000; Wang et al., 2001; Hull et al., 2003; Butler et al., 2004)
1.6 Short fragments of hIAPP form amyloid fibrils:

The first fragment of hIAPP that was shown to form amyloid fibrils *in vitro* was the decapeptide hIAPP20-29 (SNNFGAILSS) (Westermark et al., 1990). A hexapeptide fragment of hIAPP22-27 (NFGAIL) was shown to form amyloid fibrils that are very similar to those formed by the full-length polypeptide (Tenidis et al., 2000). Furthermore, rodent IAPP, which does not form amyloid fibrils *in vitro*, is almost identical to human IAPP apart from a seven amino acid block that includes most of this hexapeptide motif (Figure 1) (Hoppener et al., 2000).

![Figure 1: Sequence alignment of human vs rodent IAPP 1-37](image)

Figure 1: Sequence alignment of human vs rodent IAPP 1-37. 5 of the 6 mismatches (red) are located in the core amyloidogenic fragment SNNFGAILSS (hIAPP20-29), colored in blue, which form amyloid fibrils that are similar to the full length polypeptide.

Hence, this fragment seems to serve as the core amyloidogenic fragment of hIAPP. Previous work had demonstrated that alteration of the phenylalanine residue of this core fragment to an alanine completely abolishes the ability of the peptide fragment to form amyloid fibrils (Azriel and Gazit, 2001). On the other hand, the change of any other amino acids of the core fragment into alanine did not affect amyloid formation.
1.7 Correlation of hIAPP prefibrillar assemblies to type 2 diabetes:

Even though a direct correlation between hIAPP fibrillization and in vivo β-cell death has not yet been fully established, several studies have shown that external addition of synthetic hIAPP at low concentrations (8-10 µM) induced cytotoxic death in cell culture (Lorenzo et al., 1994; MacGibbon et al., 1997; Saafi et al., 2001; Zhang et al., 2002). A later study indicated that protofibrillar hIAPP, rather than fibrillar hIAPP, permeabilized model lipid vesicles (Anguiano et al., 2002). Protofibrillar intermediates are known to exist not only in the case of hIAPP but also in the early stages of fibril formation of various amyloidogenic proteins among them β-amyloid, α-synuclein, ABri peptide, and the N-terminal domain of HypF protein (Harper et al., 1999; Janson et al., 1999; Zhu et al., 2000; Chiti et al., 2001; El Agnaf et al., 2001; Anguiano et al., 2002; Bucciantini et al., 2002; Jimenez et al., 2002; Lashuel et al., 2002; Lashuel et al., 2002; Souillac et al., 2002; Caughey and Lansbury, 2003; Chromy et al., 2003; Hoshi et al., 2003; Kheterpal et al., 2003; Lashuel et al., 2003; Lee and Eisenberg, 2003; Volles and Lansbury, 2003; Bucciantini et al., 2004; Eakin et al., 2004; Gazit, 2004; Green et al., 2004). These soluble assemblies are rich in β-sheet structures and share similar radial dimensions of 7-12 nm (Lashuel et al., 2002). The protofibrils are transient and eventually disappear as mature fibrils grow.

1.8 Prefibrillar assemblies structure and cytotoxicity:

A recent study suggested that soluble amyloid oligomers consisting of diverse proteins might share a common structure (Kayed et al., 2003). This hypothesis was based on the observation that antibodies, raised to specifically recognize micellar structures of Alzheimer’s Aβ polypeptide, but not its soluble or amyloid forms, interacted with soluble oligomer assemblies of structurally unrelated proteins.
including insulin, IAPP, polyglutamine, and \( \alpha \)-synuclein, and inhibited their cytotoxic effect (Kayed et al., 2003). One of the crucial questions pertaining to the biological activity of protofibrils concerns the extent of their membrane interactions and significant cytotoxic effects. Inhibition of amyloid fibril formation, and especially inhibition of prefibrillar oligomers formation, is therefore considered as a key prospect therapeutic approach towards diabetes and other amyloid-related diseases.

In the present study we have focused on using our mechanistic insights into the process of hIAPP amyloid formation to search for a novel approach of inhibition using hetero-aromatic interactions. In addition we studied membrane interaction and ultra structures of soluble and nonsoluble hIAPP assemblies at high temporal resolution.

1.9 Research objectives:

There is clear evidence that islet amyloid polypeptide is related to type 2 diabetes pathogenesis. However the mechanism of amyloid formation is not fully understood. Aging and environmental reasons initiate misfolding of the normal islet amyloid polypeptide and accumulation of the amyloidogenic form of hIAPP in \( \beta \) cells, causing cell death. Preliminary results and data in the literature led us to the hypothesis that aromatic interactions are one of the driving forces of amyloid formation and can be used to inhibit fibril formation. Our overall objective was to understand the recognition elements that encounter self-assembly of hIAPP and the specific role of aromatic residues in that process. Another objective was rational design of peptide and small molecule inhibitors that will bind hIAPP molecules and will inhibit amyloid formation.
2. Experimental Procedures:

2.1 Aromatic analogue peptide synthesis:
Aromatic analogues of NFGAILSS peptide (namely NYGAILSS and NWGAILSS) were synthesized using solid-phase procedures, and purified using reverse-phase high-performance liquid chromatography (HPLC). Mass-spectrometric analysis indicated the correct molecular weight for the peptide synthesized. Peptides were synthesized on a 0.1 mmol scale using an Applied Biosystems 433A automated peptide synthesizer. Double coupling was performed for the Asn for all three peptides. Incomplete removal of Fmoc group under standard conditions for the fully assembled octapeptides necessitated a double de-protection step before cleavage from the resin. Peptides were cleaved from the resin using a cocktail containing 0.75g phenol, 0.25 ml EDT, 0.5 ml thioanisole, 0.5ml de-ionized water and 10ml TFA for 90-180 min. Peptides were purified using a C18 reversed phase column (Waters Inc.) with an acetonitrile/water/trifluoroacetic acid gradient. The final peptides as lyophilized solids were stored at –20°C.

2.2 Peptide synthesis and preparation of stock solutions:
The synthesis of all other peptides was performed using solid-phase methods by Peptron Inc (Taejeon, Korea) for hIAPP22–29 peptide analogues, and by Calbiochem for hIAPP1–37. The correct identity of the peptides was confirmed by ion spray mass spectrometry, and the purity of the peptides was confirmed by reverse phase high-pressure liquid chromatography. hIAPP22–29 and hIAPP20–29 stock solutions were prepared by dissolving the lyophilized form of the peptides in DMSO at a concentration of 100 mM. The stock solution for hIAPP1–37 was prepared by dissolving the lyophilized form of the peptide in 3,3,3,3',3',3'-hexafluoro-2-propanol
(HFIP) at a concentration of 400 mM. To avoid any preaggregation, all stock solutions were sonicated for 2 min before each experiment. MALDI-TOF mass spectrometry analysis (using an Applied Biosystems Voyager DE-STR spectrometer) revealed that no fragmentation of the polypeptide occurred after sonication.

### 2.3 MBP–IAPP fusion protein expression and purification:

This protocol is based on previous work (Mazor et al., 2002). Briefly, for expression of hIAPP fused to the C-terminus of MBP, *Escherichia coli* cells transformed with expression plasmid pMALc2x-IAPP, were grown in 200 mL of LB medium supplemented with 100 mg/mL ampicillin and 1% (w/v) glucose and induced with 0.5 mM IPTG. Cell extracts were prepared in 10 mM phosphate buffer (pH 7.5) and protease inhibitor cocktail (Sigma-Aldrich), by freezing and thawing followed by a brief sonication. The extracts were clarified by centrifugation at 20000g and stored at 4 °C. MBP–IAPP fusion protein was purified by passing the extract over an amylose resin column (New England Biolabs, USA) and recovered by elution with 20 mM maltose in 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 200 mM NaCl buffer.

### 2.4 Membrane binding assay:

An array of 19 synthetic decamers that contain the core hIAPP20–29 amyloidogenic fragment (SNNFGAILSS), with all possible natural amino acid substitutions (except cysteine) at the position of phenylalanine23 (Jerini, Germany), was used. Following blocking with 5% (v/v) nonfat milk in 25 mM Tris-buffered saline (TBS, pH 7.4 (TBS), the membrane was incubated in the presence of 25 mg/mL MBP–hIAPP at 4 °C for 12 h. The membrane was then washed repeatedly with 0.05% (v/v) Tween 20 in TBS. Interaction of the MBP–IAPP fusion protein (or MBP as a control) with the
membrane-bound peptides was detected with an anti-MBP monoclonal antibody (Sigma) and a HRP-conjugated goat anti-mouse as a secondary antibody. An immunoblot was developed using liquid 3,’3-diaminobenzidine.

2.5 Kinetic aggregation assay:

The hIAPP$_{22-29}$ or hIAPP$_{20-29}$ peptide stock solution was diluted into 10 mM Tris-HCl (pH 7.2) buffer to a final concentration of 1 mM peptide and 4% DMSO. Turbidity was measured at 405 nm at room temperature using disposable UVette cuvettes (Eppendorf, Germany) using a Scinco S-3100 spectrophotometer.

2.6 Thioflavin T fluorescence assay:

A stock solution of synthetic hIAPP$_{1-37}$ was diluted to a final concentration of 4 mM in 10 mM sodium acetate buffer (pH 6.5) with or without inhibitor (40 mM), and a final HFIP concentration of 1% (v/v). Immediately after dilution, the sample was centrifuged at 4 °C for 20 min at 20000 g, and the supernatant was used for fluorescence measurements. ThT was added to a final concentration of 3 mM, and fluorescence was measured using a Perkin-Elmer 50SB fluorimeter (excitation at 450 nm, 2.5 nm slit; emission at 482 nm, 10 nm slit). For the phenol red inhibition, samples were diluted 10-fold so that the maximal phenol red concentration did not exceed 4 mM, and measured using a Jobin Yvon Horiba Fluoromax 3 fluorimeter (excitation at 450 nm, 2.5 nm slit; emission at 482 nm, 5 nm slit).

2.7 Circular dichroism spectroscopy.

Human IAPP (4 µM) was prepared as described above, with or without inhibitor (40 µM). Spectra were recorded at 25 °C with 1 nm intervals and an averaging time of 4 s,
using an AVIV 202 CD spectrometer. For the hIAPP lipid vesicles experiments hIAPP (4 µM, 500 µL) was prepared as mentioned above. hIAPP-lipid vesicle mixtures were prepared by adding 50 µl of lipid vesicles to 445 µl acetate buffer and finally hIAPP in HFIP was added to a final concentration of 4 µM and final volume of 500 µL. Final scan values represent subtraction of the baseline (buffer in the case of hIAPP, buffer with inhibitor for the inhibition assay, and buffer plus lipid/PDA vesicles for the peptide/vesicle mixtures). The overall contribution of the inhibitors was relatively minor. The spectra of the inhibitors are available in appendix 1.

2.8 Transmission electron microscopy:

Samples (10 mL) of hIAPP_{22-29} from the aggregation assay, or hIAPP from the fluorescence assay, or samples of hIAPP-phospholipid/PDA vesicle mixtures extracted for the colorimetric assay (both pellet suspension and supernatant) were placed on 400-mesh copper grids (SPI supplies, West Chester PA) covered by carbon-stabilized Formvar film. After 1 minute, excess fluid was removed, and the grids were negatively stained with 2% uranyl acetate in water for another two minutes. Samples were viewed in a JEOL 1200EX electron microscope operating at 80 kV and high-resolution Philips Tecnai F20 field emission gun TEM operating at 200 kV.

2.9 Atomic force microscopy:

Same grids that were used for TEM were viewed using Molecular Imaging AFM PicoScan Plus. Scan size was 700nm using contact mode.
2.10 **Scanning Electron Microscopy:**

Cells were grown on glass microscope cover slips under the same conditions as for the MTT assay. Immediately after incubation with hIAPP, the cells were fixed with 2% glutaraldehyde (v/v) and stored for 24 h at 4 °C. The cells were serially dehydrated with increasing concentrations of ethanol (30, 50, 70, 90, 95, and 100%) and dried with a critical point drier. Specimen cover slips were coated with colloidal gold and viewed using a JEOL JSM 840A microscope operating at 25 kV.

