VIP Protein Aggregation

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IAPP Mimic Blocks Aβ Cytotoxic Self-Assembly: Cross-Suppression of Amyloid Toxicity of Aβ and IAPP Suggests a Molecular Link between Alzheimer's Disease and Type II Diabetes**

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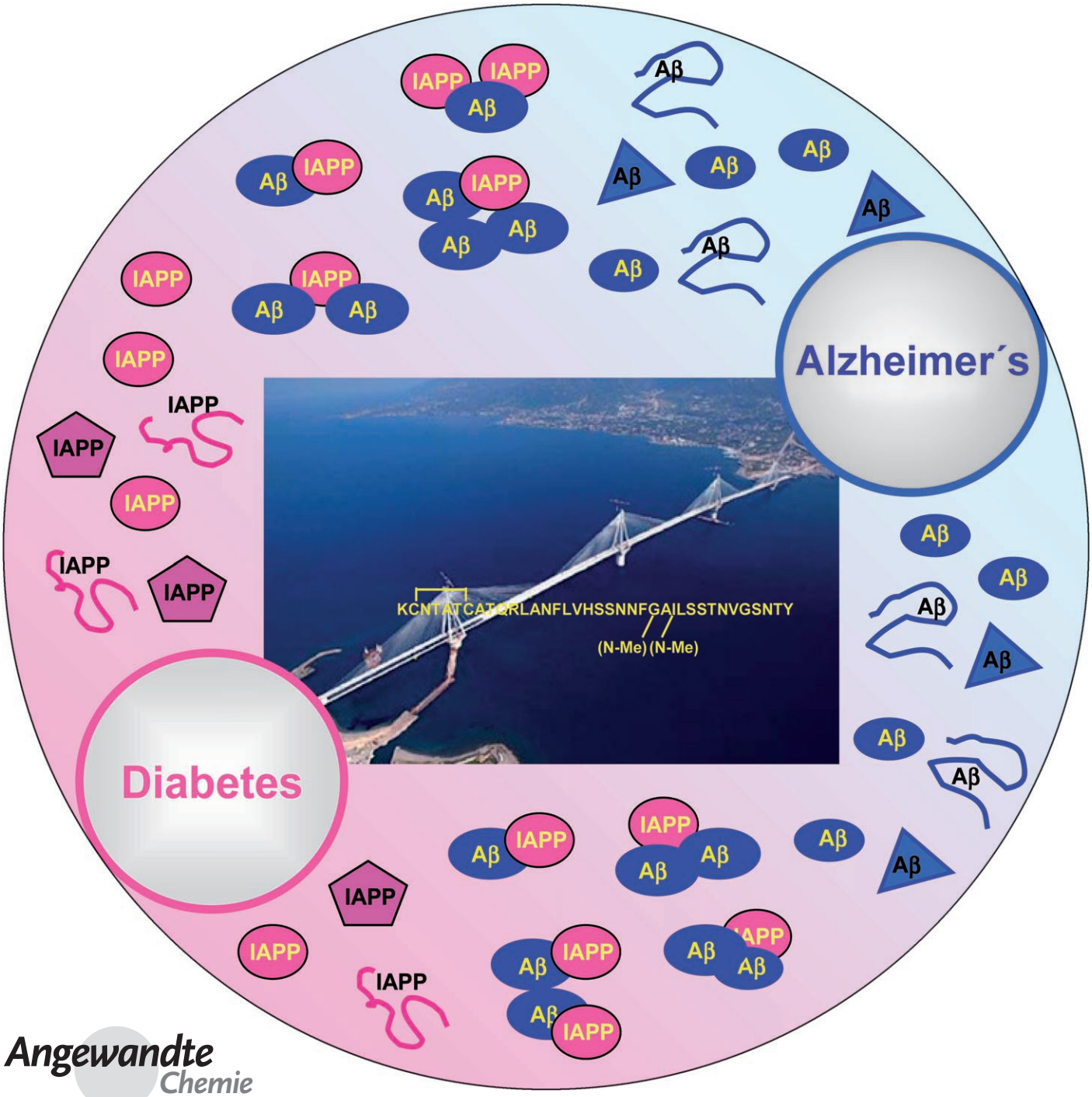

The misfolding and self-association of natively nontoxic proteins and polypeptides into cytotoxic oligomers and amyloid fibrils are linked with a number of fatal cell degenerative diseases.[1–3] Two prominent representatives of these diseases, also called protein-aggregation diseases, are Alzheimer's disease (AD) and type II diabetes (T2D).[1–3] Emerging evidence supports the hypothesis that AD and T2D might be linked to each other.[4,5] Clinical studies suggest that persons suffering from T2D might be at the risk of AD, and vice versa.[4,5] On the pathophysiological level, both diseases are characterized by localized extracellular tissue amyloid fibril deposition and progressive cell degeneration.[1] Similarities appear to be present also on the molecular level. The major component of AD brain amyloid is β-amyloid peptide (Aβ), a 40- (Aβ40) to 42-residue (Aβ42) polypeptide of yet unknown function (Figure 1).[6] The pancreatic amyloid

