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Peptide Inhibitors of the Amyloidogenesis of IAPP: Verification of the Hairpin Binding Geometry Hypothesis

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Abstract

Versions of a previously discovered β -hairpin peptide inhibitor of IAPP aggregation that are stabilized in that conformation, or even forced to remain in the hairpin conformation by a backbone cyclization constraint, display superior activity as inhibitors. The cyclized hairpin, cyclo-WW2, displays inhibitory activity at sub-stoichiometric concentrations relative to this amyloidogenic peptide. The hairpin binding hypothesis stands confirmed.

Keywords

human pancreatic amylin; Type II Diabetes; amyloid fibril formation; cytotoxicity inhibition; circular dichroism; cyclic β -hairpins; thioflavin T fluorescence

Introduction

Human pancreatic amylin (hAM, also known as IAPP, islet amyloid polypeptide) is a 37 residue peptide that is co-secreted with insulin in the body. Among its many functions, hAM has been shown to play a role in slowing down stomach emptying, inhibiting glucagon secretion and inducing a feeling of satiety after a meal. [1-3] In monomeric solution state, hAM exists as a mostly unstructured peptide with a disulfide bridge between Cys 2 and Cys 7 and an amidated C-terminus that play a central role in the native function of the peptide. [4] When bound to membranes, there is evidence that residues 5-28 adopt a helical conformation while the remaining residues continue to be unstructured. [5] There is also evidence for partial helix formation over the residue 5–20 span in aqueous buffer [6-8] which increases dramatically upon fluoro-alcohol addition. However, at certain concentrations, it has been found that hAM is capable of misfolding and forming amyloid fibrils characterized by their cross- β sheet fibrillar geometry. These fibrils, or their oligomeric precursors, likely play a role in the islet β -cell death which is associated with

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type 2 diabetes. [9-12] Multiple strategies have therefore targeted the amyloidogenesis of hAM as a lead for a therapy that is able to combat this disease. One such strategy has been to use inhibitors that are capable of slowing down the amyloidogenesis pathway or redirecting it altogether to form non-toxic aggregates. These inhibitors include small molecules such as resveratrol [13-15] and acid fuchsin[16] as well as short peptides. [17,18]

We have reported a series of peptide hairpins that inhibit hAM amyloidogenesis and traced this to the presence of Trp and Tyr residues in the β -strands of these hairpins. The most potent of these was KKLTVW-IpGK-WITVSA (peptide WW2) which, in subsequent tests also inhibits amyloid formation by α -synuclein. [19] There are also reports that trpzip-3, another peptide with a W-turn-W motif, inhibits formation of β -amyloid species by transthyretin (TTR) and the Alzheimer related A β peptide. [20] This report showed that TTR inhibition is strongly affected by the presence of tryptophans since the leucine analog of the trpzip peptide was unable to inhibit TTR amyloid formation. However, with A β , no similar preference for the trpzip peptide was noted, suggesting the possibility that a hydrophobic β -hairpin structure is a sufficient feature for amyloidogenesis inhibition in some cases. The common feature among all of these inhibitor-amyloid system interactions have been hypothesized to be the interaction of the amyloidogenic protein with structured β -strands (in the inhibitor) that has hydrophobic or aromatic residues. In the case of WW2, the stable hairpin may be acting as a prestructured strand that can interact with the early stages of amyloid formation by both α -synuclein as well as hAM.

Prior to these reports, potent peptide inhibitors of amyloidogenesis had always been sequences bearing significant homology to an amyloidogenic segment of the system to be inhibited. [17, 21, 22] In the case of hAM, rat amylin (rAM) proved to be an inhibitor albeit a weak one²³. Rat amylin differs from hAM by only 6 residues, 3 of which are proline substitutions located between residues 20 and 29. These changes result in a peptide that does not undergo amyloidogenesis in vivo or in vitro. [24–26] Studies done by Cao et al. showed that rAM was able to significantly retard the formation of amyloid species from hAM in a dose dependent manner. [27] Another study showed that proline substitution at just one residue, I26, resulted in the complete inhibition of hAM amyloidogenesis. [28] In ThT fluorescence monitored assays, I26P-hAM was able to increase the lag time of wild-type hAM amyloidogenesis by a factor of 20 (going from 14 m to 292 m) at equimolar concentrations. Other studies by Kapurniotu et al. have revealed that the full-length of the hAM peptide is not required for either amyloid formation or inhibition. [17, 18, 29]