2.11 **MTT Reduction Assay:**

βTC-tet cells (Fleischer et al., 1998) or PC12 cells were plated in 24-well plates (2\times10^5 cells/well) or 96-well plates (1\times10^4 cells/well), respectively, and allowed to adhere for 24 h. A synthetic hIAPP stock solution was diluted to a final concentration of 4 µM in serum free growth medium containing DMEM with or without phenol red. Immediately after dilution, samples were centrifuged at 4 °C for 20 min at 20000g, and the supernatant was bubbled with nitrogen for 30 min to evaporate residual HFIP. Cells were washed twice with PBS and incubated with the supernatant for 24 h. MTT was then added for 3 h, followed by addition of lysis buffer and incubation overnight. Samples were read at 570 nm. Cell viability was calculated in comparison to that of cells incubated in the absence of hIAPP, in medium with or without phenol red, respectively.

2.12 **Preparation of hIAPP aggregates and fraction separation:**

Synthetic hIAPP (CalBiochem CA, USA) was dissolved in HFIP (1.95 mg/ml) and diluted to a final concentration of 5 µM in 10 mM sodium acetate buffer (pH 6.5), and a final HFIP concentration of 1% (vol). Immediately after dilution, and every 30
minutes, 1 ml samples solutions were transferred to a microtube and centrifuged for 15 minutes in 20,000g at 4 °C. The supernatant fractions (0.6ml) were transferred to another tube and pellet fractions were gently resuspended in the remainder 0.4ml. For the higher temporal resolution experiments, the same procedure was used using 4 µM hIAPP in 10 mM sodium acetate buffer (pH 6.5) and final HFIP concentration of 1% (vol.), at 20 min intervals.

### 2.13 Polymerized lipid vesicles:

The diacetylene monomer tricosadiynoic acid was purchased from GFS Chemicals (Powell, OH). Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma (St. Louis, MO). Preparation of vesicles containing lipids and PDA has been described previously (Kolusheva et al., 2000; Kolusheva et al., 2001). Briefly, the phospholipid and monomer constituents were dissolved in chloroform/ethanol and dried together in vacuo, followed by addition of deionized water and probe-sonication for 2–3 min at 70 °C. The vesicle solution was cooled and kept at 4 °C overnight, and then polymerized in a ultra-violet (uv) oven (cross-linker) by irradiation at 220 nm for 10–20 sec. The resulting solutions exhibited an intense blue color.

### 2.14 Color reaction assay:

Vesicles were prepared at concentrations of 1 mM total lipid. Samples for the colorimetric measurements were prepared by adding 5µM hIAPP after centrifugal separation (or 4µM in the higher temporal resolution experiments, as described above) to 0.5 mM total lipid vesicles and 25 mM Tris base (pH 8) to a final volume of 1ml. Uv-vis measurements were carried out at 28 °C using a Jasco V550 uv-visible spectrophotometer, with a 1 cm optical path cell. To quantify the extent of blue-to-red
color transitions within the vesicle solutions, the colorimetric response (% CR), was defined and calculated as follows (Jelinek et al., 1998):

$$%CR = \frac{(PB_0 - PB_I)}{PB_0} \times 100,$$

where $PB = A_{\text{blue}}/(A_{\text{blue}} + A_{\text{red}})$, and $A$ is the absorbance at 640 nm, the “blue” component of the spectrum, or at 500 nm, the “red” component (“blue” and “red” refer to the visual appearance of the material, not actual absorbance). $PB_0$ is the blue/red ratio of the control sample before induction of a color change, and $PB_I$ is the value obtained after the colorimetric transition occurred.

2.15 NBD fluorescence-quenching assay:

Vesicles containing NBD-PE were prepared as described in (Ahn et al., 2000). Briefly, C6-NBD-PE was dissolved in chloroform, added to the monomers and phospholipids at 1 mol % and dried together in vacuo before sonication (see Polymerized lipid vesicles). Addition of the NBD-labeled phospholipids did not affect either the blue color of the vesicles or the blue-red transitions.

Samples were prepared by adding 4 µM hIAPP after centrifugal separation (as described above) to 0.5 mM total lipid vesicles and 25 mM Tris base pH 8 to a final volume of 1ml. The fluorescence quenching reaction was initiated by adding sodium dithionite from a stock solution (0.6 M), prepared in 50 mM tris buffer (pH 11) to a final concentration of 10 mM. The decrease in fluorescence was monitored for 5 min at 28 °C using 467 nm excitation and 535 nm emission on an Edinburgh FL920 spectrofluorimeter. The fluorescence decay was calculated as a percentage of the initial fluorescence measured before the addition of dithionite. A control curve represented NBD fluorescence decays induced by dithionite only, while other curves were recorded following addition of hIAPP after different aggregation periods.
3. Results:

3.1 Mechanism of amyloid formation

3.1.1 Self-assembly of the hIAPP core amyloidogenic peptides:

Human IAPP\textsubscript{20-29} is considered to be the main core module of hIAPP fibril formation, and was previously shown to assemble amyloid fibrils that resemble hIAPP\textsubscript{1-37} fibrils. In order to follow the self-assembly process of the minimal amyloidogenic fragment of hIAPP we measured the aggregation of the NFGAIL hexamer fragment (hIAPP\textsubscript{22-27}), the NFGAILSS octamer, and SNNFGAILSS decamer (hIAPP\textsubscript{22-29}, hIAPP\textsubscript{20-29} respectively) and studied their ultrastructural morphology using transmission electron microscopy (TEM). We compared these ultrastructures to the morphology of hIAPP (hIAPP\textsubscript{1-37}) fibrils. Aggregation assay using high concentrations of these peptides (1-2 mM) showed that residue length affects aggregate formation kinetics in an opposite manner, so that longer residues form aggregates more slowly. NFGAIL and NFGAILSS residues aggregate within seconds at 1mM concentration, SNNFGAILSS aggregates within a few hours at 1mM, and hIAPP\textsubscript{1-37}, which is used in 4-10\textmu M concentration range, aggregates within days.

In order to verify that the measured aggregation indeed reflects amyloid formation, TEM was used to view aggregate morphology. NFGAIL aggregates were composed of relatively small amounts of 10-20 nanometer wide fibrils (Figure 2A). NFGAILSS aggregates were relatively dense with 10-50 nanometer wide fibrils with more rigid morphology (Figure 2B). The SNNFGAILSS decamer peptide aggregates were also relatively less dense fibrils with morphology that resembled hIAPP fibrils (Figure 2C, 2D). These fibrils had 10-15 nm width and \textmu meter range length with less rigid morphology.
Figure 2: Ultrastructural morphology of fibrillar structures formed by various hIAPP fragments. NFGAIL, NFGAILSS and SNNFGAILSS were dissolved in DMSO and diluted to final concentration of 1mM in Tris-HCl (pH 7.2). hIAPP1-37 was dissolved in HFIP and diluted to final concentration of 4µM in Sodium acetate (pH 6.5). All samples were negatively stained in 2% uranyl acetate and viewed in TEM. Bars represent 100nm.

Figure 3: Ultrastructures of hIAPP using AFM. Human IAPP from the TEM experiment above was scanned using atomic force microscopy (AFM). (A) 2D phase mode. (B) 3D landscape mode. Scan size 700nm.
Atomic force microscopy (AFM) was also used to study hIAPP ultrastructural morphology (Figure 3). The same sample that was used for TEM micrographs was scanned using AFM. Elongated fibrils were viewed using the phase mode (Figure 3A), and 10 nanometer diameter fibrils were measured using 3D landscape mode (Figure 3B).

3.1.2 Alanine scan for the NFGAIL peptide, the minimal core-recognition element of hIAPP:

Previous results from our laboratory (Azriel and Gazit, 2001) have shown that phenylalanine is crucial for amyloid formation using alanine scan of hIAPP_{22-29} (NFGAILSS) residue. Substitution of phenylalanine_{23} residue by an alanine completely abolished the ability of the peptide fragment to form amyloid fibrils. On the other hand, substitution of any other amino acid of this residue into alanine did not affect amyloid formation. In order to have a better understanding on the role of phenylalanine_{23} we have performed an alanine-scan for the hexapeptide fragment NFGAIL (hIAPP_{22-27}). This fragment was suggested to serve as the minimal core-recognition element that mediates fibril formation by hIAPP (Tenidis et al., 2000). Each amino acid in this fragment was systematically replaced with alanine. The ability of each peptide to form amyloid fibrils under the wild-type peptide conditions (Tenidis et al., 2000) was determined using TEM, Congo red birefringence and fourier transformed infrared spectroscopy (FTIR). Figure 4 presents the morphology of structures formed by each of the peptides and Figure 5 the birefringence of stained deposits observed between cross-polarizers, and Figure 6 the FTIR spectra for each solid sample.

As expected, the typical fibrillar morphology of amyloid structures was observed only for the wild type NFGAIL peptide (Figure 4A), showing sharp and long
structures. These results are analogous to those reported previously for this hexapeptide (Tenidis et al., 1999). None of the other synthesized analogues showed formation of canonical amyloid fibrils. However, small differences in the aggregated clusters point to some degree of microscopic organization in some of the studied sequences. Despite the limitations of the TEM analysis, as can be observed in Figures 4D and 4F, in both NFAAIL and NFGAIA peptides the aggregate morphologies suggest a certain degree of ultrastructural order. Unlike this ordered-like organization, the AFGAIL mutant presented some aggregate formation but with what appeared to be a very low degree of order. The TEM images revealed small elongated aggregates that a priori could not definitely be identified as ordered fibrils (Figure 4B).

Figure 4: Ultrastructural Morphology of NFGAIL Peptide Analogues Using TEM. Samples of 2 mM peptide were incubated for 5 days in 10 mM Tris-HCl (pH 7.2) and 4% DMSO, negatively stained with uranyl acetate (2%w/v), and visualized using transmission electron microscopy. (A) NFGAIL, wild-type peptide; (B) AFGAIL analogue; (C) NAGAIL analogue; (D) NFAIL analogue; (E) NFGAAL analogue; (F) NFGAIA analogue. Scale bar = 100 nm.
Finally, peptides NAGAIL and NFGAAL did not show any type of ordered aggregation, presenting amorphous structures in both samples. We believe that the different morphologies observed by TEM are significant since our negative controls showed no deposition of any kind (data not shown).

To assess which analogues presented a significant level of organization that could be associated with the images observed by TEM, we performed two assay types. We inspected the birefringence of our solid samples upon congo red staining to detect any trace of cross-β organization. Additionally, we obtained the FTIR spectra for all samples to ensure the presence of β-sheet conformations in our potentially ordered aggregates.

As expected, the characteristic green-yellow birefringence was clearly observed only for the wild type peptide (Figure 5A). The rest of the samples presented a very low response, as can be seen when these are compared with the negative control, probably due to the small size of the studied aggregates. However, we could confirm the presence of a certain degree of organization in one of the analogues that we suspected was not amorphous. As can be seen in Figure 5D, the NFAIL analogue presented a moderate level of birefringence, mostly visible at the edge of the sample, indicating some ultrastructural organization.

Finally, the secondary structure analysis using FTIR is in agreement with the results described above. Both the wild type and the NFAIL analogue revealed absorption peaks in the amide I region, characteristic of β-sheet structures. The wild type presented a distinguishing peak at 1634 cm⁻¹ while the NFAIL analogue presented a strong peak at 1630 cm⁻¹ (Figure 5H). None of the other analogues presented specific signals in the amide I region.
Figure 5: Congo red Birefringence of NFGAIL Analogues. A 10 µl sample of 2 mM peptide was incubated for 48 hr in 10 mM Tris-HCl (pH 7.2) and 4% DMSO and was allowed to dry overnight on a glass microscope slide. Staining was performed by the addition of a 10 µl suspension of saturated CR and NaCl in 80% (v/v) ethanol, filtered via a 0.45 µm filter. (A) NFGAIL, wild-type peptide; (B) AFGAIL analogue; (C) NAGAIL analogue; (D) NFAAIL analogue; (E) NFGAAL analogue; (F) NFGAIA analogue; (G) negative control; (H) NFIAIL analogue, 1/5 of magnification.