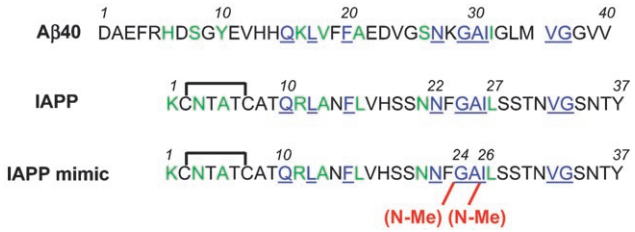

Figure 1. Primary structures of Aβ40, IAPP, and the IAPP mimic IAPP-GI.[13] Identical residues between sequences are indicated in blue and are underlined, and similar residues are indicated in green.[9]

plaques in T2D consist of islet amyloid polypeptide (IAPP), a 37-residue polypeptide that acts as a neuroendocrine regulator of glucose homeostasis (Figure 1).[1] Thus, in both AD and T2D, two short, linear, and conformationally flexible or "natively unfolded" but highly aggregation-prone polypeptides are key amyloidogenic molecules.[7,8] Notably, both polypeptides self-assemble into cytotoxic aggregates at nanomolar concentrations. Interestingly, the sequences of Aβ40 and IAPP are 25% identical and 50% similar, while high degrees of identity and similarity are shared between the short sequences Aβ(15–21) and IAPP(10–16) or Aβ(26–32) and IAPP(21–27), which are believed to participate in Aβ40 or IAPP self-assembly (Figure 1).[9–12] Moreover, it was recently reported that Aβ40 fibrils can cross-seed fibrillization of IAPP.[9] This finding indicated that Aβ40 and IAPP might populate states that cross-interact. However, there has been no direct evidence for such an interaction so far.

We have recently designed a conformationally constrained analogue of IAPP as a mimic of a non-amyloidogenic and nontoxic IAPP conformation.[13] The mimic, [(N-Me)G24, (N-Me)I26]-IAPP (IAPP-GI), was generated by N-methylation of two amide bonds on the same "side" of the β-strand in the amyloid core sequence IAPP(22–27) (NFGAIL sequence) of full-length IAPP (Figure 1). Interstrand amide hydrogen-bond formation, a process necessary for β-sheet-mediated self- or heteroassociation, was thus only possible through one "side" of the N-methylated sequence.

IAPP-GI has proved to be a highly soluble, non-amyloidogenic, and non-cytotoxic IAPP analogue. Moreover, IAPP-GI has been shown to bind soluble, prefibrillar IAPP species with low nanomolar affinity and to block and reverse IAPP cytotoxic self-assembly and amyloidogenesis.[13] Here, we show that IAPP-GI is also able to bind prefibrillar Aβ40 and that it blocks and reverses Aβ40 cytotoxic self-assembly. We also show that early prefibrillar IAPP species bind Aβ40 and that Aβ40-IAPP heteroassociation attenuates cytotoxic self-association by both Aβ40 and IAPP.

We first investigated whether IAPP-GI could block formation of cytotoxic Aβ40 assemblies using cultured rat pheochromocytoma PC-12 cells.[14] Aged solutions of Aβ40 or mixtures of Aβ40 with IAPP-GI (1:1) were added to the cells (Figure 2a). Cell viabilities were determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay and by assessment of apoptosis.[14,15] IAPP-GI completely blocked Aβ40 toxicity over a 100-fold concentration range of Aβ40 (Figure 2a,b). Note that rat IAPP (rIAPP), a natively occuring non-amyloidogenic IAPP analogue, did not significantly affect Aβ40 cytotoxicity, indicating that a specific interaction might underlie the inhibitory effect of IAPP-GI (Figure 2a). Titrations of cytotoxic Aβ40 with IAPP-GI yielded an IC_{50} of 78 nM, demonstrating that IAPP-GI is an inhibitor of Aβ40 toxicity with low nanomolar activity (Figure 2c).

We next investigated whether IAPP-GI could interfere with Aβ amyloidogenesis by using the amyloid specific thioflavin T (ThT) binding assay and transmission electron microscopy (TEM).[16] Aβ40 fibrillogenesis exhibited a lag time of approximately 48 h and reached completion at 72–96 h (Figure 2d,e). In the presence of an equimolar amount of IAPP-GI, however, Aβ40 fibrillogenesis was completely blocked (Figure 2d,e). The Aβ40/IAPP-GI mixtures consisted mainly of spherical oligomers with diameters of between 20–50 nm and nonfibrillar, strongly beaded multimeric assemblies (Figure 2e).