The 23-27 residue fragment of hAM is one of the shortest segments that have been reported to form aggregates recognized as fibrils by AFM, electron microscopy and Congo red binding. [29,30] N-methylation at residues G24 and I26, in either the full-length sequence [22] or in the hexapeptide fragment comprising residues (22-27) [17,21] yielded potent hAM aggregation inhibitors. The double N-methylated hexapeptide fragment display some suppression the aggregation, and cytotoxicity, of hAM when coincubated at inhibitor/hAM molar ratios of 10/1 to 0.1/1. [17] A full length hAM analog, N-methylated at the same two residues, completely suppressed hAM fibrillization for at least 14 days which, together with its nanomolar IC₅₀ on its inhibitory effect on hAM cytotoxicity, makes it one of the most potent inhibitors ever reported. [22]

Most prior peptidic inhibitors of hAM amyloid formation have been derived from, or mimic, the hAM sequence. [17,21,22] The idea of using hairpin peptides as inhibitors follows from a mechanistic hypothesis for the earliest stages of amyloidogenesis and a mode of inhibition: based on the hypothesis that a combination of aryl sidechain hydrophobic cluster formation and a prestructured β -strand for intermolecular association with other β -strands, could result in the interception of preamyloid β states. [19] The aryl-rings on the prestructured β -strand “inhibitor” were intended to provide binding affinity to the hydrophobic hot spots in the amyloidogenic nucleus. With nascent preamyloid β -strand concentrations thus reduced, the self-self recognition that leads to the formation of toxic β -oligomers [19] could be reduced. In the present work, we examine additional hairpins as well as a cyclized hairpin as inhibitors and provide data that suggest that these inhibitory agents bind in a hairpin formation as suggested by Fig. 1.

Materials and Methods

Peptide synthesis and characterization

Peptide hairpins were synthesized and purified as previously described. [31] The hAM sequence was synthesized by standard Fmoc solid-phase peptide synthesis methods. Wang resin preloaded with C-terminal amino acids as well as Rink Amide-MBHA resin was employed. Pseudoprolines were used at positions 19-20 and 27-28. Peptides were cleaved from the resin using a 95:2.5:2.5 trifluoroacetic acid (TFA):triisopropylsilane: water mixture. Sequence and conformational preferences of peptides were confirmed by 2D NOESY spectra recorded at 298 K. A selection of the NMR data that supports a common hairpin conformation assignment for these peptides appear in the Supporting Information (SI Fig. S5).

hAM oxidation and purification

The crude product from peptide synthesis, presumably a solely cysteine-containing peptide mixture, was dissolved in 30% acetonitrile solution and lyophilized. This step was repeated 6 times. The resulting mixture was then dissolved in 6M guanidinium chloride before being purified by reverse-phase HPLC (Varian ProStar 220 HPLC, Agilent 21.2 $\times$ 50 mm C18 column, 10 mL/min, eluent A: water with 0.1% TFA, eluent B: acetonitrile with 0.085% TFA). The central peak from HPLC, concentrated by lyophilization, was dissolved in a solution of 30% DMSO and allowed to air oxidize overnight. The mixture was then centrifuged and the supernatant was removed. Any precipitate was re-dissolved in 6M Guanidine. HCl, and both solutions were purified by reverse-phase HPLC. The lyophilizate of the collected fractions was dissolved in neat HFIP affording a final concentration of 5mM hAM in the stock solution. The sample was vortexed for 5 minutes to ensure complete dissolution of peptide.