3.1.3 Molecular recognition of hIAPP20-29 peptide analogues to hIAPP1-37:

In order to systematically explore the molecular determinants that facilitate recognition between hIAPP and its amyloidogenic core (hIAPP20-29), a non-biased peptide array screen was used. We probed the capacity of hIAPP fused to the C terminus of maltose binding protein (MBP) to interact with an array of 19 membrane-bound decamer peptides (SNNXGAILSS, where X represents position 23), in which the phenylalanine23 position was altered with any of the natural amino-acids excluding cysteine (Figure 7). The decamer system was used as it was previously demonstrated to allow a detection of high affinity binding between hIAPP and peptide fragments (Mazor et al., 2002). Thus, it allowed sensitive analysis of molecular determinant that mediate this interaction.
Figure 6: Analysis of secondary structures using Fourier transform infrared spectroscopy. Peptide analogue, were dried in CaF2 slides after 5 days of incubation. The amide I region between 1600 and 1700 cm⁻¹ was measured.

Binding was clearly observed to peptides that contained the aromatic tryptophan, phenylalanine and tyrosine residues (Figure 7). Binding was also observed with the positively charged analogues. In marked contrast, no binding was observed with any of the four hydrophobic substitutions in this position (leucine, isoleucine, valine, and alanine). As a control, an identical peptide array membrane was incubated with MBP alone under the same experimental conditions (Figure 7). In clear contrast to the MBP-IAPP binding assay, no significant binding could be observed with the MBP incubation.
Figure 7: Molecular recognition of hIAPP\textsubscript{20-29} peptide analogues to hIAPP\textsubscript{1-37}. A synthetic peptide array containing analogues of hIAPP\textsubscript{20-29} (SNNFGAILSS) with all the possible natural amino acid substitutions (except cysteine) at the position of phenylalanine\textsubscript{23}, was synthesized on a cellulose membrane matrix. hIAPP\textsubscript{1-37} was fused to the C-terminus of maltose binding protein (MBP) and incubated with the peptide array membrane to analyze its recognition to the bound peptides. The level of interaction was detected using an anti-MBP antibody. The letters indicate one letter code of the amino acid residue used to substitute phenylalanine\textsubscript{23} at each spot. (Top) Incubation of the membrane with MBP-IAPP. (Bottom) Incubation of the membrane with MBP as control.

3.1.4 Substitution of phenylalanine\textsubscript{23} in NFGAILSS peptide with hydrophobic residues:

When we studied the amyloid-forming potential of peptide analogues containing substitutions to all four naturally occurring hydrophobic amino-acids using aggregation assay, all the hydrophobic analogue peptides revealed a very low aggregation capacity, as reflected by solution turbidity (Figure 8A). To determine whether aggregation of the hydrophobic analogues occurs with extremely slow kinetics, solutions of the peptides under the same experimental conditions were incubated for one week, presenting relatively low turbidity for all hydrophobic analogues (Figure 8B). Ultrastructural visualization of the analogues using TEM also revealed that well-ordered fibrils were only observed with hIAPP\textsubscript{22-29} peptide NFGAILSS (Figure 8C). Considerably less ordered aggregates could be detected with the leucine analogue and to a much lower extent with the alanine analogue (NLGAILSS and NAGAILSS, respectively). However, these structures were
significantly less abundant, and did not have the typical amyloid structure. No ordered structures were observed with the isoleucine and valine hydrophobic analogues (NIGAILSS and NVGAILSS, respectively).

**Figure 8: Aggregation and morphology of hIAPP22-29 peptide and hydrophobic analogues.**
Aggregation was initiated by diluting peptide stock solution in Tris-HCl buffer (pH 6.5) to a final peptide concentration of 1 mM and 4% DMSO. Hydrophobic analogues were a replacement of phenylalanine23 with valine, alanine, isoleucine and leucine. (A) Time-dependent turbidity of hydrophobic analogues of hIAPP22-29 peptide at 405 nm. (B) End-point turbidity of the same peptides after one-week incubation. (C) TEM micrographs of the aggregated peptides after 48 hours incubation. Samples were negatively stained with 2% uranyl acetate. The letters represent one letter code of the amino acid residue used to substitute phenylalanine23 in the NFGAILSS peptide context. Bar represents 100nm.

### 3.1.5 Substitution of phenylalanine23 in NFGAILSS peptide with aromatic residues:
To gain further insights into the role of aromatic residues in the process of amyloid fibril formation by very short peptide fragments, we studied amyloid formation upon replacement of the phenylalanine23 residue of hIAPP core...
amyloidogenic fragment - NFGAILSS with the two other natural aromatic residues, tyrosine and tryptophan. For that purpose, three peptide analogues NFGAILSS, NYGAILSS, and the three synthetic peptides were studied for their amyloidogenic potential, using turbidity measurements, Congo Red birefringence, and electron microscopy.

The results presented here clearly demonstrate that the wild-type NFGAILSS peptide fragment and the substituted NWGAILSS peptide are highly amyloidogenic but the NYGAILSS shows a very low amyloidogenic potential (Figures 9, 10). This assessment is based on the three independent parameters mentioned above which are commonly used for the study of amyloid fibril formation. In order to prevent amyloid formation in the stock solution, fresh peptide stock (100 mM in DMSO) was prepared and stock solutions were sonicated for 5 minutes prior to dilution. The aggregation response, as observed by increase in turbidity, showed very fast kinetics for both the NFGAILSS wild-type peptide and NWGAILSS analogue. Significant amounts of aggregated structures were formed by both peptides in less than a minute (Figure 9). The decrease in turbidity, which was observed for the NWGAILSS analogue, is probably a result of precipitation of large aggregates in the ELISA plate well. The NYGAILSS analogue on the other hand did not show any significant aggregative behavior during the entire course of the measurements (Figure 9). Even after a week of incubation no aggregation could be detected by turbidity (data not shown).
The birefringence of the studied peptides was consistent with the turbidity results. After twenty-four hours of incubation, congo red stained samples were examined with cross-polarizer stereoscope. The samples were dried overnight on glass microscope slides and stained with Congo red solution, containing saturated congo red in 80% ethanol saturated with NaCl. Figure 10B shows the intensive birefringence of NWGAILSS peptide as compared to the birefringence of the wild-type NFGAILSS peptide (Figure 10D). Tyrosine analogue NYGAILSS did not show any birefringence within twenty-four hours (Figure 10F). Only after one week of incubation was some minor birefringence observed. Electron microscopy micrographs revealed a major difference in fiber morphology. While the NFGAILSS peptides form amyloid fibrils that are similar to the full-length IAPP both in diameter and form, the NWGAILSS assemblies are rather different in morphology, presenting thicker and less ordered structures (Figures 10C, 10A respectively). This change in morphology may be an intrinsic property of the substituted peptide or reflect the fact that amyloid formation by the NWGAILSS peptide had much faster kinetics (Figure 9).
examination of the NYGAILSS micrographs did not reveal any fiber pattern and only amorphous aggregates were observed at low abundance (Figure 10E). After one week of incubation of the NYGAILSS peptide, no amyloid structures could be observed by electron microscopy.

![Figure 10: Morphology and birefringence of the various aromatic analogues.](image)

3.2 Inhibition of amyloid fibril formation by NYGAILSS peptide

3.2.1 Inhibition of hIAPP fibril formation with tyrosine analogue peptide:

The fact that tyrosine analogue NYGAILSS could interact with hIAPP protein (Figure 7), but did not form amyloid fibrils itself (Figures 9, 10) led us to the notion
that this peptide may inhibit amyloid formation. This peptide was particularly attractive since, despite the completely different amyloidogenic potential, it is almost chemically identical to the highly amyloidogenic native peptide. Thus, the peptide may preserve all the molecular recognition parameters on one hand, and may prevent further amyloid assembly on the other.

To examine this idea, we studied the ability of the tyrosine-peptide analogue (NYGAILSS) to inhibit amyloid formation by the full-length hIAPP1-37. Thioflavin T (ThT) fluorescence assay confirmed the inhibitory effect of this peptide (Figure 11A). After a lag phase of approximately 20 hours, hIAPP1-37 alone showed a logarithmic increase in fluorescence, while the addition of NYGAILSS had a strong inhibitory effect, showing reduced linear increase in fluorescence levels (Figure 11A). Inhibition level of the tyrosine peptide analogue was significantly higher compared to that of β-breaker peptide methodology (Soto et al., 1998). Even after incubation of one week, hIAPP with NYGAILSS peptide inhibitor displayed lower fluorescence hIAPP alone and hIAPP with the recognition motif peptide conjugated to proline β-sheet breaker (NFGAILPP) as inhibitor (Figure 11B). Circular dichroism analysis revealed that hIAPP1-37 alone underwent a transition from a random coil conformation to β-sheet conformation (Figure 11C). An initial structural transition to β-sheet structure was evident after 6 hours, reaching a maximum after 26 hours (Figure 11C). However, when the NYGAILSS peptide was added to hIAPP1-37, no transition to β-sheet conformation was evident in the first 24 hours, and an initial transition was observed only after 4 days (Figure 11D).
Figure 11: Inhibition of hIAPP$_{1-37}$ fibril formation by NYGAILSS peptide. Human IAPP$_{1-37}$ was dissolved in HFIP and diluted into sodium acetate buffer (pH 6.5) to a final concentration of 4 µM and 1% HFIP, and non-soluble peptide was separated by centrifugation. (A) Thioflavin T fluorescence values of hIAPP$_{1-37}$ and 40 µM NYGAILSS peptide. (B) End point fluorescence values after 5 days incubation of hIAPP$_{1-37}$ in the absence or presence of a β-sheet breaker NFGAILPP and tyrosine analogue NYGAILSS peptide. (C) Circular dichroism of 4µM hIAPP$_{1-37}$ shows transition from random coil conformation to β sheet conformation within 6 hours (D) Circular dichroism of 4µM hIAPP$_{1-37}$ with 40 µM NYGAILSS, show an inhibited transition to β-sheet. The spectra of inhibitor in buffer (see Appendix 2) were subtracted from the corresponding final spectra.

Although non-quantitative, the ultrastructural analysis using TEM was also consistent with the spectroscopic data. While hIAPP$_{1-37}$ alone formed distinct characteristic fibrils within 30 hours, and aggregated mature fibrils within 48 hours (Figure. 12A, B respectively), addition of NYGAILSS had a significant inhibitory effect. The amount of fibrillar structures on the TEM grid was much lower and less distinct characteristic amyloid morphology was evident after 30 hours (Figure 12C). Some fibrils that resembled hIAPP alone at an earlier stage (30 hrs) were evident after
48 hours (Figure 12D). The observation of small amount of fibrils using TEM reflects the high-sensitivity of the microscopy analysis. Although this method is not quantitative, it allows the detection of single molecular assemblies that are obviously undetectable by bulk methods.

3.2.2 Octamer peptide control for NYGAILSS inhibition:

Addition of NFGAILSS peptide to hIAPP under the same conditions accelerated fibril formation, as previously reported by Scrocchi et al. (2002) for the NFGAIL hexapeptide, and mature fibrils were clearly observed on the TEM grid after 30 hr (Figure 12E). Addition of NRGAILSS peptide, which exhibited some level of interaction to hIAPP in the molecular recognition assay (Figure 7), had no inhibitory effect and fibrils, of typical wild type morphology, were observed after 30 hr (Figure 12F).