The ability of IAPP-GI to bind prefibrillar Aβ40 was confirmed and characterized by far-UV circular dichroism (CD) spectroscopy, pull-down assays, and fluorescence spectroscopy. The CD spectrum of the mixture of freshly dissolved Aβ40 with IAPP-GI (1:1) differed markedly from the sum of

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

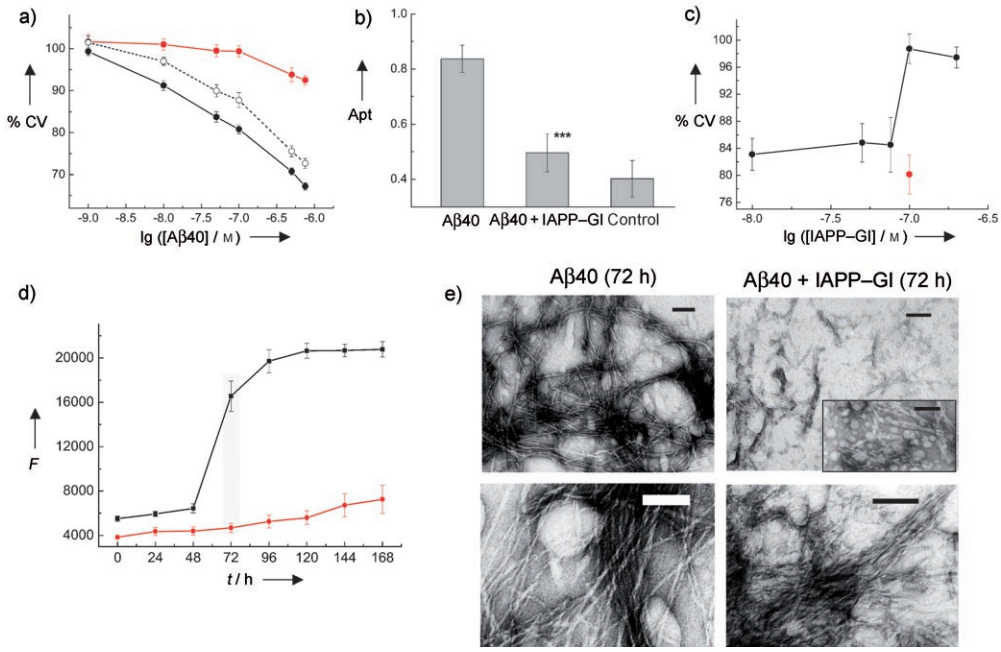

Figure 2. Inhibition of Aβ40 cytotoxic self-assembly and fibrillogenesis by IAPP-GI. a) Effects of Aβ40 (black circles) versus a mixture (1:1) of Aβ40 with IAPP-GI (red circles) or with rIAPP (unfilled circles) on PC-12 cell viability (% CV; % of control) as assessed by MTT reduction. Data are the average (± standard error) from three to six assays ($n = 3$ each). (b) Inhibition of Aβ40-mediated PC-12 cell apoptosis (Apt) by IAPP-GI (1:1; 1 μM each). (*** $P < 0.001$ by ANOVA.) Data are the average (± standard error) from three to six assays ($n = 3$ each). c) Determination of the IC_{50} of the inhibitory effect of IAPP-GI on PC-12 cell toxicity of Aβ40 by titration of Aβ40 (100 nM) with various amounts of IAPP-GI (Aβ40: red circle; mixtures: black circles) as assessed by the MTT assay. Data are the average (± standard error) from three to six assays ($n = 3$ each). d) ThT binding assay (*F*: ThT fluorescence) of the fibrillogenesis of Aβ40 (16.5 μM; black circles) versus a mixture of Aβ40 and IAPP-GI (1:1; red circles). The gray bar shows the time point of TEM examination. e) TEM images of fibril formation of Aβ40 (left) versus a mixture of Aβ40 and IAPP-GI (1:1; right). Aliquots of the incubations (part (d)) were examined by TEM at 72 h (scale bars: 100 nm). The inset shows spherical oligomeric assemblies.

the spectra, indicating that IAPP-GI interacted with Aβ40 (Figure 3a). The shape of the spectrum indicated formation of ordered heterocomplexes. N^{α}-amino-terminal biotinylated IAPP-GI (Biotin-IAPP-GI) was then incubated with freshly dissolved Aβ40 (1:1 or 1:2), and Biotin-IAPP-GI/Aβ complexes were isolated by binding to streptavidin-coated magnetic beads. Following complex dissociation and NuPAGE (electrophoresis), Western blotting revealed significant amounts of Aβ40 monomers and dimers in the mixture (Figure 3b) demonstrating that Biotin-IAPP-GI bound Aβ40.