Circular dichroism (CD) assay

The hAM stock solution is employed to make CD assay samples with a final concentration of 50 μ M hAM, with or without 5 – 200 μ M inhibitor, in 50mM pH 2.0 phosphate buffer containing 25 mM NaCl and 2 vol-% hexafluoroisopropanol (HFIP). For each assay, a 400 μ L sample in a 2mL vial with a screw cap closure and a 7 mm stirbar is warmed to 37°C

in a water bath with constant stirring. At the onset, and every 45 minutes thereafter, a 40 μ L aliquot is removed and diluted to a final volume of 200 μ L with 2% HFIP, 50mM pH 2 phosphate buffer, producing a 10 μ M hAM concentration sample for CD measurement. At the end of 3 hours, aliquots were taken every hour up to 6 hours. All spectra were recorded on a Jasco J-720 Circular Dichroism instrument using parameters as previously described³¹. Changes in the amplitude of the CD spectra of the hairpin inhibitors were recorded to ascertain and compare fold stability and possible effects of pH changes. The CD traces appear in the Supporting Information (SI Fig. S4).

Thioflavin T (ThT) fluorescence assay

The stock solution of hAM was diluted to make assay sample with a final concentration of 50 μ M hAM, with or without 5 - 200 μ M inhibitor, in 50mM pH 2.0 phosphate buffer containing 25mM NaCl, 2vol-% hexafluoroisopropanol (HFIP) and 25 μ M ThT; 40 μ L aliquots were taken every half hour and dissolved in 50mM Phosphate pH 2.0 buffer containing 2 vol-% HFIP to make a sample with a final concentration of 10 μ M hAM for the assay. The fluorescence was measured on a Perkin Elmer LS50B, with the following parameters: emission wavelength 482nm and excitation wavelength 450nm.

Each inhibitor was tested in triplicate at all concentrations. In order to get a more complete time course plot, the runs were staggered so that the initial measurement was taken at 0 min for run 1, at 10 min for run 2 and at 20 min for run 3. This allows us to populate a graph with data points every 10 min for the first 180 min, then every 20 min up to 6 hours. The smoothness of the resulting plots (Figure 2 and 3, *vide infra*) attests to the reproducibility of the assay and inhibitory activity. The non-inhibited control is shown as a solid line in the ThT assay figures.

Cell viability assays

The rat insulinoma cell line RIN5fm was employed; the cells were cultured and plated as previously described. [17,19] Freshly made solutions of hAM (5 μ M) alone or mixtures of hAM (5 μ M) with various amounts of peptide inhibitors as indicated were made in 10 mM sodium phosphate buffer, pH 7.4 containing 1% HFIP and incubated at room temperature for ~20 h. Thereafter, the solutions were diluted with cell culture medium and added to the cells at the indicated final hAM concentrations. Following incubations with the cells for 20 h, cell damage was assessed by measuring the cellular reduction of MTT as previously described. [29] Of note, under the experimental conditions employed, hAM was in a nonfibrillar state at the beginning of the pre-incubation. [22]

Results

Previously, our hairpin peptides were tested against low concentrations (5-8 μ M) of hAM in order to determine the activity of these peptides as inhibitors of amyloidogenesis. [19] Since the key early steps of amyloidogenesis are highly concentration dependent oligomerization processes, and we were also aiming for conditions which will allow NMR determination of binding sites, we developed a hAM amyloid assay using higher concentrations of hAM for quantitating relative inhibitor potencies. This assay employs 50 μ M of hAM in 2% HFIP,

50mM (pH 2.0 phosphate buffer with 25mM NaCl, conditions suitable for NMR studies. At this pH and with this level of added HFIP, the complete amyloidogenesis time course is less than 3 hours with maximal β -sheet signal detected by CD from 135 minutes onward (Fig. 4A, vide infra). ThT fluorescence (Fig. 2) confirmed the 135 min time course to maximum “amyloid signal” and provided quantitative data that allowed us to determine relative potencies for different peptide inhibitors more readily.