3.3 Inhibition of amyloid fibril formation by aromatic small molecules

Our mechanistic insights of amyloid formation, the reported data on the suppressive effect of aromatic compounds (Aitken et al., 2003; Ono et al., 2003), and the results above on the inhibitory effect of tyrosine analogue, led us to search for non-toxic, small molecular weight aromatic compounds that could inhibit hIAPP amyloid formation. Due to the apparent mode of interaction between phenol and benzene moieties in the peptide inhibitor system, we launched a comprehensive search for inhibitory polyphenol molecules using a series of synthetic and natural polyphenol compounds. Phenol red, a non-toxic aromatic compound, was found to be an effective compound for amyloid inhibition.
Figure 12: Ultrastructural morphology of hIAPP fibrils and peptide inhibition effect. TEM micrographs of hIAPP₁₋₃₇ samples from the inhibition assay in the initial phase of fibril formation (30 hr) and after 48 hours. (A, B) hIAPP without inhibitors after 30 hr and 48 hr (respectively). (C, D) hIAPP with NYGAILSS peptide after 30 hr and 48 hr respectively. (E) hIAPP with NFGAILSS peptide after 30 hr. (F) hIAPP with NRGAILSS peptide after 30 hr. Samples were negatively stained with 2% uranyl acetate. Bar represents 100 nm.

3.3.1 Inhibition effect of phenol red molecule on the core amyloidogenic peptides of hIAPP - NFGAILSS and SNNFGAILS:

The core amyloidogenic fragments of hIAPP (hIAPP₂₀₋₂₉ or hIAPP₂₂₋₂₉) aggregate relatively fast in aqueous solution, and this aggregation is a preliminary assay for amyloid formation. In order to have a preliminary evaluation of the inhibitory effect of phenol red on hIAPP fibril formation, we compared aggregation of 1 mM hIAPP₂₂.
and hIAPP\textsubscript{20-29} with or without 10mM phenol red. To verify the specificity of phenol red molecule towards hIAPP core peptides, a very similar molecule – phenolphthaleine, which differs only by the lack of the sulfon group, was used as control (Figure 12).

As previously described in chapter 3.1.1 hIAPP\textsubscript{20-29} aggregation rate is lower than hIAPP\textsubscript{22-29}, and an approximately two hour lag time is evident in all samples. No inhibition effect was evident using phenolphthaleine molecule and its aggregation curve is similar to hIAPP\textsubscript{20-29} aggregation which increased dramatically after three hours (Figure 14B). Using phenol red as an inhibitor decreased all changes in aggregation and very minor elevation in aggregation levels is evident after 3 hours.

hIAPP\textsubscript{22-29} aggregated within seconds and its turbidity reached a plateau after 10 minutes (Figure 14A). Aggregation of hIAPP\textsubscript{22-29} with phenol red was practically stopped and much lower and constant levels of turbidity were present during the entire assay (Figure 14A).

Figure 13: Chemical structures (2D) of the inhibitors phenol red and phenolphthaleine.
Figure 14: Inhibition of hIAPP<sub>20-29</sub> and hIAPP<sub>22-29</sub> peptides with phenol red using aggregation assay. Peptides were dissolved in DMSO and diluted in Tris buffer (pH 7.2) to a final concentration of 1mM and 4% DMSO. Phenol red and phenolphthaleine were dissolved in ethanol and diluted in the same buffer conditions to a final concentration of 10mM and 1% ethanol. Turbidity at 650nm was measured continuously for each sample and background values of the buffer or phenol red were reduced from the relevant measurement.// represents vortexing of the samples.

3.3.2 Morphology of hIAPP core peptide fibrils with phenol red using TEM:

To verify that aggregation results represent amyloid fibril formation, samples of hIAPP<sub>20-29</sub> and hIAPP<sub>22-29</sub> taken from the aggregation assay were viewed using electron microscopy. The hIAPP<sub>22-29</sub> samples were viewed 3 and 72 hours after initiation of aggregation (Figure 15). Distinct and well-defined amyloid fibrils were present in both samples for the hIAPP<sub>22-29</sub> peptide, with minor increase in fiber density and width after 72 hours. In contrast to the distinct morphology of the peptide alone when phenol red was used as inhibitor, no fibrils were visualized after 3 hours and some fibrils, with different morphology, were visualized after 72 hours.

hIAPP<sub>20-29</sub> aggregates were visualized after 24 hours with phenol red as inhibitor and phenolphthaleine as control (Figure 16). Characteristic amyloid fibrils which resemble hIAPP<sub>1-37</sub> fibrils were formed by the peptide hIAPP<sub>20-29</sub> alone (Figure 16A). Addition of 10mM phenolphthaleine did not interrupt with fibril formation, and mature fibrils, which were assembled in big bundles, were present on the TEM grid.
(Figure 16B). This result confirmed the non-inhibitory effect of the phenolphthaleine molecule obtained in the aggregation assay. In contrast to the characteristic morphology of hIAPP_{20-29} peptide alone, addition of phenol red inhibited fibril formation and no fibrils were visualized after 24 hours (Figure 16C). The aggregates formed by addition of phenol red to hIAPP_{20-29} show some degree of order, but do not resemble the characteristic amyloid fibrils formed by the peptide alone or in the presence of phenolphthalein.

**Figure 15: Morphology of hIAPP_{22-29} fibrils with phenol red inhibitor:** peptides were prepared as described for the aggregation assay and final concentration of 1µM was inhibited with 10µM phenol red. TEM samples were prepared by staining 10µL aggregated peptide with uranyl acetate. Distinct morphological differences are evident between the peptide alone and after addition of phenol red. Bar represents 100nm.
Distinct morphological differences are evident for all peptides, which show a kinetic inhibitory effect of fibril formation by phenol red. In contrary, no inhibition effect is evident by phenolephthaleine molecule.

3.3.3 Inhibition of hIAPP<sub>1-37</sub> fibril formation and cytotoxicity with phenol red molecule:

Addition of phenol red to hIAPP<sub>1-37</sub> had a strong inhibitory effect <em>in vitro</em> on fibril formation that exceeded that of the tyrosine peptide analogue. ThT fluorescence assay for variable concentrations of phenol red demonstrated concentration-dependent inhibition. Higher phenol red concentration (higher than 4 fold of phenol red to hIAPP) showed constant low fluorescence levels for at least 48 hours, while hIAPP had a logarithmic increase in fluorescence levels after 24 hours (Figure 17A). Even after 5 days incubation the concentration-dependent inhibition was similar (Figure 17B), and inhibition level of ~ 90% was achieved for phenol red concentration of 20 µM and above. CD results showed that addition of phenol red to hIAPP caused evident inhibition of transition from random coil conformation to β-sheet
conformation for at least 46 hours, and a very low transition after 96 hours (Figure 17C). Electron microscopy showed that in contrast to the distinct dense morphology of the fibrils formed by the peptide alone (Figure 17E, 30 hr), no fibrils were visualized after 30 hours when phenol red was included (Figure 17F). Only after 48 hours of incubation some fibrils were observed which were much less abundant on the TEM grid as compared to the IAPP control (Figure 17G). However, these fibrils lacked the typical dense morphology as observed with the IAPP control amyloidogenic fibrils (Figures 17E, 12A). Such changes in morphology of the fibrils were observed upon the inhibition of fibril formation by the Aβ protein by its pentapeptide fragment (Tjernberg et al., 1996). This pentapeptide currently serves as a lead for the development of Aβ inhibitor drugs. To verify the effect of phenol red inhibition on hIAPP cytotoxicity a general PC12 cell line was used to measure cytotoxicity in a concentration-dependent manner. Cells were grown in a 96 well plate with or without gradual concentration of phenol red, and fresh hIAPP was added to the growth medium. After 24 hours incubation cell viability was measured by MTT reduction assay, and calculation was done by substitution of IAPP samples absorbance with non IAPP controls containing the same phenol red concentrations. The MTT cell viability assay showed a concentration-dependent rescue of the cells (Figure 17D).
Figure 17. Inhibition of hIAPP_{1-37} fibril formation and cytotoxicity by phenol red molecule.

Human IAPP was dissolved in HFIP and diluted to a final concentration of 5µM with gradual concentration of phenol red molecule as an inhibitor. (A) Thioflavin T fluorescence kinetic values of hIAPP_{1-37} and gradual concentration of phenol red result in a dose-dependent inhibition. (B) End point fluorescence of the same samples after 5 days incubation. Values are mean±SD (n=3). (C) Circular dichroism spectra of 4µM hIAPP_{1-37} with 40 µM show an inhibited transition from random coil conformation to β-sheet. Only after 70-96 hr was there some transition to β-sheet conformation. The spectra of phenol red in buffer (see appendix 1) were subtracted from the corresponding final spectra. (D) Cytotoxicity assay of hIAPP_{1-37} and gradual concentrations of phenol red towards PC12 cells. Values are mean±SD (n=4). (E-G) Ultra-structural morphology using TEM of 4 µM hIAPP_{1-37} and 40 µM phenol red from the fluorescence inhibition assay. hIAPP_{1-37} without inhibitor after 30 hr incubation shows distinct characteristic dense fiber formation (E), while in the presence of phenol red no fibrils were visualized after 30 hours when phenol red was included (F). After 48 hours of incubation some fibrils were observed that were much less abundant on the TEM grid (G).
To evaluate the specificity of phenol red molecule towards hIAPP a very similar molecule – phenolphthaleine was used as control. The ThT assay was performed under the same conditions that were described above (hIAPP 4 μM, phenolphthaleine and phenol red 40 μM) and as shown in Figure 18 phenolphthaleine had a relatively low inhibition effect on hIAPP as compared to the inhibition effect of phenol red. This result is in agreement with the previously shown results in Figure 16 for the SNNFGAILSS peptide. Furthermore, TEM micrographs of the same samples clearly show that amyloid fibrils were performed while using phenolphthaleine as an inhibitor (Figure 19B) and as previously shown in Figure 17G only rare and non-characteristic fibrils were formed while using phenol red (Figure 19C).

Altogether, these results suggest that phenol red is a very effective and specific inhibitor towards hIAPP.

![Figure 18: Inhibition of hIAPP with phenol red or phenolphthaleine using fluorescence assay.](image)

hIAPP stock solution was dissolved in sodium acetate buffer (pH 6.5) to a final concentration of 4µM and 1% HFIP. Phenol red and phenolphthaleine were dissolved in ethanol and diluted in the same buffer conditions to a final concentration of 40µM and 1% ethanol. Samples were diluted 10x in the cuvette and ThT was added to a final concentration of 0.3 µM.
Figure 19: Ultrastructural morphology of hIAPP and polyphenol inhibitors. Samples from the fluorescence assay were placed on 400 mesh grids, negatively stained with uranyl acetate and viewed using TEM. Dense characteristic fibrils were visible both for hIAPP alone and after addition of phenolphthaleine molecule. In contrast, only rare and morphological altered fibrils were visible after inhibition with 40µM phenol red.

3.3.4 Inhibitory effect of phenol red on various amyloidogenic polypeptides and evaluation of inhibition coefficient (IC50):

To address the question whether phenol red inhibition effect is generic, we measured amyloid formation by two additional well-known amyloidogenic polypeptides, β-amyloid and insulin. Insulin amyloidogenicity is performed using relatively high insulin concentration (50 µM), high temperature (60 °C) and low pH conditions (pH=2). In these conditions, addition of a double molar ratio of phenol red (100 µM) efficiently inhibited amyloid formation as measured in ThT fluorescence and CD (Figures 20, 21). On the other hand, inhibition of β-amyloid1-42 (10 µM) with the same molar ratio of phenol red (10 µM) in Tris buffer (pH 7.2) had a minor inhibition effect as measured with ThT fluorescence (Figure 20).

Similar results were obtained by evaluation of IC50 values of phenol red as an inhibitor of IAPP, Isulin and β-amyloid1-40 (Figure 22 and data not shown). In both
IAPP and insulin phenol red inhibited fibril formation in relatively low concentrations, and evaluated inhibition coefficient values were: IC50(IAPP) ≅ 0.8 µM, IC50(Insulin) ≅ 2 µM. However, relatively high concentrations of phenol red were needed to inhibit β-amyloid1-40 and evaluated inhibition coefficient value was IC50(β-amyloid) ≅ 30 µM.