The affinity of the IAPP-GI–Aβ40 interaction was quantified by fluorescence spectroscopy.[13,17] N^{α}-amino-terminal fluorescein-labeled IAPP-GI (Fluos-IAPP-GI; 1 nM) was titrated with Aβ40, and fluorescence emission spectra were recorded.[13] Binding of 100-fold molar excess of Aβ40 to Fluos-IAPP-GI caused a fluorescence enhancement of about 50% (Figure 3c). A sigmoidal binding isotherm was obtained, and curve fitting yielded an apparent affinity of interaction (app. K_d) of (41.2 ± 3.9) nM (Figure 3d). As nanomolar Aβ40 solutions may contain both monomers and low-molecular-weight oligomers, our data suggest that IAPP-GI bound monomers and/or low-molecular-weight prefibrillar Aβ40 oligomers.[18]

We next examined at what stages of the Aβ40 self-assembly pathway IAPP-GI interfered with. IAPP-GI blocked formation of cytotoxic Aβ40 oligomers and fibrils when it was added (1:1) to Aβ40 species present before nucleation of fibrillogenesis (Figure 4a,b). When it was added after the start of Aβ40 cytotoxic self-assembly and fibrillogenesis, IAPP-GI (1:1) completely blocked further Aβ40 fibrillogenesis. Moreover, it redissociated Aβ40 cytotoxic assemblies thus reversing Aβ40 cytotoxicity (Figure 4a–c). Importantly, addition of IAPP-GI (1:1) to early prefibrillar and nontoxic Aβ40 species completely blocked formation of cytotoxic assemblies and fibrils for at least 7 days, suggesting that IAPP-GI binds prefibrillar nontoxic Aβ40 species and blocks their further conversion into cytotoxic oligomers and fibrils (Figure 4a,d).

As IAPP-GI represents a stabilized non-amyloidogenic IAPP conformation, our findings indicated that there might be at least one nonfibrillar IAPP conformation that might interfere with Aβ40 cytotoxic self-assembly. To test this hypothesis, we followed the kinetics of formation of cytotoxic oligomers and fibrils of freshly dissolved Aβ40, IAPP, and mixtures of both peptides (1:1). The ThT assay revealed that nucleation of fibrillization of Aβ40/IAPP mixtures was delayed by about 24 h as compared to Aβ40 and by about

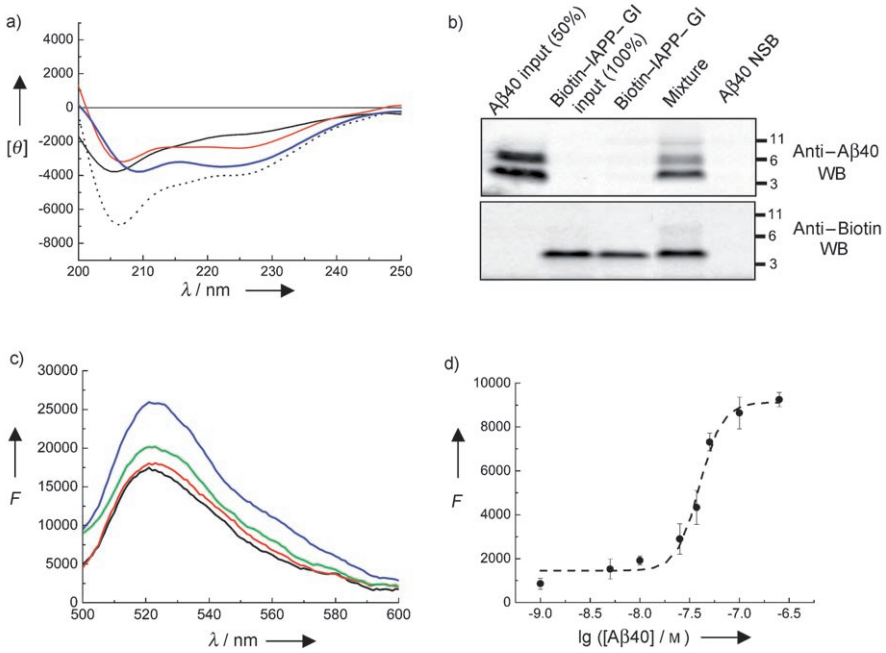

Figure 3. Binding of IAPP-GI to Aβ40. a) Far-UV CD spectra (mean residual ellipticity: $[\theta]$/deg cm^2 dmol^{-1}) of Aβ40 (5 μM; black), IAPP-GI (5 μM; red), a mixture of Aβ40 and IAPP-GI (5 μM each; blue), and the sum of the spectra of Aβ40 and IAPP-GI (black dotted line). b) Biotin pull-down assay. Top: Anti-Aβ40 Western blot (WB) analysis of a mixture of Aβ40 (5 μM) and Biotin-IAPP-GI (2.5 μM) versus Aβ40 alone (5 μM) following biotin pull-down and peptide dissociation. "Aβ40 input (50%)": 50% input, freshly dissolved peptide (4 μg) not incubated with beads; "Biotin-IAPP-GI input (100%)": 100% input (4 μg); "Biotin-IAPP-GI": Biotin-IAPP-GI alone; "Mixture": 1:2 mixture of Biotin-IAPP-GI and Aβ40; "Aβ40 NSB": Aβ40 alone (nonspecific binding for lane "Mixture"). Bottom: Anti-biotin WB analysis of the same mixtures as in upper panel. c) Fluorescence emission spectra (F) of Fluos-IAPP-GI alone (1 nM; black) and after titration with Aβ40. Fluos-IAPP-GI/Aβ40 molar ratios were 1:1 (red), 1:25 (green), and 1:100 (blue). d) Binding curve (dashed line) of Fluos-IAPP-GI obtained upon titration with Aβ40 (see part (c); F: fluorescence at 522 nm with that of Fluos-IAPP-GI alone subtracted). Data are the average (± standard error) from three binding curves.