The present study focuses on three peptide sequences as inhibitors. Herein we report data for the original discovery system, peptide WW2 (the KKLTVW-**Ip**GK-WITVSA sequence) and two sequences that are held in a more stable and rigid β -hairpin conformations: β -cap-WW2 (AcW-KKLTVW-**Ip**GK-WITVSAWTG-NH₂) and cyclo-WW2, cyclo(-GKWITVS-**Ip**PK-KLTVW**Ip**). Note that in these sequences and henceforth a bold lowercase **p** denotes D-Pro. The conformations of the three peptides at pH 2 were confirmed by detailed NMR studies at 298K; the pattern of downfield shifts for cross-strand-directed H α and H β sites (intramolecularly H-bonded) confirmed hairpin formation and register (SI Fig. S5). In the case of cyclo-WW2 and β -cap-WW2 comparable studies near pH 7 were complicated by reduced peptide solubility. The increased fold stability of β -cap-WW2 at pH 7, and significantly greater stability of cyclo-WW2 at both pH's was confirmed by CD melting studies (SI Fig. S4).

We anticipated that aggregation inhibition would require more potent agents, or higher inhibitor concentrations, to counter the higher concentrations of hAM in the current assay. In prior assays against 8 μ M hAM (5 mM pH 7.2 phosphate, 2% HFIP), WW2 displayed significant partial inhibition at equimolar concentrations (an increase in aggregation lag time, from 50 to 150 min), with nearly complete inhibition in the ThT assay with a 2-fold amount of WW2 relative to hAM. [19] In the new 50 μ M-hAM aggregation assay, only a very modest (perhaps not a significant effect) delay was observed at equimolar ratio of WW2 (data not shown). With 2 equivalents of WW2, significant inhibition was observed: aggregation was still incomplete at the 400 minute end-point. Nearly complete inhibition of 50 μ M hAM required 4 equivalents of WW2.

Having confirmed our expectations regarding the assay and the activity of peptide WW2 in the new assay, we moved on to an examination of the two new analogs, Figure 3. The ThT data for β -cap-WW2 appears in panel A of Figure 3. It is apparent that 0.5 equivalents of the capped analog is nearly as effective as 2 equivalents of WW2. At 1 and 2 molar equivalents β -cap-WW2 showed increasing inhibition but, interestingly, it did not achieve the degree of inhibition observed with 4 equivalents of WW2 (Fig. 2). In contrast, cyclo-WW2 was remarkably more potent (panel B, Fig. 3). Cyclo-WW2 was able to inhibit amyloidogenesis even at substoichiometric concentrations. The ThT fluorescence data shows that, even with only 0.5 equivalents cyclo-WW2 present, there is no significant increase in fluorescence intensity over the course of the 6 hour experiment, a complete lack of amyloid fibril development. The superior activity of cyclo-WW2 is also evident in CD assays.

The CD assay is based on the development of a β -structure signature, an ellipticity maximum at 194 nm replaces the random coil minimum and a new minimum appears at 216 nm. Panels A and B of Figure 4 compare representative time courses of the appearance of

the CD signal for an uninhibited control and in the presence of 0.25 molar equivalents of cyclo-WW. Replicate assays of β -structure formation (measured as $[-\theta]_{216}$) for the control and with 0.25 and 0.5 equivalents of cyclo-WW2 added appear as Figure 4C. The data shows that in the absence of inhibitor, the minima at 216 nm rises to approximately $25000 \pm 1335 \text{ mdeg.mol}^{-1} \text{ cm}^{-1}$ over a 3-h period. In the presence of 0.25 equivalents of cyclo-WW2 less than one-half of this level is reached over a longer time course (6 h). With 0.5 equivalents, complete suppression of β -sheet formation is observed. With 0.1 equivalents of the peptide inhibitor only a modest, but still significant, increase in the time required for the amyloidogenesis is observed (Supporting Information Fig. S1). Comparative CD data for inhibition by 4 equivalents (and ineffectively by 1 equivalent) of WW2 appear in SI Fig. S2.

