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The Combination of Kinetically Selected Inhibitors *in Trans* Leads to the Highly Effective Inhibition of Amyloid Formation

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Amyloid fibril formation plays a role in at least 20 different diseases, including Alzheimer's disease, Parkinson disease and type-2 diabetes[1–6]. The design of inhibitors of amyloid formation is an extremely active area of research and a broad range of strategies have been applied to the problem[7–11]. Unfortunately, many inhibitors are effective only in molar excess and typically either lengthen the time to the onset of amyloid formation, (the lag time), while having modest effects on the total amount of amyloid fibrils produced, or decrease the amount of fibrils without significantly reducing the lag time. Improved inhibitors are needed but, given the considerable effort expended to date, it is not obvious what approach will be generally applicable. In this communication we demonstrate a general strategy whereby two moderate inhibitors of amyloid formation can be rationally selected via kinetic assays and combined *in trans* to yield a highly effective inhibitor, which dramatically delays the time to the appearance of amyloid and drastically reduces the total amount of amyloid formed. A key feature of the approach is that the selection of the components of the mixture is based on their effect on the time course of amyloid formation rather than on just the amount of amyloid produced.

The approach is demonstrated using islet amyloid polypeptide, (IAPP, also known as Amylin), which is the causative agent of islet amyloid in type 2 diabetes and the $A\beta_{1\text{-}40}$ peptide of Alzheimer's disease (Figure 1)[12–18]. The development of effective inhibitors of amyloid formation by IAPP is a challenging test case since the polypeptide is extremely amyloidogenic and aggregates even faster than the Aβ peptide *in vitro*. Considerably less work has been reported on the development of IAPP inhibitors than has been reported on the development of Aβ inhibitors.

The point mutation I26P, (I26P-IAPP), has been shown to convert IAPP into a moderately effective inhibitor of amyloid formation by wild type IAPP[19]. We now report that the G24P point mutant, (G24P-IAPP), is also a moderately effective inhibitor of wild type IAPP amyloid formation and, more importantly, demonstrate a striking synergy when the two inhibitors are combined in *trans*. We show that the combination is also a potent inhibitor of amyloid formation by the $A\beta_{1\text{-}40}$ peptide.

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Supporting Information Available: Experimental procedures. Thioflavin-T kinetic assays and TEM images of the G24P-IAPP peptide and I26P IAPP peptide. Analytical ultracentrifugation and gel filtration of I26P and G24P IAPP and a mixture of the two peptides. Additional kinetic experiments conducted for various ratios of IAPP with each point mutant and for various mixtures of the two inhibitors with wildtype IAPP. CD spectra of wild type IAPP and Aβ peptide in the presence and in the absence of the inhibitors. This material is available free of charge via the Internet at http://pubs.acs.org.

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Thioflavin-T fluorescence monitored kinetic assays of the time course of amyloid are shown in Figure 2A. The data obtained for wild type IAPP in the absence of inhibitor displays the classic sigmoidal curve expected for amyloid formation. Neither the G24P variant nor the I26P variant form amyloid and both appear to be monomer as judged by analytical ultracentrifugation and gel filtration (Supporting Information). A 1:1 molar mixture of wild type IAPP with either inhibitor behaves very differently than does the wild type peptide in the absence of inhibitor. Like the I26P peptide, the G24P mutant is an inhibitor of amyloid formation. It lengthens the lag phase and decreases the value of final thioflavin-T fluorescence intensity, but it does not completely abolish amyloid formation by IAPP. We have observed some experiment to experiment variability for the effects of the G24P peptide on wild type IAPP, but the same trend relative to I26P is always observed. The G24P mutant always has a larger effect on the lag phase of wild type than does the I26P mutant. In contrast, the G24P peptide always has a smaller effect on the final value of the thioflavin-T fluorescence of the 1:1 mixture. The general features of the kinetic assays are robust and similar results have been obtained by different investigators using different preparations of peptides. Both peptides also inhibit amyloid formation when added at substoichiometric levels, albeit less effectively (Supporting Information).