72 h as compared to IAPP alone (Figure 5a). Fibrillogenesis of mixtures, however, was accomplished after 7 days (Figure 5a). These results were confirmed by TEM, which showed that nonfibrillar round oligomeric assemblies, likely hetero-oligomers, were main species at early time points (see the Supporting Information and Figure S1 therein). Aliquots were added to PC-12 cells, and cell viabilities were assessed (Figure 5b). Conversion of nontoxic prefibrillar species into cytotoxic assemblies in the Aβ40/IAPP mixtures was found to be significantly attenuated relative to the solutions of Aβ40 and IAPP alone (Figure 5b). In contrast to the Aβ40/IAPP-GI mixtures, however, Aβ40/IAPP solutions became as toxic as aged (7 days) IAPP and Aβ40 alone. Thus, interaction of early prefibrillar and nontoxic IAPP and Aβ40 species attenuated, although it did not block, formation of cytotoxic oligomers and fibrils by both Aβ40 and IAPP. More detailed studies showed that only interactions between early prefibrillar IAPP and Aβ40 species could delay cytotoxic self-assembly of the two polypeptides (see the Supporting Information and Figure S2 therein). Direct evidence for Aβ40/IAPP heterocomplex formation was obtained by CD spectroscopy (data not shown), Aβ pull-down assays, and fluorescence spectroscopy (Figure 5c–e). The affinity of the Aβ40/IAPP interaction (app. K_d), as assessed by fluorescence titration of N^{α}-amino-terminal fluorescein-labeled IAPP (Fluos-IAPP; 1 nM) with Aβ40, was (48.5 ± 4.2) nM (Figure 5d,e).

Next, Aβ40/IAPP-GI and Aβ40/IAPP heterocomplexes were stabilized by cross-linking with glutaraldehyde and characterized by NuPAGE and Western blot analysis. Western blotting with anti-Aβ40 showed twice as many trimers in the mixtures than in the Aβ40 incubation alone and significant amounts of low-molecular-weight oligomers, including tetra-, penta-, and hexamers which were completely absent from the Aβ40 incubations (Figure 5f; see also Figure S3 in the Supporting Information). Western blotting with anti-IAPP also revealed increased amounts and downward shifts of the trimers to hexamers in the mixtures (Figure 5f). These results suggest that trimers to hexamers were the main hetero-oligomeric species in the mixtures.

Our results have the following biomedical implications: 1) A designed 37-residue polypeptide, IAPP-GI, is identified as a ligand for Aβ40 with low nanomolar affinity and an inhibitor of Aβ40 cytotoxic oligomerization and fibrillogenesis with nanomolar activity (Figure 6a). IAPP-GI is thus one of the most potent Aβ40 ligands and inhibitors of Aβ40 cytotoxic self-assembly reported so far.[19,20] 2) IAPP-GI is the only known peptide-derived compound which binds, with high affinity, both IAPP and Aβ40 and blocks and reverses cytotoxic self-assembly of both polypeptides. As the incidence