Cell cytotoxicity assays have played a large part in determining if an amyloidogenesis inhibitor is actually suppressing the negative effects of hAM aggregation which appear to be associated with the oligomer formation process rather than final fibril concentrations. [32–34] In our previous study, we have shown that in the presence of 2 eq. of WW2 formation of cytotoxic assemblies by IAPP was suppressed albeit not fully blocked. [19] A stoichiometric level of WW2 appeared to have no effect of cytotoxicity but increases in the WW2 ratio to hAM (to 6 and 10-fold) during the pre-incubation period resulted in strong to nearly complete suppression of hAM-induced cytotoxicity. [19] The same experiment was performed in the current study using the β -cap- and cyclo-WW2 peptides.

The results indicate that both peptides block hAM-associated toxicity even at molar concentration ratios (hAM/peptide) of 1 to 0.5 (Fig. 5A and B). Thus, both stabilized hairpins display significantly greater hAM-induced cytotoxicity inhibition than WW2 itself. In the case of cyclo-WW2 the cytotoxicity assays mirror (in inhibitor/hAM mole ratio effectiveness) the in vitro amyloid inhibition assay results. This correlation breaks down to some extent for β -cap-WW. Although it is essentially as potent as cyclo-WW2 by the cytotoxicity-reduction assay, β -cap-WW2 addition does not appear to produce complete elimination of the enhanced ThT-fluorescence signal due to β -amyloid formation: the ThT signal grows in slowly (Fig. 3A) when a stoichiometric and even 1-fold excess (relative to hAM) amount of β -cap-WW2 is present.

Discussion

This study was prompted by two questions. Is there a relationship between hairpin fold stability and anti-amyloid potency? Do WW2 peptides bind with their hairpin-like conformation intact? β -cap-WW2 was expected to have greater hairpin stability than WW2, and it is a significantly more potent inhibitor of amyloidogenesis than uncapped WW2. The increased stability was expected due to the insertion of a stabilizing feature, the terminal β -cap. [35] However, this feature would not necessarily stabilize the structure in the turn-flanking region bearing the W/W cluster and does not preclude unfolding and restructuring to another conformation, including more linear arrangements, for binding to hAM. In contrast, backbone cyclization, particularly with a superior β -turn unit (the heterochiral D-Pro-L-Pro, **pP**) forces the peptide to remain in a conformation with both of the original β -strands still in the β -conformation and associated by cross-strand H-bonds. We also viewed cyclo-WW2 as potentially less likely to be targeted for proteolytic digestion due to its cyclic

structure, another favorable feature. Cyclo-WW2 was able to completely inhibit formation of amyloid even at substoichiometric concentrations in the in vitro assays. Cyclo-WW2 has also proven to be a more potent inhibitor of β -oligomer formation by α -synuclein. [31]

NMR studies (SI Fig. S5) at pH 2 – 3, indicated that both of the new peptides (as well as WW2) were well-folded with the same backbone conformation (with the hairpin state mole fractions exceeding 0.92 at 25°C) and indicated very similar geometries for the edge-to-face Trp/Trp cluster flanking the turn (SI Fig. S6A). These studies could be extended to pH 6.5 for peptide WW2 but cyclo-WW2 and β -cap-WW2 were less soluble at this pH and we needed to rely on CD comparisons. These also allowed us to examine fold stability at higher temperatures.

As previously noted, there is a distinct difference in the relative potencies of cyclo-WW2 and β -cap-WW2 in the ThT fluorescence (and CD) monitored inhibition of β -amyloid structure formation versus cytotoxicity amelioration assays. Since these assays were performed at two quite different pH values, we needed to examine the pH-effect on hairpin structures and fold stability. The effects of higher temperatures and the pH change were examined by CD melts (SI Fig. S4). The CD comparisons implied the same fold at both pHs for all three peptides with an increase in fold stability at >45°C at pH 7.4 compared to pH 2, particularly for β -cap-WW2. The enhanced melting of β -cap-WW2 (versus cyclo-WW2) does not, however, result in a significant change (pH 2 versus 7.4) in the fold population at the assay temperature. As a result, we posit that there is some selectivity for the cytotoxicity suppression in the case of β -cap-WW2: a differential effect on toxic oligomer formation versus amyloid fibril formation. There may, however, also be pH-induced differences in the amyloid formation pathway such as the lifetime and concentration of toxic intermediates. Specific NMR parameters (SI Fig. S6) associated with the Trp/Trp clusters and their dynamics do indicate that the cluster of cyclo-WW2 has a slightly different geometry (see Supporting Information); this could also be a factor.