**Figure 20: Inhibition of insulin and β-amyloid with phenol red using fluorescence assay.** Insulin was dissolved in ddW pH=2 to a final concentration of 50µM and incubated in 60°C. β-amyloid was dissolved in 5% acetic acid to a final concentration of 10µM and incubated at room temperature.

**Figure 21: CD secondary structure analysis of insulin amyloid inhibition by phenol red.** Same insulin samples from fluorescence assay above were diluted to final concentration of 1.25µM in the same buffer and were measured after incubation of 14 days.
3.3.5 Inhibition of IAPP with green tea polyphenols:

In order to have more conformational understanding regarding polyphenol inhibitors we have compared the inhibitory effect of six green tea polyphenols (Figure 23), that two of them were previously reported to inhibit β-amyloid fibril formation and to protect β-amyloid cytotoxicity (Levites et al., 2001; Ono et al., 2003). ThT fluorescence was used to determine the inhibitory effect of green tea polyphenol compounds on hIAPP1-37 fibril formation. As is shown in Figure 24A, all polyphenol compounds had a long-term inhibitory effect on hIAPP1-37 fibril formation as compared to the hIAPP alone. This inhibition resembled the inhibitory effect of congo red. Fluorescence values of IAPP alone increased after 48 hr while an initial increase of hIAPP1-37 fluorescence in the presence of inhibitors was detectable only after 72 hr.

A more detailed observation of the inhibitory effect revealed that all poly phenols with the gallate group (i.e., containing an additional phenolic ring) are better inhibitors (Figure 24B). This may suggest that there is an importance to the additional aromatic ring for the inhibitory effect.
3.4 Phenol red inhibition effect on IAPP cytotoxicity towards β cells.

We studied the ability of phenol red to modulate cytotoxicity of hIAPP$_{1-37}$ amyloid assemblies to pancreatic β cells in culture. We used a highly-differentiated murine β-cell line (βTC-tet) (Fleischer et al., 1998) with a normal insulin secretory response to glucose. Cells were grown with or without phenol red, and fresh hIAPP was added to the growth medium. An MTT cell viability assay clearly revealed that the presence of phenol red in the medium protected β cells from the cytotoxic effect of hIAPP assemblies and increased cell viability from 50% to 80% (P<0.05) (Figure 25A). Scanning electron microscopy (SEM) analysis of β-cells that were grown in the presence of hIAPP showed an extensive membrane blebbing (Figure 25B), as previously reported for hIAPP cytotoxicity (Saafi et al., 2001), and a collapse of typical cellular morphology in the vast majority of cells (Figure 25D).
Figure 24: Histograms depicting inhibition of hIAPP fibril formation by green tea polyphenols. Inhibition was determined by Tht fluorescence assay. Color code for Figure 5A: Blue – 48 hr., red 3 days, yellow – 8 days, green 12 - days. B, Detailed observation of the inhibitory effect following 3 days of incubation in the presence of green tea polyphenols revealed high efficacy for the gallate group.
Moreover, practically no significant difference could be observed between untreated cells (Figure 25H) and cells grown in the presence of hIAPP and phenol red (Figure 25F). In both cases, most of the cells maintained normal morphology, no blebbing was visible, and membrane extensions of microspikes and lamellipodia were present. Furthermore, at low magnification SEM examination, normal arrays of β-cells could be observed with the phenol red protected cells and control cells (Figures 25E, G respectively). In marked contrast, only isolated and morphologically altered cells could be observed upon IAPP incubation with no phenol red protection (Figure 25C).

To study the ability of NYGAILSS peptide to inhibit hIAPP cytotoxicity we have added 40µM NYGAILSS peptide to the growth medium. Apparently, this concentration of NYGAILSS was cytotoxic to βTC-tet cells and decreased β-cell viability to 20% of control, without hIAPP addition (Figure 25A). Therefore, NYGAILSS could not be used as cytotoxicity inhibitor.

3.5 The human islet amyloid polypeptide forms transient membrane-active prefibrillar assemblies:

Studying the membrane interaction properties of hIAPP is crucial to understanding one of the most common amyloid-related cytotoxic effects. However, the aggregation of hIAPP is significantly more rapid than aggregation of other known amyloidogenic proteins such as α-synuclein or β-amyloid, which makes the analysis of membrane association difficult. Specifically, compared to fibrillization processes of days or even weeks observed for other amyloidogenic peptides, hIAPP fibrillization is completed in a few hours even at very low concentrations and, thus, the separation and
Figure 25: Inhibition of hIAPP cytotoxicity towards β-cells. βTC-tet rodent β-cells (31) were incubated for 24 hrs with 4 µM hIAPP1-37 in serum free DMEM with or without phenol red, and viability was measured using MTT reduction assay. (A). β-Cell viability compared to cells incubated in the absence of hIAPP, in medium with or without phenol red, respectively, and NYGAILSS control without IAPP. Values are mean±SD (n=4), * p=0.03, ** p<0.005; SEM analysis of β-cells grown on microscope coverslips under the same conditions.(B-D) β-cells after addition of hIAPP alone display membrane blebbing and collapse. (E, F) β-cells after addition of hIAPP in the presence of 40µM phenol red in the growth medium. (G, H) Control β-cells that were incubated under the same conditions without addition of hIAPP.
extraction of putative membrane-active species by chromatography is impractical. Here, we incubated hIAPP monomers at different durations and determined the membrane interaction profiles of the assembled aggregates using a biomimetic lipid/PDA vesicle assay. Colorimetric, fluorescence, and electron microscopy experiments facilitated high temporal resolution of the membrane-active species in the amyloid solutions.

3.5.1 Membrane interactions of hIAPP aggregates using a colorimetric assay:

To study the membrane interactions of the soluble and insoluble hIAPP assemblies, we recorded the color transitions induced by fibrils and prefibrils upon interaction with chromatic phosphatidylcholine PC/PDA vesicles (2:3 molar ratio). Lipid/PDA vesicles have been shown to serve as a versatile platform for detection and analysis of membrane interactions (Jelinek et al., 1998; Kolusheva et al., 2000; Kolusheva et al., 2001). To initiate the aggregation process, synthetic hIAPP (5 µM) was dissolved in 1% HFIP and 10 mM acetate buffer (pH 6.5). Within short intervals, non-soluble aggregates were separated by centrifugation, and after addition of the soluble supernatants or the resuspended pellets to the lipid/PDA vesicle solutions, the induced colorimetric transitions were recorded. Figure 26 demonstrates that a distinct difference in the induced colorimetric transitions was apparent between the supernatant and pellet of hIAPP. Specifically, an increase in the colorimetric response induced by the supernatant was observed, reaching a maximal %CR value after approximately 1 h, followed by a rapid decrease (Figure 26A). The blue-red color transformations induced by the suspended pellet were small at all times, showing experimentally insignificant variations (Figure 26A).
To achieve higher temporal resolution, we repeated the colorimetric experiment using a slightly lower hIAPP concentration and shorter time intervals (Figure 26B). An almost identical trend of a transient increase in the extent of the color reaction was observed for the supernatant fractions with a maximal colorimetric response approximately 80 min after the initial peptide solubilization (Figure 26B). Again, the colorimetric changes induced by the resuspended pellets were consistently low for the duration of the measurements. The higher temporal resolution depicted in Figure 26B allowed a clear observation of a gradual rise in the level of membrane binding followed by a subsequent decrease in membrane activity. This result implies the formation and ensuing disappearance of transient membrane-active prefibrillar assemblies.

3.5.2 Fluorescence quenching of bilayer surface NBD:

To confirm the existence of transient membrane-active assemblies of hIAPP, we carried out a fluorescence quenching experiment, utilizing C6-NBD-PE incorporated within the phospholipid/PDA vesicles (Figure 27). In these experiments, we examined the effect of membrane-active species formed in the hIAPP suspensions upon fluorescence quenching of C6-NBD-PE by sodium dithionite (Langner and Hui 1993). In principle, greater bilayer perturbation by membrane-reactive aggregates in solution would give rise to faster fluorescence quenching of the NBD label. In the experiments depicted in Figure 27, samples of freshly dissolved hIAPP were prepared following centrifugation every 30 min and added to NBD-PE/DMPC/PDA (0.01:2:3 mole ratio) vesicles, and the quenching reaction was initiated by a reaction with
sodium dithionite to a final concentration of 10 mM. The fluorescence emission at 535 nm was then monitored for 3.5 min.

Figure 26: Membrane interaction of fibrils and prefibrillar assemblies using the colorimetric lipid/PDA vesicle assay. Human IAPP was dissolved in sodium acetate buffer and HFIP (1%). Fractions were separated using centrifugation and added separately to the vesicles in Tris-HCl (pH 8): (●) supernatant and (Δ) pellet. Color response values were measured in three independent repeats, and error bars represent the standard deviation. (A) Color response of 5 µM hIAPP fractions at 30 min intervals. A significant increase is observed for the supernatant fraction, which contains mostly prefibrils, within 1 h. (B) Same procedure as for panel A using 4 µM hIAPP and 20 min intervals to enhance the temporal resolution.
The fluorescence decays shown in Figure 27 are presented as a percentage of the initial fluorescence measured before addition of dithionite. The topmost decay curve in Figure 27 was recorded for the control sample (without adding peptide suspensions). The slower decay in the control vesicle solution was due to the slow penetration of the water-soluble dithionite quencher ion into the intact vesicles. Faster fluorescence decays were observed when fractions of the supernatant suspensions were added to the NBD-PE/DMPC/PDA vesicles (Figure 27). Importantly, the most pronounced quenching occurred after addition of the supernatant fraction collected 60 min after dissolution of hIAPP (lowest curve in Figure 27). This enhanced quenching most likely corresponds to the better access of quencher molecules into the bilayer, as a result of perturbation of the lipid surface by hIAPP prefibrils. As aggregation proceeded, the quenching rates became slower (Figure 27), consistent with fewer lipid interactions by the aggregating fibrils.

Figure 27: Lipid bilayer perturbation by hIAPP using the NBD marker. The NBD fluorescence was measured after addition of 4 µM hIAPP and the dithionite quencher, and fluorescence values represent the percentage of initial emission reading. The control curve represents the fluorescent decay without addition of hIAPP, while the other curves depict the fluorescence decays induced by supernatant fractions (containing mostly prefibrils) at various time points of aggregation. The maximum quenching effect was measured for the soluble fraction after incubation for 1 h.
Overall, the fluorescence quenching data shown in Figure 27 are consistent with the colorimetric analysis (Figure 26) and indicate that the most active membrane-reactive prefibrils assembled in the hIAPP solution after approximately 1 h. The fluorescence quenching data further demonstrate the existence of transient, stronger membrane binding soluble prefibrillar assemblies formed ~1 hr after peptide incubation.

3.5.3 Ultrastructural TEM visualization:

To visualize the hIAPP assemblies and their effect on the phospholipid/PDA vesicles, an ultrastructural analysis was performed using TEM (Figure 28) and HR-TEM (Figure 29). The TEM images in Figure 28 show lipid/PDA vesicles mixed with hIAPP suspensions extracted at different times. In the experiments depicted in Figure 28, synthetic hIAPP was dissolved, separated using centrifugation, and mixed with the lipid/PDA vesicles, as described in the colorimetric analysis (Figure 26). The samples were then negatively stained using uranyl acetate and analyzed by TEM. Figure 28A features the control DMPC/PDA vesicle solution. The polymerized vesicles adopt elongated rectangle shapes due to the ordered PDA framework. Addition of hIAPP supernatant fraction extracted 60 min after the initial dissolution of the peptide induced a significant degradation of the vesicles (Figure 28B). This result resembles the effect of mixing the DMPC/PDA vesicles with polymyxin B, a potent membrane-disrupting antimicrobial peptide (Figure 28C). A similar degradation of phospholipid/PDA vesicles following lipid disruption by various membrane-reactive compounds was previously observed (S. Kolusheva and R. Jelinek, unpublished results). Significantly different appearances were detected in the vesicle samples following addition of a supernatant fraction collected 4 h after the
initial dissolution (Figure 28D) or a pellet suspension 60 min after peptide dissolution (Figure 28E). Both images clearly feature elongated fibrils, some attached to the rectangular lipid/PDA particles. Importantly, the vesicles did not appear to be disrupted or deformed by the fibrils, in contrast to the apparent pronounced degradation following addition of the supernatant suspension extracted after 1 h (Figure 28B).