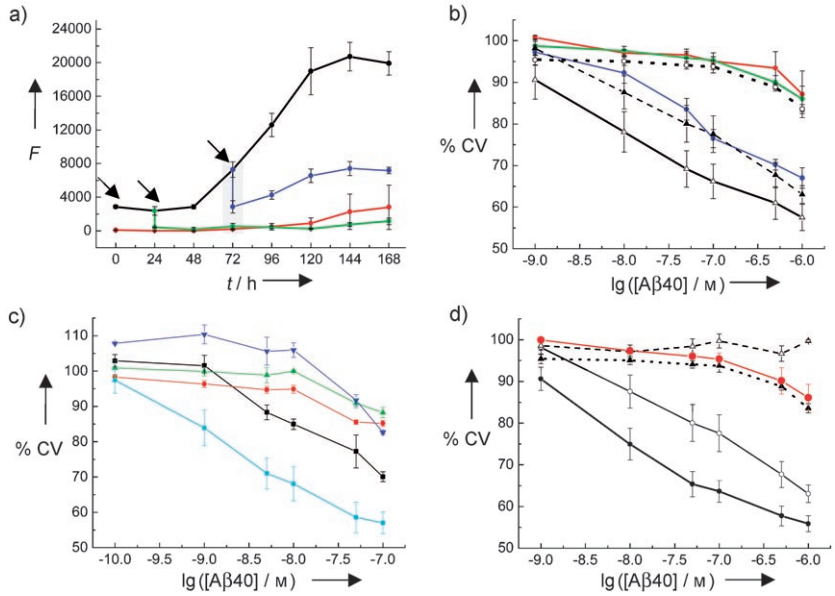

Figure 4. Inhibitory effects of IAPP-GI on different stages of Aβ40 cytotoxic oligomerization and fibrillogenesis. a) IAPP-GI completely blocks Aβ40 fibril formation both before and after the nucleation event. Aβ40 (16.5 μM) was aged for 7 days. At the indicated time points (arrows), aliquots were mixed with IAPP-GI (1:1) and fibrils were quantified by the ThT assay. Black line: Aβ40 alone; red line: IAPP-GI + non-aged Aβ40; green line: IAPP-GI + 24 h aged Aβ40; blue line: IAPP-GI + 72 h aged Aβ40. The gray bar indicates the time point of addition of solutions to cells for assessment of cytotoxicities (part (b)). Data are the average (± standard error) from three assays. b) IAPP-GI blocks formation of cytotoxic Aβ40 assemblies and redissociates already formed ones when added both before and after nucleation of Aβ40 fibrillogenesis. Solutions from part (a) were added to PC-12 cells at 72 h, and cell viabilities were assessed with the MTT assay. Black unfilled circles and dotted line: non-aged Aβ40; black filled triangles: 24 h aged Aβ40; black unfilled triangles: 72 h aged Aβ40; red symbols and line: IAPP-GI + non-aged Aβ40; green symbols and line: IAPP-GI + 24 h aged Aβ40; blue symbols and line: IAPP-GI + 72 h aged Aβ40. Data are the average (± standard error) from three assays ($n = 3$ each). c) IAPP-GI is able to completely redissociate already formed cytotoxic Aβ40 assemblies. IAPP-GI was added (50-fold molar excess) to cytotoxic and fibrillar Aβ40 (1.65 μM) aged for 7 days. The mixture and aged Aβ40 alone were then incubated for an additional 7 days. At various time points, aliquots were added to PC-12 cells and cytotoxicities were assessed by the MTT assay. Pale blue line and symbols: 7 days aged Aβ40 alone after additional 0–7 days; black line and symbols: mixture at 0 h; red line and symbols: mixture at 1 day; green line and symbols: mixture at 3 days; blue line and symbols: mixture at 7 days. No changes in cytotoxicity of the already aged Aβ40 solution were observed after the additional incubation for 7 days. By contrast, near-complete disappearance of cytotoxic Aβ40 species was observed 24 h after the addition of IAPP-GI. Data are from three determinations. d) IAPP-GI blocks conversion of nontoxic prefibrillar Aβ40 species into cytotoxic species. Aβ40 alone (16.5 μM), IAPP-GI alone (16.5 μM), and mixtures of Aβ40 with IAPP-GI (16.5 μM each) were aged for 7 days, and fibrillogenesis was followed by the ThT assay (part (a)). Solutions were added at various time points to PC-12 cells, and cytotoxicities were assessed by MTT reduction. Black open triangles and dashed line: IAPP-GI alone; black closed triangles and dotted line: Aβ40 alone at 0 h; black open circles and solid line: Aβ40 alone at 24 h; black closed circles and solid line: Aβ40 alone at 72 h to 7 days.

of both AD and T2D strongly increases with age and as AD patients may also suffer from/be at the risk of T2D, and vice versa, IAPP-GI could become the first representative of a novel class of peptide-derived cross-amyloid disease therapeutics.[4,5] 3) Our finding that Aβ40 binds IAPP with low nanomolar affinity and that Aβ40/IAPP heteroassociation attenuates cytotoxic self-assembly of both peptides offers a novel potential molecular link between AD and T2D. Our results support the hypothesis that in vivo Aβ40 and IAPP, two intrinsically unfolded polypeptides with extremely high self-association propensities, might mutually "protect" themselves from cytotoxic misfolding and self-association through heteroassociation of early prefibrillar and yet nontoxic conformations (Figure 6b). Such a cross-amyloid disease "suppression mechanism" would be consistent with reports linking the onsets of AD and T2D to each other.[4,5] Because Aβ40 and IAPP are present in serum and cerebrospinal fluid in comparable subnanomolar concentrations, an in vivo cross-interaction would be possible.

In conclusion, our studies identify a previously unrecognized interaction between the two key amyloidogenic molecules of AD and T2D suggesting a potential molecular link between the two diseases and offer a highly potent lead compound and a molecular basis for designing novel therapeutic compounds and concepts targeting both as-yet incurable amyloid diseases.