The two inhibitors clearly have different relative affects on the lag phase and on the final fluorescence signal, suggesting that the two molecules target different steps in the pathway of amyloid fibril formation with different efficiencies. We reasoned, based upon their differential effects upon the kinetics, that a combination of the two inhibitors would prove much more effective than the individual inhibitors. The results of combining the two inhibitors *in trans* are striking; the combination is a far more effective inhibitor than either mutant alone and no change in thioflavin-T fluorescence is observed over the time course of the entire experiment (Figure 2A). Thioflavin-T fluorescence intensity is an imperfect reporter of the total amount of amyloid produced, but in the simplest case when there are no synergistic effects, one might expect that the reduction in the final thioflavin-T fluorescence intensity relative to wildtype would be on the order of the product of the effects of the individual inhibitors. However, the effect is clearly significantly larger. It is also clearly larger even if the log of the final fluorescence intensities are compared. The dramatic effect of the mixture is not due to an increase in the total amount of inhibitor since experiments were conducted with the total inhibitor concentration, (single inhibitor or combination), held constant. Note that the concentration of each inhibitor is 16 μM in the experiments involving the 1:1 mixture of wildtype with a single inhibitor, but only 8 μM in the samples which contained the mixture of both inhibitors. Even more pronounced synergistic effects are observed if the mixture of the two inhibitors is compared to samples which contain an individual inhibitor at 8 μM (Supporting Information). We also observed significant synergistic effect at lower ratios of the total inhibitor to IAPP (Supporting Information).

We confirmed the results of the thioflavin-T studies by recording TEM images of the end points of the kinetic experiments (Figure 2B–2E). TEM images were recorded of aliquots removed 600 minutes after the start of the reaction. This corresponds to a time that is 20-fold longer than that required for IAPP to form amyloid under these conditions. The images collected for the sample of wild type IAPP in the absence of inhibitor display numerous amyloid fibers with the morphology commonly observed for IAPP-derived amyloid (Figure 2B). The TEM images of the 1:1 molar mixtures of IAPP with either point mutant are very different (Figure 2C, 2D). Significantly fewer amyloid fibers are observed and those which are detected have a distinctly thinner appearance compared to the wild type amyloid fibers. The TEM image of the 1:0.5:0.5 mixture of wild type IAPP with G24P-IAPP and I26P-IAPP is very different from the images of the binary mixtures and no fibers were detected on the grid (Figure 2E). Far UV circular dichroism, (CD), spectra were also recorded 600 minutes after the start of the reaction and provide a third independent probe of the effects of

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the various inhibitors (Supporting Information). The CD spectrum of IAPP indicates considerable β-structure. In contrast, the spectrum of the 1:0.5:0.5 mixture of IAPP with the G24P-IAPP and I26P-IAPP point mutants shows no evidence of β–structure.

We tested the generality of the synergistic effects by examining the ability of the combination of inhibitors to inhibit amyloid formation by a different polypeptide. There has been at least one report of a peptide based inhibitor of IAPP amyloid formation inhibiting amyloid formation by the $A\beta_{1\text{-}40}$ Alzheimer's polypeptide[20]. Helical intermediates have been proposed to play a role in amyloid formation by IAPP and Aβ, at least under some circumstances, thus there might be some similarity in the early intermediates populated by both polypeptides[21–24]. In addition, recent studies have shown that the regions of IAPP which are important for self-association are also important for hetero-association with Aβ. The converse is also true; regions of Aβ that are important for self-association are involved in the binding interface of Aβ with IAPP[25]. The segment of IAPP corresponding to residues 8 to 20 was found to be important for self-association. The G24P and I26P point mutants are expected to bind to this region since the mutants are outside of this segment. Thus, the overlap of the regions important for self-association of IAPP with the binding interface for hetero-association with Aβ, suggests that the G24P and I26P IAPP point mutants might inhibit Aβ amyloid formaton. This is exactly what was observed. Thioflavin-T monitored kinetic assays reveal that each peptide is a moderate inhibitor of amyloid formation by $A\beta_{1\text{-}40}$ and show that the two IAPP point mutants exert different relative effects on the lag phase and the final thioflavin-T fluorescence intensity (Figure 3A). The combination of the two inhibitors again proved to be much more effective than either inhibitor alone, even though the total inhibitor concentration was kept constant. TEM images collected of aliquots removed at the end of kinetic experiments, (20 hours), are fully consistent with the thioflavin-T studies. Dense mats of fibers are observed for the $A\beta_{1\text{-}40}$ sample in the absence of inhibitors, while a 1:1 mixtures of $A\beta_{1\text{-}40}$ with either point mutant leads to much shorter aggregates (Figure 3B–3D). Fewer aggregates are observed in the 1:0.5:0.5 mixture of $A\beta_{1\text{-}40}$ with G24P-IAPP and I26P-IAPP (Figure 3E). CD measurements show that the mixture inhibits β-sheet formation (Supporting Information).