Figure 28: Ultrastructural morphology of lipid vesicles after addition of hIAPP. Vesicle samples (10 µL) used for the color reaction assay at various time points were negatively stained with uranyl acetate and visualized using transmission electron microscopy. (A) Control DMPC/PDA (2:3 mole ratio) vesicle sample, without addition of hIAPP. (B) DMPC/PDA vesicle after addition of a supernatant fraction incubated for 1 h. (C) DMPC/PDA vesicles after addition of 30 µM polymyxin B. (D) DMPC/PDA vesicles after addition of a supernatant fraction collected 4 h after the initial dissolution. (E) DMPC/PDA vesicles after addition of a pellet fraction collected 1 h after the initial dissolution. All scales are 100 nm in length.

To gain a better understanding of the morphology of the soluble prefibrillar assemblies present in the supernatant solution, we used high-resolution TEM analysis
(HR-TEM) to visualize the soluble fraction after 60 min without the addition of lipid vesicles (Figure 29). Synthetic hIAPP was dissolved, separated using centrifugation, and visualized after 60 min. The supernatant fraction of the peptide contained spheroid assemblies, 15-20 nm in diameter (Figure 29A) with a morphology very similar to that of early assemblies that were observed with the β-amyloid polypeptide (20). TEM analysis of the supernatant fraction further revealed the early stages of fibrillar assemblies, or in the more common term “protofibril assemblies” (Figure 29B). The protofibrillar assemblies exhibited different morphologies compared with the mature fibrils (Figure 29C), having less distinct contours of thicker and shorter assemblies. The TEM analysis provides clear visual evidence for the membrane activity of the transient prefibrils assembled in the hIAPP suspensions, reaching their maximal activity approximately 1 h after the dissolution of the peptide in aqueous solutions.

![Figure 29](image)

**Figure 29: Ultrastructural morphology of hIAPP prefibrillar assemblies and mature fibrils.** hIAPP was dissolved in HFIP and diluted in 10 mM acetate buffer (pH 6.5) to a final concentration of 4µM and 1% HFIP. After 60 min, a sample was separated using centrifugation (20000g), and 10 µL of the supernatant was negatively stained with uranyl acetate and visualized using transmission electron microscopy (TEM) and high-resolution field-emission gun TEM (HR-TEM). (A) HR-TEM micrograph of a spheroid prefibrillar assembly, 20 nm in diameter, present in the supernatant fraction after 60 min. (B) TEM micrograph of protofibrils (an initiation of elongated fibrils) present in the supernatant fraction after 60 min. (C) TEM micrograph of mature hIAPP fibrils after 24 h. The sample was visualized without separation through centrifugation.
3.5.4 Secondary structure analysis of membrane-hIAPP complexes using CD:

To further evaluate the correlation between the structural properties of the hIAPP assemblies and their membrane interactions, we recorded CD spectra of the peptide-suspension supernatants extracted at different time points, after mixing with the lipid/PDA vesicles (Figure 30). hIAPP has been shown to adopt a random-coil conformation in aqueous solutions, transforming slowly into a β-sheet structure (Jaikaran and Clark, 2001). In the presence of lipid/PDA vesicles, however, we observed that the transition from a random coil to a β-sheet structure was already apparent after only 20 min, becoming the dominant peptide structure after little more than 1 h (Figure 30A). A comparison of the molar ellipticity values of hIAPP and an hIAPP/lipid/PDA vesicle mixture at 218 nm (Figure 30B) clearly shows that β-sheet formation was significantly enhanced shortly after peptide dissolution in the presence of the vesicles. Figure 30B shows that the molar ellipticity of hIAPP decreased rapidly to approximately -10000 deg cm²/dmol⁻¹ when the peptide was suspended with the vesicles, while a much slower adoption of a sheet structure was apparent in a vesicle-free aqueous solution (Figure 30B). The CD spectral analysis thus confirms that significant membrane binding occurs for prefibrillar assemblies formed in the peptide suspension within approximately 1 h, giving rise to the expected β-sheet structures.
**Figure 30:** Secondary structure analysis of lipid vesicles and hIAPP using CD spectroscopy. hIAPP (4 µM) was dissolved in 10 mM sodium acetate buffer (pH 6.5) and 1% HFIP. (A) CD spectra of hIAPP in the presence of 50 mM DMPC/PDA vesicles. Peptide samples were collected after the indicated incubation times. (B) Molar ellipticity at 218 nm (corresponding to formation of β-sheet structures) of hIAPP in an aqueous solution (2) and hIAPP in a solution containing DMPC/PDA vesicles (b).
Discussion:

The formation of amyloid fibrils is a hallmark of a variety of unrelated diseases. Despite its central public health importance, the mechanism of amyloid-related pathogenesis is not fully understood. Genuine understanding of the mechanism that leads to the formation of amyloid fibrils, its inhibition, and the effect on the pathogenesis process would be important both for basic understanding of the phenomenon as well as for development of future therapeutic approaches.

Type 2 diabetes is the most common amyloidogenic disease and human islet amyloid polypeptide (hIAPP) was shown both in vitro and with animal models to be one of the main factors for β-cells dysfunction. The core amyloidogenic module of the hIAPP serves as an excellent model system to study the molecular mechanism of amyloid fibril formation (Westermark et al., 1990; Tenidis et al., 2000; Azriel and Gazit, 2001). This is due to the fact that such a small fragment contains all the structural information needed to mediate the self-assembly and molecular recognition processes that lead to the formation of amyloid structures. Initially, it is shown here that the fibrillar morphology of three of the core amyloidogenic modules of hIAPP (hIAPP22-27, hIAPP22-29, and hIAPP20-29) resembles the morphology of hIAPP fibrils (Figures 2, 3) and therefore, serves as a strong initial assessment of hIAPP behavior. Furthermore, it is shown that the self-assembly process rate is inverse to peptide length and, therefore, longer peptides give a more detailed evaluation of hIAPP characteristics.

Previous results from our laboratory (Azriel and Gazit, 2001) have shown that phenylalanine23 is crucial for amyloid formation using alanine scan of hIAPP22-29 (NFGAILSS) residue. Substitution of phenylalanine23 residue by an alanine
completely abolished the ability of the peptide fragment to form amyloid fibrils. On the other hand, substitution of any other amino acid of this residue into alanine did not affect amyloid formation. Here, it is demonstrated that substitution of the key phenylalanine\textsubscript{23} residue to the other natural aromatic amino acids still enabled the molecular recognition between the full-length hIAPP\textsubscript{1-37} and its core module SNNFGAILSS. However, substitution of the phenylalanine\textsubscript{23} with any of the other natural hydrophobic amino acids completely prevented the molecular recognition process (Figure 7).

Furthermore, to study the effect of substitution of phenylalanine\textsubscript{23} on the fibril formation process we have substituted this residue with various hydrophobic residues (Figure 8) and aromatic residues (Figures 9, 10) in the context of NFGAILSS core amyloidogenic peptide of hIAPP (hIAPP\textsubscript{22-29}). Substitution of the phenylalanine\textsubscript{23} to the leucine, valine or isoleucine residues, where their hydrophobicity is comparable to that of phenylalanine, significantly reduces amyloid formation by the NXGAILSS peptide (X represents substitutions of phenylalanine\textsubscript{23}). Different hydrophobicity scales do vary significantly with regard to the estimated relative hydrophobicity of phenylalanine, tryptophan, leucine, valine, and isoleucine. However, it appears that the amyloidogenic and non-amyloidogenic peptides could not be sorted by hydrophobicity \textit{per se}.

Substitution of phenylalanine\textsubscript{23} with aromatic tryptophan and tyrosine has shown interesting results. No decrease in amyloid formation, but rather acceleration, was observed upon substitution of the phenylalanine residue to the tryptophan amino acid, and a sharp decrease in all the amyloid formation parameters for the tyrosine substitution. This result obtained with the NYGAILSS peptide is quite striking since
NFGAILSS and NYGAILSS are almost identical, apart from the phenolic hydroxyl in the latter peptide. While the NYGAILSS peptide is slightly less hydrophobic than the NFGAILSS peptide, this does not seem to be the basis for such a major difference in their amyloidogenic potential.

Therefore, the effect of these substitutions is unlikely to merely reflect a change in hydrophobicity. In addition, this effect does not seem to reflect the ability to form β-sheet structures, the common structural component of amyloid fibrils, as isoleucine and valine are considered to be more efficient β-sheet formers than phenylalanine. Taken together, we suggest that aromatic interactions are important factors in the process that leads to amyloid fibril formation. This notion is in agreement with recent studies on de novo designed peptides that suggested that hydrophobic interactions could not simply account for the formation of fibrils and that specific interactions are crucial in the stabilization of fibrillar aggregates (Lopez De La Paz et al., 2002).

Since the hydrophobic nature does not seem to be a key issue, other reasons should be considered. We speculate that it may be the electronic character of the different aromatic rings, which is the basis for the difference between the peptides. The electronic character of the π-system of phenol and that of the benzene ring are significantly different. This is due to the fact that the lone electron pair of the OH groups can conjugate to the aromatic ring to form a π-donor entity. This difference in the electronic character of the ring may be reflected in the energetically favored configuration of the system. A recent molecular dynamics study (Chelli et al., 2002), clearly indicated a differential preference in the configuration of phenylalanine-phenylalanine and tyrosine-tyrosine pairs in apolar environment. While the phenylalanine-phenylalanine pair clearly prefer a stacked orientation (as is ideal for fibrillization), a T-shaped configuration is observed for the tyrosine-tyrosine pair.
under the molecular dynamics simulations. Phenylalanine pairs were reported to be preferentially arranged in a parallel-displaced geometry that is consistent with aromatic stacking. The simulation revealed one predominant (91% of the population) phenylalanine-phenylalanine energetically favored stacked state, with 4.5 Å distance between the two ring centroids, which is consistent with β-sheet stacking. On the other hand, tyrosine-tyrosine pair had eight different states of minimum energy with no predominant structure. The most common structure that represented 26.1% of the population had a T-shape orientation with 5.9 Å distance (Chelli et al., 2002). However, the increased aggregative and amyloidogenic potential of the tryptophan-substituted peptide may reflect a more energetically favorable stacking π-donor acceptor rings as compared to bare-aromatic rings. Unfortunately, no molecular dynamic simulations were available for the tryptophan-tryptophan pair. Another possibility is that the additional hydroxyl can engage in hydrogen bonding interactions with other residues, and thus avoid the specific directionality contributed by the aromatic interaction. Aromatic stacking interactions energetic contribution is only (-0.6) - (-1.3) kcal/mole, as compared to -5 kcal/mole or more contribution of hydrogen bonds. Therefore, even if stacking does occur in tyrosine-tyrosine pairs, random ectopic hydrogen bonding may result in an ensemble of energetically-favored non-fibrillar species (i.e., amorphous aggregates).

It is worth mentioning that other recent studies have shown that single amino acid substitutions may have a marked effect on the amyloidogenic potential of much larger proteins and polypeptides (Chiti et al., 2000; Thakur and Wetzel, 2002; Wurth et al., 2002). These studies further support the notion that a specific pattern of molecular recognition, rather than nonspecific hydrophobic interactions, directs the process of self-assembly that lead to the formation of well-ordered amyloid fibrils. The work of
Wurth et al (2002) is especially relevant to the current discussion as it demonstrates the importance of the specific phenylalanine residues in the case of amyloid fibrils self-assembly by the β amyloid polypeptide.