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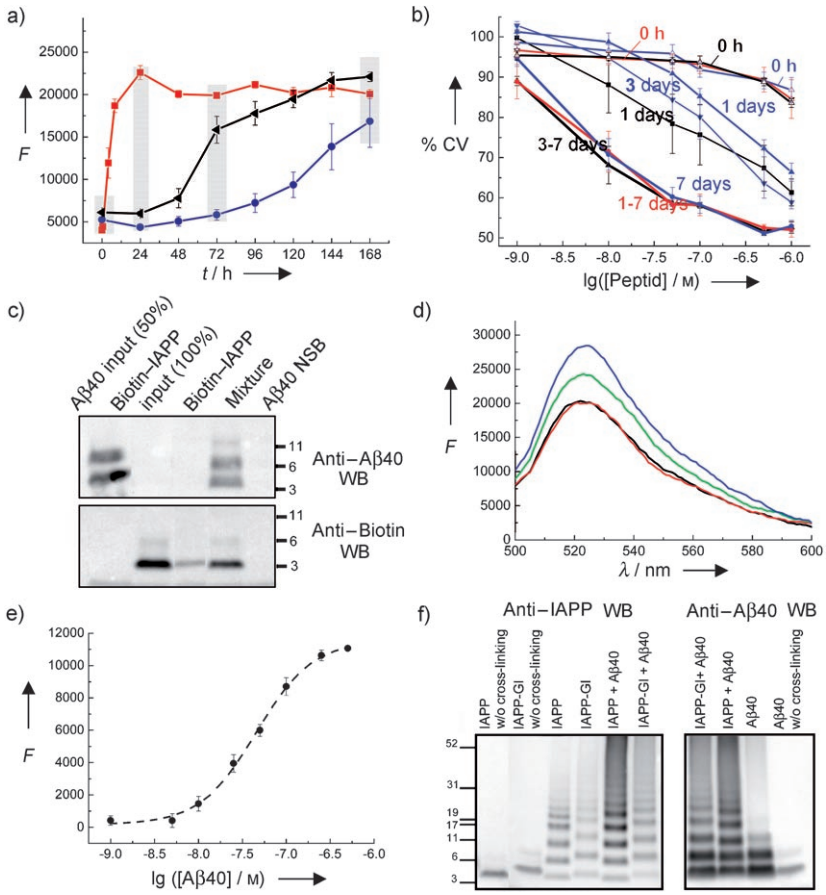

Figure 5. Attenuation of formation of cytotoxic Aβ40 and IAPP oligomers and fibrils by formation of high-affinity soluble Aβ40-IAPP heteroassemblies. a) IAPP delays Aβ40 fibrillogenesis, and IAPP fibrillogenesis is delayed by Aβ40. ThT binding assay of fibrillogenesis of Aβ40 alone (black; 16.5 μM), IAPP alone (red; 16.5 μM), and a mixture of Aβ40 with IAPP (blue; 16.5 μM each). Data are the average (±standard error) from eight assays. Gray bars indicate time points of addition of the solutions to PC-12 cells (part (b)). b) Interaction of Aβ40 with IAPP delays but cannot block formation of cytotoxic Aβ40 and IAPP species. Incubations of Aβ40 (black), IAPP (red), and mixtures of prefibrillar Aβ40 with IAPP (1:1; blue) were aged for 7 days, and fibrillogenesis was followed by ThT binding (part (a)). Solutions were added at the indicated times to PC-12 cells, and cytotoxicities were assessed by MTT reduction. Data are the average (±standard error) from three to four assays except for the 7-days-aged mixtures (two assays, $n=3$ each). c) Binding of Aβ40 to Biotin-IAPP as assessed by a biotin pull-down assay. Top: anti-Aβ40 Western blot analysis of a mixture of Aβ40 (5 μM) and Biotin-IAPP (2.5 μM) versus Aβ40 alone (5 μM) following biotin pull-down and peptide dissociation. "Aβ40 input": input (50%, freshly dissolved peptide (4 μg) not incubated with beads); "Biotin-IAPP input": input (100%, 4 μg); "Biotin-IAPP": Biotin-IAPP alone; "Mixture": 1:2 mixture of Biotin-IAPP and Aβ40; "Aβ40 NSB": Aβ40 alone (NSB for lane "Mixture"). Bottom: Anti-biotin Western blot analysis of the same mixture as in upper panel. Blots shown are representative of three experiments. d) Binding of Aβ40 to Fluos-IAPP as assessed by fluorescence spectroscopy: Spectra of Fluos-IAPP (1 nM) alone (black line) and after titration with various amounts of Aβ40 (Fluos-IAPP/Aβ40 molar ratios were 1:1 (red), 1:25 (green), and 1:100 (blue). e) Binding curve (dashed lines) of Fluos-IAPP (1 nM) obtained upon titration with Aβ40 (F: fluorescence at 522 nm; see part d). Data are the average (±standard error) from three binding curves. f) Characterization of heteroassemblies of Aβ40 with IAPP-GI or IAPP by cross-linking with glutaraldehyde, NuPAGE, and Western blot analysis. Left panel: Western blot with anti-IAPP antibody. Lanes (from left to right): IAPP and IAPP-GI input (3 μg, 100%) without cross-linking and without incubation; IAPP alone and IAPP-GI alone, incubated and cross-linked (controls for mixtures); mixtures (1:1) of Aβ40 with IAPP and IAPP-GI, incubated and cross-linked. Right panel: Western blot analysis with anti-Aβ40 antibody. Lanes (from left to right): mixtures (1:1) of Aβ40 with IAPP-GI and IAPP incubated and cross-linked; Aβ40 alone, incubated and cross-linked (control for mixtures); Aβ40 input (3 μg, 100%), without cross-linking and without incubation. Equal amounts of IAPP, IAPP-GI, and Aβ40 were loaded in all lanes. Blots are representative of four assays.