In any case, we have now found, for small peptides that bear no similarity to hAM, substoichiometric inhibition of hAM amyloidogenesis-induced cytotoxicity. These structure-stabilized analogs of peptide WW2 are additions to a limited set of potent peptide inhibitors of this amyloidogenesis pathway that have been reported to date. Others, including a new series of ISM inhibitors, [18] IAPP-GI and NFGAILS-GI [17] are also able to suppress hAM fibrillogenesis and hAM-induced toxicity at substoichiometric rates with IC₅₀ values in the nanomolar range. Cyclo-WW2 (and β -cap-WW2) are the only peptides in this group that bear no sequence similarity to hAM. Also, with the confirmed and enhanced stability of cyclo-WW2, there can no longer be any doubt that a hairpin-like conformation is involved in the binding interactions of the WW2-related peptides that are responsible for the inhibition of toxic oligomer formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

hAM	human amylin
IAPP	islet amyloid polypeptide
MTT	3-4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide
ThT	thioflavin T
HFIP	hexafluoroisopropanol

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Highlights

- A strategy that stabilizes the hairpin conformation increases inhibitor potency
- Substoichiometric amounts of a cyclic β -hairpin peptide are able to inhibit IAPP amyloidogenesis
- Verification that hairpin-like conformation is involved in inhibition of IAPP cytotoxicity

Figure 1. Diagram illustrating the mechanistic hypothesis for the earliest stages of amyloidogenesis and its inhibition by a cyclic hairpin.

Figure 2. ThT fluorescence assay data for “amyloid” formation from hAM in the absence (circles) and presence of 2 molar equivalents of WW2 (triangles) as well as in the presence of 4 molar equivalents of WW2 (squares). The inhibited runs are triplicate determinations (see Methods).

Figure 3.

ThT fluorescence assays data for “amyloid” formation from hAM. Panel A - Effects of β -cap-WW2 on hAM at 0.5:1 (squares), 1:1 (circles), and 2:1 (crosses) inhib:hAM molar ratios. Panel B - Effects of substoichiometric concentrations of cyclo-WW2 on hAM at 0.5:1 (squares), 0.25:1 (diamonds), and 0.1:1 (circles) inhib:hAM molar ratios. The control response with no inhibitor (from Figure 2) is shown as a solid line in both panels.

Figure 4. Panel A - CD spectra showing the progression of hAM secondary structure from random coil to β -sheet over the course of the assay (135 minutes) in the absence of inhibitors. Panel B – CD spectra depicting the progression of hAM secondary structure over 300 minutes in the presence of 0.25eq cyclo-WW2. In panel B, even at 300 min., the formation of β -structure is far from complete. Panel C – Graphs of mean residue ellipticity at 216 nm, $[-\theta]_{216}$, versus time. These show changes in β -sheet character of hAM throughout the time course of the assay in the absence of cyclo-WW2 (circles) and in the presence of 0.25 (triangles), as well as 0.5 molar equivalents (squares) of cyclo-WW2. All data points represent an average of 3 runs.

Figure 5.

Panel A - Effects of cyclo-WW2 on the cytotoxicity of hAM. Aliquots of 24-hr aged incubations of hAM with cyclo-WW2 at 1/1, 1/2, 1/6 and 1/10 molar ratios were diluted with cell medium and added to RIN5fm cells. Cell viability was measured after 20 hrs of incubations. Panel B – Effects of sub-stoichiometric amounts of cyclo-WW2 and β-cap WW2 on the cytotoxicity of hAM. Aliquots of 24-hr aged incubations of hAM with cyclo-WW2 or β-cap-WW2 at 1/1, 1/0.5 and 1/0.1 molar ratios were diluted with cell medium and added to RIN5fm cells. Cell viability was measured after 20 hrs of incubations. Results are means (+SEM) from 3 experiments (n=3 each).