It is natural to ask why the combination of the two inhibitors is more effective than a single inhibitor. An indirect physio-chemical effect on the bulk properties of the solution is unlikely, especially since other variants of IAPP are much less effective inhibitors even when added in significantly large excess[22]. One possibility is that the two peptides form a stable complex which is a more effective inhibitor than either peptide in isolation; however gel filtration and analytical ultracentrifugation experiments strongly argue against this scenario. Another possibility is that the inhibitors target different stages of the amyloid fibril formation pathway. The different kinetic curves are consistent with this notion. In this scenario one inhibitor may preferentially bind to species which are populated early and inhibit their progression to the next stage, while the second inhibitor may target structures which are formed latter and whose production was slowed by the first inhibitor, or which formed from molecules that escaped the first inhibitor. Alternatively, amyloid formation may proceed by parallel pathways and the two inhibitors may target separate pathways with different efficiencies. The process of amyloid formation has yet to be defined at high resolution for any system and in the absence of a residue specific description of the kinetic mechanism of amyloid formation, it is impossible to deduce exactly what stages the two inhibitors target. Irrespective of the mechanistic details, the data presented here is striking, and illustrates strong synergistic effects with the combination strategy.

In the present study, we used peptide based inhibitors to illustrate a combination approach for inhibiting amyloid formation and we have provided a second example of an IAPP inhibitor that exerts strong inhibitory effects on amyloid formation by the Aβ peptide.

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The concept is generalizable to non-peptide inhibitors. The key feature of the approach is that the selection is based upon assays which monitor the time course of amyloid formation and not just the total amount of amyloid produced. These real time measurements allow one to select molecules which have different effects on different aspects of the self-assembly reaction. In contrast, traditional assays which rely on the read out of a single time point, such as the final thioflavin-T fluorescence, are unable to select such combinations. The approach outlined here has the potential to greatly expand the number of hits for a given set of compounds and is well suited for high throughput screens since thioflavin-T based assays are readily conducted in microtiter plates[27].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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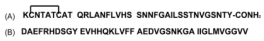

Figure 1.
(A) The primary sequence of human IAPP. The polypeptide contains a disulfide bridge between Cy-2 and Cys-7 and the C-terminus is amidated. (B) The primary sequence of the 1-40 isoform of the Aβ polypeptide.