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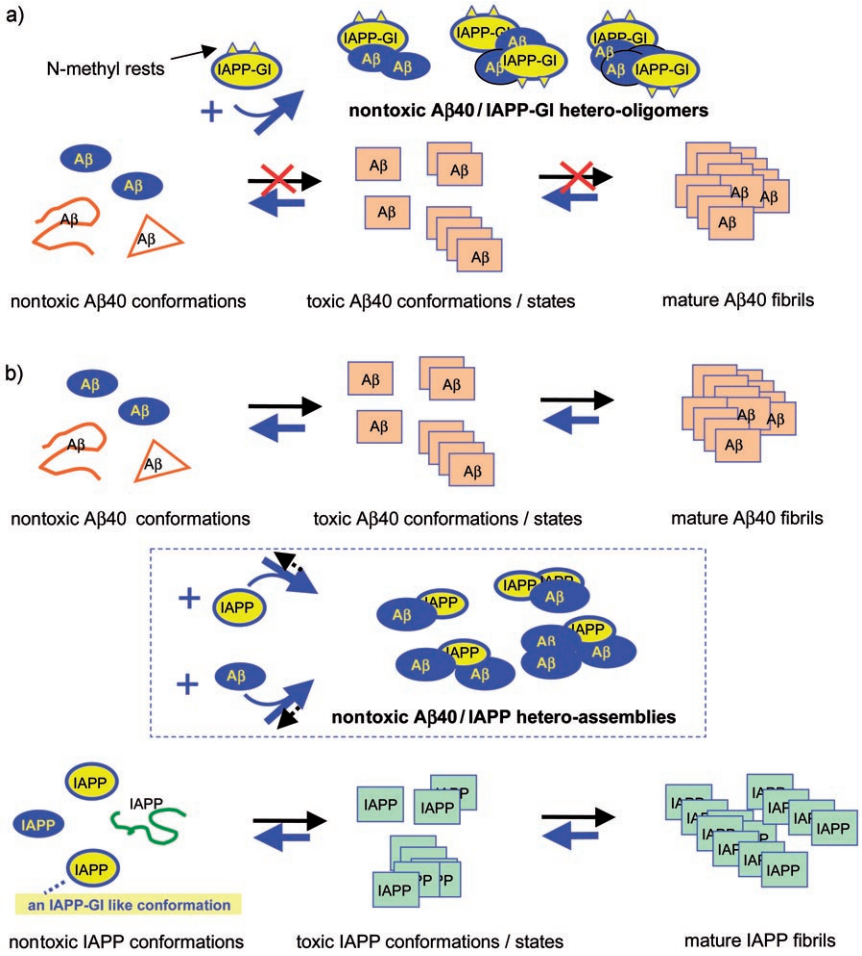

Figure 6. Proposed molecular models of interaction of a) IAPP-GI with early prefibrillar Aβ40 species resulting in inhibition and reversal of Aβ40 cytotoxic oligomerization and fibril formation (blue arrows), and b) early prefibrillar IAPP and Aβ40 species resulting in attenuation (blue arrows) of cytotoxic self-assembly and fibrillogenesis of both Aβ40 and IAPP. Sequesteration of early prefibrillar and nontoxic precursors of cytotoxic Aβ40 assemblies in the form of soluble and nontoxic hetero-oligomers is suggested to occur through their high-affinity binding to IAPP-GI (a) or to an early prefibrillar and nontoxic IAPP conformer, that is, an IAPP-GI-like conformer, in a competitive manner to Aβ40 or IAPP self-association (b). Owing to the high conformational flexibility and the strong β-sheet and self-association propensity of both IAPP and Aβ40, however, competitive self-association, seeding, and cross-seeding events are expected to interfere with Aβ40/IAPP heterocomplex formation and stability shifting thus heteroassociation equilibria towards self-association (b). In contrast, Aβ40/IAPP-GI hetero-oligomers will not propagate β-sheet structure and cytotoxic self-association and fibrillogenesis of Aβ40 as a result of the N-methylations in the amyloid core of IAPP-GI, consistent with the high potency of the inhibitory effect of IAPP-GI (a).

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