Interestingly, the tyrosine peptide analogue, NYGAILSS, demonstrated a clear recognition of hIAPP in the peptide array assay (Figure 7), but had no amyloidogenic activity (Figures 8, 9). It is speculated that the difference in the molecular nature of tyrosine pair interactions, as compared to phenylalanine pairs, accounts for the difference in the amyloidogenic potential. The concept of differential geometry of aromatic interactions led us to test the ability of the NYGAILSS peptide to serve as an inhibitor of amyloid fibril formation by hIAPP. Molecular dynamic studies of the hetero-aromatic phenylalanine-tyrosine pair (Chelli et al., 2002) revealed that the most common phenylalanine-tyrosine geometry (64.7 %) has an oblique orientation with an angle of a 31.7 º between the two normals to the ring (Figure 31). The stacked and T-shape geometries were much less common (31.4 % and 3.9% respectively). This may explain a limited interaction between phenyl alanine and tyrosine which enables molecular recognition but does not favor continues stacking (Figure 31).

Indeed, the NYGAILSS peptide demonstrated a very significant inhibitory activity as revealed independently by ThT fluorescence (Figure 11A, B), circular dichroism secondary structure analysis (Figure 11C), and electron microscopy (Figure 12). On the other hand, NFGAILSS peptide had an accelerating affect on fibril formation, as previously reported for the NFGAIL peptide (Scrocchi et al). NRGAILSS peptide, which had some level of molecular recognition to IAPP, had no inhibition effect. We assume that NYGAILSS peptide ability to interact with IAPP, yet in a geometry that
is inconsistent with amyloid formation, may account for such significant inhibitory effect.

Figure 31: Possible mechanism of aromatic inhibition

Another approach for amyloid fibril inhibition is the use of small molecular weight inhibitors. Our mechanistic insights of amyloid formation, the reported data on the inhibitory effect of aromatic compounds (Kuner et al., 2000; Harroun et al., 2001; Lashuel et al. 2002; Aitken et al. 2003), the aromatic nature of amyloid specific dyes such as congo red and ThT, and the results above on the inhibitory effect of tyrosine analogue, led us to search for non-toxic, small molecular weight aromatic compounds that could inhibit hIAPP amyloid formation. Due to the apparent mode of interaction
between phenol and benzene moieties in the peptide inhibitor system, we launched a comprehensive search for inhibitory polyphenol molecules using a series of synthetic and natural polyphenol compounds.

Three groups of aromatic compounds were tested: the first group included one aromatic ring compound, such as phenol and benzene (Appendix 2), another group of two aromatic rings, such as 1,5 naphthalendisulfonic acid and catechin, and a group including three aromatic ring compounds, such as phenolphthaleine, phenol red, and epigallocatechin gallate (Appendix 2). None of the mono-aromatic compounds have shown any inhibition of hIAPP amyloid formation (data not shown), furthermore among the di and tri- aromatic compounds there was a clear inhibitory advantage to the tri-aromatic compounds (figure 24). These results may suggest that direct interaction with the aromatic ring is not sufficient for inhibition and that there is importance to the additional aromatic ring on the inhibitory effect. This result was independently confirmed by two other groups (Lashuel et al. 2002; Kocisko et al. 2003) describing the inhibitory effect of Apomorphines on β- amyloid and the inhibitory effect of various polyphenols on scrapie –associated prion protein.

Phenol red, a non-toxic tri-aromatic polyphenol compound, was found to be an effective compound for inhibition of hIAPP fibril formation in vitro. Phenol red exhibited a concentration dependent inhibition of hIAPP fibril formation using ThT fluorescence assay, prevented transformation of hIAPP from random coil conformation to β-sheet as seen in circular dichroism analysis, and inhibited the formation of characteristic amyloid fibrils for at least 48 hours as seen on TEM micrographs (Figure 17).
On the other hand, phenolphthaleine, a tri-aromatic compound which differs from phenol red only by lack of the sulfon group, had no inhibitory qualities whatsoever, not on the amyloidogenic peptides hIAPP$_{22-29}$ (Figure 14A) and hIAPP$_{20-29}$ (Figures 14B and 16), nor on hIAPP$_{1-37}$ (Figures 18, 19). This result implies the importance of the sulfon group which is predominant in the aromatic groups of the amyloid specific Congo red dye. Another option for the difference in the inhibitory effect of phenol red and phenolphthaleine might be the fact that these two molecules have different 3D structures. The main difference is in the angles between the two phenol rings plane to the central carbon atom (Figure 32). Characterization of several other polyphenol inhibitors, such as pyrocatechol, dihydrodibenzothiepin and green tea extract compounds, such as epigallocatechin gallate, is necessary for better understanding of the overall structural and chemical factors influencing the inhibition process. Taken together, these results imply that the interaction of phenol red molecule to hIAPP fibrils is highly specific and this issue will be important for future therapeutic approach.

Figure 32: 3D structure of phenol red (A) and phenolphthaleine (B).

The issue of the generic nature of phenol red molecule to inhibit other amyloidogenic proteins is another major aspect. So far only one small molecule, the
main green tea polyphenol epigallocatechin gallate, was described as an inhibitor of more than one amyloidogenic protein. This molecule was independently reported as an inhibitor of β-amyloid (Ono 2003) and PrPsc (Kocisko 2003), however, in both cases clear mechanism of inhibition was not suggested. Epigallocatechin gallate was also shown to inhibit IAPP, calcitonin and insulin in other work that was done in our lab (data not shown).

Phenol red molecule displays a more complex picture. Here it is shown that phenol red efficiently inhibits IAPP and insulin with high affinity and low effective concentrations, IC50(IAPP) ≅ 0.8µM, IC50(insulin) ≅ 2µM (Figure 22). However, a much lower affinity and higher concentration for effective inhibition of β-amyloid (IC50(β-amyloid) ≅ 30µM). Although the results for insulin were made by extrapolation and are not accurate, the fact that there is such a difference between IAPP and β-amyloid does not suggest that there is a generic nature of inhibition but rather more specific interaction between phenol red and IAPP monomers or intermediate oligomers.

A more advanced way to study the efficiency of amyloidogenic protein inhibitors is the inhibitory effect of these inhibitors on live cells hIAPP cytotoxicity. As an initial step, PC12, a rodent adrenal cell line, was used as a general assay to study hIAPP cytotoxicity. A dose-dependent rescue of PC12 cells was observed with IC50 of about 40 µM. This high concentration may be explained by lower activity of the phenol red molecule in the hydrophobic environment of cell membrane.

As a second step, phenol red inhibition was measured using βTC-tet cell line, a rodent β-cell line with a normal insulin secretory response to glucose (Fleischer et al., 1998) which serves as the natural target for IAPP cytotoxicity. Phenol red demonstrated a very efficient protection of pancreatic β-cells, at a concentration of 40
μM, from the cytotoxic effect of hIAPP (Figure 25). This result is quite intriguing as phenol red is a simple, safe, non-toxic, and non-carcinogenic compound that has been used for many years in tissue culture with no adverse effect.

The long term effect of the peptide and polyphenol inhibitors was measured in this work with one week limit due to technical difficulties. We have demonstrated that this inhibition is basically kinetic and does not represent an absolute inhibition. On the other hand, our experimental conditions utilized relatively high concentrations of amyloidogenic protein that normally are not produced in vivo. Furthermore, since amyloidogenic processes are dependent with high order in concentration these processes are relatively much slower than in the test tube and might take very long time, even up to several years. Therefore, even small effect on the aggregation process may lead to prolonged inhibition in the fibril formation process.

Another important aspect of amyloidogenic proteins is the cytotoxicity mechanism and especially the molecular organization of the amyloidogenic molecules while affecting the cells. In the past few years there has been an accumulation of evidence showing that the most cytotoxic phase is the oligomers or prefibrillar assembly structure of the protein. Here, membrane binding and ultrastructures of soluble and nonsoluble hIAPP assemblies were studied at high temporal resolution.

Convincing experimental evidence for the existence of transient soluble membrane-active intermediate hIAPP species, which appear in aqueous solutions prior to fibril formation, are shown here. The observation of a gradual increase in the extent of lipid bilayer perturbation reaching a maximum after approximately 1 hour is a clear indication that membrane interactions are not mediated by monomeric hIAPP, but rather that a process of assembly is necessary for the formation of membrane-active species. Furthermore, the progressive decrease in the extent of membrane
interactions after the peak following incubation for 1 hour (Figures 26 and 27) as well as the detection of membrane activity only in the supernatant fractions suggests that the transient assemblies consist of prefibrillar assemblies rather than the large fibrillar structures.

Ultrastructural TEM analyses in Figures 28 and 29 provided observable evidence of the existence of prefibrillar assemblies and their morphologies. Electron microscopy data indicated the formation of spherical prefibrillar assemblies, 1 hour after hIAPP solubilization, and demonstrated the transformation of these assemblies into elongated mature fibrils (Figure 29). The appearance of soluble spherical assemblies with a very similar ultrastructure was previously reported for other amyloidogenic proteins, such as β-amyloid and α-synuclein (Lashuel et al., 2002; Hoshi et al., 2003) and is consistent with a pore formation mechanism of toxicity (Lashuel et al., 2002). Detection of rapid formation of β-sheet structures upon association with the lipid/PDA vesicles (Figure 30) is a further indication for the enhanced membrane interactions of the transient prefibrillar assemblies. Importantly, the assembly of many amyloid fibrils in vivo occurs in the intracellular space, in which cellular membranes are readily accessible for molecular interactions. Our observation of membrane interaction and bilayer disruption induced by prefibrillar assemblies of amyloidogenic polypeptide is highly relevant for the development of therapeutic agents aimed at preventing and treating amyloid related diseases. Indeed, the emerging molecular mechanism of amyloid toxicity suggests that the disassembly of the larger fibrils that were already formed may be actually more harmful than beneficial. This is because amyloid fibril disruption might actually result in an increased concentration of monomeric peptides and prefibrillar assemblies that will exert higher cytotoxic activity. According to our study, a therapeutic effort might be
better directed toward inhibition of the earlier stage of transient prefibril formation. This could be achieved by targeting the molecular recognition determinants that may play a role in the very early stages of oligomerization. The apparent role of aromatic interactions in the molecular recognition and self-assembly of IAPP (Azriel and Gazit, 2001; Mazor et al., 2002; Porat et al., 2003; Porat et al., 2004) and other amyloidogenic polypeptides (Gazit, 2002; Reches et al., 2002; Reches and Gazit, 2003) may point to specific design criteria leading to inhibitors that will target the early stages of molecular association leading to formation of prefibrillar assemblies.

Taken together, this study provides further experimental support on the role of aromatic interactions in the self-assembly of hIAPP amyloid fibrils, using various biophysical and cytotoxicity methods. It also demonstrates the ability of a short tyrosine-modified peptide and a small polyphenol molecule to effectively inhibit the formation of amyloid fibrils by hIAPP. These results, taken together with the demonstration of amyloid formation inhibition by polycyclic molecules and the formation of amyloid fibrils by short aromatic peptides, further implies to the use of aromatic recognition motifs as targets for molecular design. We assume that the inhibitory aromatic compound competes with polypeptide monomers for interaction with the growing oligomers or fibrils. So far, current methods described in this work could not determine whether the inhibitory peptide NYGAILSS or the polyphenol compounds affect fibril formation at the nucleation phase or at the elongation phase. Several new techniques may be implemented in order to have a better determination of the exact inhibition module. Some possible techniques include immunological techniques, such as specific antibodies for the oligomer structures (Kayed et al., 2003; O'Nuallain and Wetzel 2002) or dotblot assay with antibodies that recognize
monomers or fibrils only. Separation techniques, such as native gel filtration, mass spectroscopy with cross-linking and gel filtration using organic solvents as running buffer, can also help in determining inhibition efficiency and improving the initial monomer separation for the inhibition assays. New electron microscopy techniques such as Quantomix wet cell capsules may enable following (on line) the morphological changes of cells after addition of amyloidogenic peptides and the inhibition effect of cytotoxicity inhibitors. Gold labeling of amyloidogenic protein monomers will enable following membranal interaction by TEM. Irreversible or improved interaction of the amyloidogenic monomer by the inhibitor should result in an efficient halt of the nucleation process and might have the best therapeutic effect.