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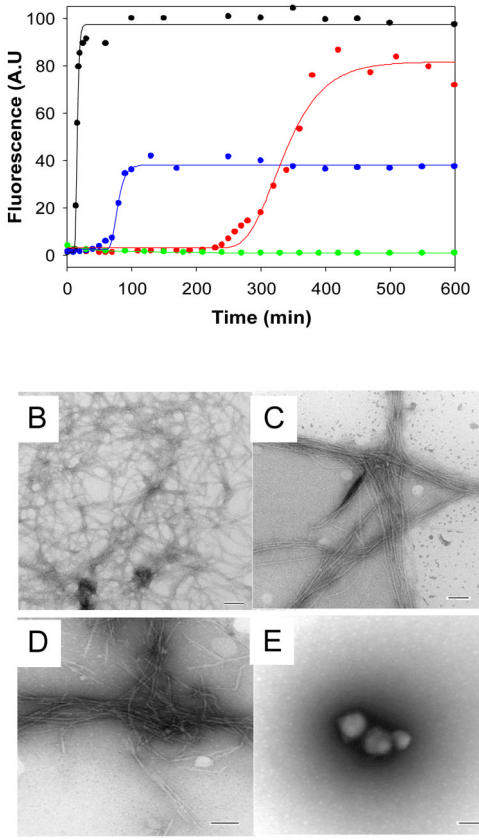

Figure 2.

Synergistic inhibition of amyloid formation by IAPP. (A) The results of fluorescent detected thioflavin-T binding assays are displayed. Black, wild type IAPP; Red, a 1:1 mixture of wild type IAPP with G24P-IAPP; Blue, a 1:1 mixture of wild type IAPP with I26P-IAPP; Green, a 1:0.5:0.5 mixture of wild type IAPP with G24P-IAPP and I26P-IAPP. (B) TEM image of wild type IAPP alone. (C) TEM image of a 1:1 mixture of wild type IAPP and G24P-IAPP. (D) TEM image of a 1:1 mixture of wild type IAPP and IAPP-I26P. (E) TEM image of a 1:0.5:0.5 mixture of wild type IAPP, G24P-IAPP and I26P-IAPP. Scale bars represent 100 nm. Aliquots were removed from the kinetic experiments 600 minutes after amyloid formation was initiated and TEM images collected. The kinetic assays depicted in panel (A) were carried out in 20 mM Tris-HCl (pH 7.4), 2% HFIP (v/v) with continuous stirring at 25°C. The total concentration of inhibitor was the same in the 1:1 mixtures and in the 1:0.5:0.5 mixtures and was equal to 16 μM. Wild type IAPP was at 16 μM.

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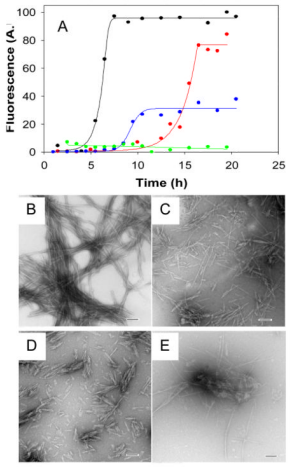

Figure 3.

Synergistic inhibition of amyloid formation by the $A\beta_{1\text{-}40}$ polypeptide. (A) The results of fluorescent detected thioflavin-T binding assays are displayed. Black, $A\beta_{1\text{-}40}$; Red, a1:1 mixture of $A\beta_{1\text{-}40}$ with G24P-IAPP; Blue, a 1:1 mixture of $A\beta_{1\text{-}40}$ with I26P-IAPP; Green, a 1:0.5:0.5 mixture of $A\beta_{1\text{-}40}$ with G24P-IAPP and I26P-IAPP. (B) TEM image of $A\beta_{1\text{-}40}$ alone. (C) TEM image of a1:1 mixture of $A\beta_{1\text{-}40}$ and G24P-IAPP. (D) TEM image of a 1:1 mixture of $A\beta_{1\text{-}40}$ and I26P-IAPP. (E) TEM image of a 1:0.5:0.5 mixture of $A\beta_{1\text{-}40}$, G24P-IAPP and I26P-IAPP. Scale bars represent 100 nm. Aliquots were removed from the kinetic experiments 20 hours after amyloid formation was initiated and TEM images collected. The kinetic assays depicted in panel (A) were carried out in 100 mM Tris-HCl (pH 7.4) with continuous stirring at 25°C. The total concentration of inhibitor was the same in the 1:1 mixtures and in the 1:0.5:0.5 mixtures and was equal to 24 μM. $A\beta_{1\text{-}40}$ was at 24 μM.