In this thesis we have used our understanding of the molecular interactions that lead to hIAPP fibril formation in order to apply novel inhibition strategy based on aromatic interactions. Direct inhibition of amyloid fibrils is promising therapeutic approach to type 2 diabetes and may be relevant to other amyloidogenic diseases. Specifically for type 2 diabetes, early diagnosis of high hIAPP concentration is relatively feasible and inhibition of the nucleation process at this stage may inhibit or even prevent β-cell damage by hIAPP at the acute stage of the disease.

Summary:
In this work we have sown that aromatic interactions are important factor in the molecular interactions that lead to type 2 diabetes related hIAPP amyloidogenic fibril formation and may be used to develop efficient inhibitors for direct inhibition of fibril formation. Our main findings are:
• Phenylalanine residue is crucial for amyloid formation by both NFGAIL and NFGAILSS core peptide fragments of hIAPP.

• Aromatic interactions rather than non specific hydrophobic interactions are important factor in the specificity and directionality needed for the fast self assembly which characterizes hIAPP fibril formation.

• Substitution of phenylalanine with tyrosine in the context of NFGAILSS (NYGAILSS) core peptide resulted in substantial halt of fibril formation but did not affect molecular recognition to hIAPP. We suggest that these differences stem from different stacking patterns of phenol and benzene aromatic rings.

• Tyrosine peptide analogue (NYGAILSS) was used as efficient peptide inhibitor for hIAPP fibril formation in vitro although toxic to cells

• The small aromatic poly phenol compound was shown to be very efficient inhibitor of hIAPP fibril formation in vitro and decreased the cytotoxic effect of hIAPP on β-cells.
Publications resulting from this work:

Research articles:


Meetings:


**Patent:**

Porat, Y. and Gazit, E. Inhibition of Amyloid Formation using the Phenol Red Aromatic Compound. Provisional patent application filed on 25 September 2003 in the US and received serial No. 60/505,425.
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Appendix 1:

Figure A1: Background levels of sodium acetate buffer (pH 6.5), 40µM phenol red in sodium acetate buffer, and 40µM NYGAILSS peptide in sodium acetate buffer. None of these showed any secondary structure conformation during the whole assay.
Appendix 2:

| Benzene | Phenol | Phenol red | Phenolphthalein | 1,5 Naphthalendisulfonic acid |

| Catechin | Epicatechin | Gallicatechpin |
| Epigallocatechin | Epicatechin Gallate | Gallicatechin Gallate |
| Epigallocatechin Gallate |

**Figure A2**: 2D structure of various one ring, two ring, and three ring aromatic compounds used as inhibitors of hIAPP.
Appendix 3:  

List of abbreviations

AFM – Atomic Force Microscopy  
CD – Circular Dichroism  
CR – color reaction  
DMSO – di methyl sulfoxid  
DMEM – Dulfbeco’s Minimum essential Medium  
IAPP – Islet Amyloid Polypeptide  
hIAPP – human Islet Amyloid Polypeptide  
HFIP – 3,3,3,3’, 3’, 3’, hexafluoro – 2 - propanol  
HPLC - High Performance liquid Chromatography  
MBP – Maltose Binding Protein  
MBP-IAPP - Maltose Binding Protein fusion with Islet Amyloid Polypeptide  
MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide  
NBD-PE – 7-nitrobenz-2-oxa 1, 3-diazone phosphatidylethanolamine  
PDA - polydiacetylene  
TBS – Tris Buffer Saline  
SEM – Scanning Electron Microscopy  
TEM – Transmission Electron Microscopy  
ThT- Thioflavin T

List of peptides used in this work

NFGAIL - hIAPP22-27  
NFGAILSS - hIAPP22-29  
SNNFGAILSS - hIAPP20-29  
NYGAILSS - hIAPP22-29 with F→Y mutation  
NWGAILSS - hIAPP22-29 with F→W mutation  
NFGAILPP - hIAPP22-29 with SS→PP mutation
עדרניאזיה של עמילואיד בسكر-II: שלמות והקרבה להכאות

 מציעים מחקרים יותר ובר�ב על העまりים-universe prospects ובעיון ערכי

 יאיר פורט

הוורדות סיביות עמילואידית תכנית ומוקפת בין הטבליון והאבטיס למספר מחקר שאמ

 בינויה קשר רגיש, ונ載וגה, ואלצוהים. פורניזון מחלות פורירונט סוכרת מוסון II. סיווט לעבר

 התלך יוניז הסביבות העמילואידית הם צל בלוע הליטוב בלילב במעל הנה. מפריש וגית

 תיפעלת המחלות עמילואידית במסטור על עיכוב יישר של עזר הסיבים עיינו פִּזיזים זרעים

 המכיל צבעים המבעלים יוניז משחת בווט, (β-sheet), וגדייס ספיגיפזים ומולקולות כמות.

 הלומד שא הנחיתים ברגה חומץ האמינו של החלבונים עםילואידים שנייה, קיים דמיון בר

 בבגנה במקנות הפיסיקוקימיה. תכונת המשויתת הכרה בחלבי המנגנון במעל הטבליון

 דמיון בין הסיבות הש暮ら ברשת מביתו החיניק בין תכונת אפתומות שבו האורבר בצפיפות ש

 למלבנים עמילואידים. החלבונים איזו גו חכוני ישות קידון חיומי המאפית ובלקאלציה

 רואשים ישל אולוגיציה שלגית דומם להלך פִּזיזים בר.

 באחת המחקרים העמילואידית המפורטות בחומץ היא סוכרת מוסון II. משキャンペ על עמילואידים בלבל

 הממסדים מחבלון המונגול בשמ ממוסון על עיל מ-90% מחולות סוכרת מוסון II. הווה

 הייתה כי הפקת הפִּזיזים של משקיעים על בז גורם לתאי בבלב בלבל בלבל המתקדמים של

 המחלות. תאלון ב- II שוחזרו המפורשות חוכי איסטנפליגים וסיויסים במעל המחלוב בן הבלב והז

 הראו כי הזר סיבים עמילואידים כי הוסף של החלבון זה לטייה להכרה את β-בלבל בלבל בלבל

 התשערה הרוחות עבירה היית כי הסיבים העמילואידיםski הונוגמה של עǤיים הליטוב ההיא, אולז

 הוכחת ודושה הרה כי מוכני ביניים פרף-סיבים וסיבים להדר מבוד станет الواלבון עǤיים הליט

 להשתמש בבוגר. מיסיבות אלה יוכדיע צירית הסיבים העמילואידים ולא על משמעות טיפולי רהב

 למחלות סוכרת מוסון II. (NFGAILSS) מתוח האזר חשש לש

 תוצאת קדום מהת ראיה כי מחקרים של שנות תוצאת ומיני IAPP עורג סיבים עמילואידיםقودים ל sito שגרים בחלק הוה

 החלבונים על. הווה עימו פִּזיז

 אולימ במקעה זה יחידי חותם צירית הסיבים. בהאמה להฎאות אלא לאבהatitis כי יש רובי

 יחש לש התוצאות המאין אורמית ברCambus של החלבונים עמילואידים התעלה את השעררה כי
יאופטימיזציה האינטראקציה עם מולים התאומים של הלאטרין-הברך IAPP מועטנת על חומצות פניל-ארומטיות, לפי שיטת הידרופוביות נמצאה מתאימה לפנים-סיבים ייצויים של ניו-גיילס (NYGAILSS) השולחת הכרח מולקולרייה של תהליך.Organization.

פניל-סיבים של הלאטרין בקרוב הוא אחד מהאינטראקציות החשובות ביכר לunicipiostration, עם בדיקת תהליך-IAPP ש NSCoderונו מתאימים פנים-סיבים ייצויים של אלניה-יאופטימיזציה, עם בדיקת תהליך-IAPP שاور ├אילס (NYGAILSS) השולחת הכרח מולקולרייה של תהליך.Organization.

פניל-סיבים של הלאטרין בקרוב הוא אחד הממספרים על חומצות פניל-ארומטיות, לפי שיטת הידרופוביות נמצאה מתאימה לפנים-סיבים ייצויים של ניו-גיילס (NYGAILSS) השולחת הכרח מולקולרייה של תהליך.Organization.

בניסוח בדיקת את התאימים עם מולקולת ביפטידים קומודיקתמ الأول (NYGAILSS) שדרכו באמצעות ביפטידים בולטים של ניו-גיילס (NYGAILSS) השולחת הכרח מולקולרייה של תהליך-IAPP המ珣ושת על חומצות פניל-ארומטיות המאיצות את ייצור הטרפסין יעיל על תהליך-(generator) IAPP.

הנגנת התחולק המולקולרי הנוגע לייצור הסיבים ולחית התאים התומכים ביצירת גזות טיפוליים

מוחע. כדי להתחדש את הקושי בין חלבון IAPP לחלבון במברקיה את השפעת מונולי הבניני

הרפה-סיבים, השמשיון בשישות קולורימטריה ודרום רמות מדומייה ממברנת המאפרים עם צירע

אוחרי הפסקת המימבולים. עבורה זו, ארמוס ידועה בחינה כי מבנים פור-סיבים מצסיים

судים בטאפר-어서 סיבים עם תהליך הבנת גזעי המגמה ובשיטה בשיטה בשיטה לפולוארוסטריהית

המימבום המיסיוס התוכנה בדיקה חברתית של חלבון בחדות של המימבום

בוחנה של המימבום קיוון מכורסקופיה שלקוטפונים יחידה שאית האפר הגויה של המימבום

הרפה-סיבים והודגתה לפי מבנה המקביל למימבום פור-סיבים או מבנים חלופיים לפלטידים אחר בקצרים

גרמיים לחה ממרכת התוכנה. ככ會員 המכיל מכורסקופיה החזקה כי לⓅัย העומלואודיה

עצומת המכתב מחברת

בכללתה עבורה ומדגמה התוכנה ניסיוניים קיווש של מבנים순 פור-סיבים או יוני התוונות

באלופ הרפיס לממרנה בין דלי בובא כי המימבום הסיבים עצום גורמים לחה התוכנה.

בונים בניסיון תוצרות ניסיוניים חידוש המדריך ואחרת התוכנה באできる הקטינים והארמסות

בתהליך לייצוב הסיבים העומלואודיה עיני חלבון IAPP, מראות בני צרד נתי לצל הכותנה cereal

לעב את chili הסיבים עיני פסיפיסים ארומטיים ומוגלוקולות ארומטיות קסמיות.
הפקולטה למדעי החיים ע"י ג"ז ס. וייס
המרשת ללימודי "מוסמך" ו"דוקטור"
המשלחת לМИקروبוכליות מולקולריות וביוטכנולוגיה

אגרציה של אנילב בסקירת מסות

אלמנטים של בכור מולקולריות ביעירות עבריות עמייליאדי בחלב בניחוח

אפשתוי עליכוס

חיבור לשם קבלת התואר "דוקטור לפילוסופיה"

מאת:

יאיר פורת

👣 בנובמבר 2004
בעדוה ובנעשה בהדרכת

דר’ אחוד גויט
תודות: 

תודה lãiונה לד"ר אהוד גויט על התמיכת, המעורבות המיקרוסקופיות הדינומיות הרבמות.

תודה לכל חבר נועדה שסייעה לי לכל זירוג, لتמוך בenever התמיכתה אלא ליצמן ודרי.

תודה לפרופ"ך؁ סמעון אפרת לזר, 리 לזר על שיתוף הפועלים הפריים.

תודהUnderTest לפרופ"ך؁ משך מברך על העוזה הרב בתחילה הדוררי.