Inhibition of Protein Aggregation

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Exploiting Cross-Amyloid Interactions To Inhibit Protein Aggregation but not Function: Nanomolar Affinity Inhibition of Insulin Aggregation by an IAPP Mimic**

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In vivo protein aggregation is linked to the pathogenesis of a number of incurable cell- and neurodegenerative diseases.[1,2] As self-association into cell-toxic aggregates appears to be a common property of polypeptide chains, natural inhibitory mechanisms might exist.[2,3] Such mechanisms may include interactions with other proteins or even cross-interactions between the amyloidogenic polypeptide sequences per se.[4–8] In vitro protein aggregation precludes or complicates storage and therapeutic application of several bioactive polypeptides.[9,10] A prominent representative of such biomolecules is insulin; the 51-residue polypeptide is applied in the treatment of diabetes and is one of the most commonly used biopharmaceuticals.[11,12] Insulin is prone to aggregation, in particular when it is in a partially unfolded state.[12–15] Non-native insulin aggregation does not occur in vivo. Insulin aggregation in vitro, however, leads to nonfunctional aggregates, thus complicating its therapeutic application.[11]

A potential complication in the development of potent inhibitors of aggregation of a bioactive or therapeutically applied protein is that these compounds should not affect protein function. We reasoned that a good candidate might be a compound that mimics a natively occuring protein-aggregation inhibitor as such a molecule should not intervene with protein function. A natively occuring insulin-binding polypeptide is islet amyloid polypeptide (IAPP).[5,16,17] This 37-residue glucose regulatory polypeptide shares a remarkable sequence similarity with insulin and is synthesized, stored, and secreted from the pancreatic β-cells together with insulin (Figure 1).[17–19] However, IAPP is extremely amyloidogenic and aggregates into pancreatic amyloid in type 2 diabetes (T2D).[17,18] IAPP has been shown to interact with insulin, and this interaction strongly delays IAPP fibrillization in vitro.[5] Insulin has been thus proposed to be a native IAPP aggregation inhibitor.[5,6,20,21] However, the question whether IAPP—in a non-aggregated and nontoxic state—might be a native inhibitor of insulin aggregation has not been addressed yet, most likely because IAPP is not accessible in a stable nontoxic state.

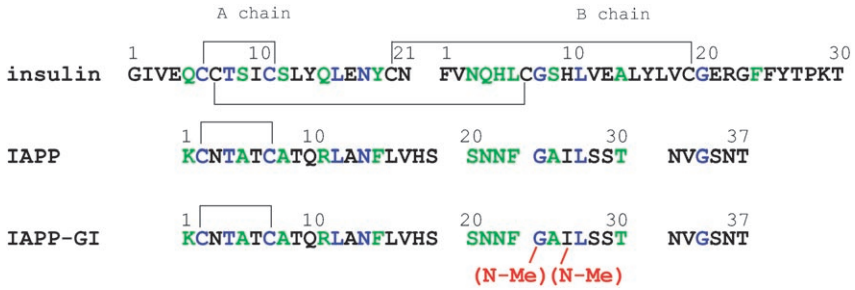

Figure 1. Primary structures of insulin, IAPP, and the IAPP mimic IAPP-GI. Residues common to all three sequences are in blue, and and similar residues are in green.[23]

We have recently designed a conformationally constrained IAPP analogue as a mimic of a non-amyloidogenic IAPP conformation.[22] This peptide, [(N-Me)G24, (N-Me)I26]-IAPP (IAPP-GI; Figure 1), has proved to be a highly soluble, non-amyloidogenic, and nontoxic IAPP analogue which bound IAPP with nanomolar affinity and blocked its cytotoxic self-assembly (Figure 1).[22] Here, we show that IAPP-GI is also a nanomolar-affinity inhibitor of insulin aggregation and that it does so without affecting insulin function. Our studies also suggest that a high-affinity interaction between nonfibrillar and nontoxic insulin and IAPP species attenuates cytotoxic self-assembly by both polypeptides.

We first addressed the question whether IAPP-GI could interfere with insulin fibrillogenesis. Insulin was incubated

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alone or with IAPP-GI (1:5) under previously established in vitro fibrillogenesis-inducing conditions (pH 2, 60 °C; see the Supporting Information), and fibrillogenesis was followed by thioflavin T (ThT) binding and transmission electron microscopy (TEM).[12–15,24,25] Insulin fibrillogenesis had a lag time of roughly five days and was accomplished after an additional four to five days (Figure 2a,b). However, in the presence of IAPP-GI, fibrillization was strongly suppressed (Figure 2a, Figure 2b). Maximum inhibition was obtained when a five- to tenfold molar excess of IAPP-GI was used, while at an 1:1 ratio a weaker inhibitory effect was observed (see Figure S1 and the Supporting Information).

Because non-native self-assembly of insulin also leads to cytotoxic species, we next investigated whether IAPP-GI might also intervene with this process.[26] Insulin or insulin/IAPP-GI mixtures (1:10) were incubated (24 h, pH 2, 60 °C) and added to cultured rat insulinoma RIN5fm cells, and cell viabilities were determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Figure 2c,d). We found that IAPP-GI completely blocked formation of cytotoxic species over a 100-fold range of insulin concentrations (Figure 2c). Maximum inhibition was obtained by a tenfold molar excess of IAPP-GI (Figure 2d). Importantly, an IC_{50} of 205 nM was determined (Figure 2d), and the validity of this result was confirmed by two additional assays (see Figure S2 and the Supporting Information). Thus, IAPP-GI is a nanomolar-activity inhibitor of cytotoxic self-assembly of insulin.

To characterize the insulin/IAPP-GI interaction, far-UV CD and fluorescence spectroscopy were applied. The CD spectrum of insulin (pH 2) indicated significant amounts of α-helical structure (Figure 3a).[14,25] The spectrum of the mixture (1:5) indicated the presence of ordered heterocomplexes with less helical content than insulin (Figure 3a). N^{α}-amino-terminal fluorescein-labeled IAPP-GI (Fluos-IAPP-GI) was then titrated with insulin, and fluorescence emission spectra were recorded.[22,27] A 100-fold molar excess of insulin caused a fluorescence enhancement of 61 % and an apparent affinity of interaction ($K_{d,app.}$) of (100 ± 9) nM was obtained which was in very good agreement with the determined IC_{50} value (Figure 3b, inset). As nano- to low micromolar insulin solutions contain mainly monomers and dimers, this data suggested that IAPP-GI binds insulin monomers and/or dimers with high affinity.[14,15]

We next addressed the question whether IAPP-GI could also intervene with formation of nonfibrillar insulin oligo- and multimers.[14,15] Insulin aggregation occurs by dissociation and partial unfolding steps.[14,15] At pH 2 and room temperature

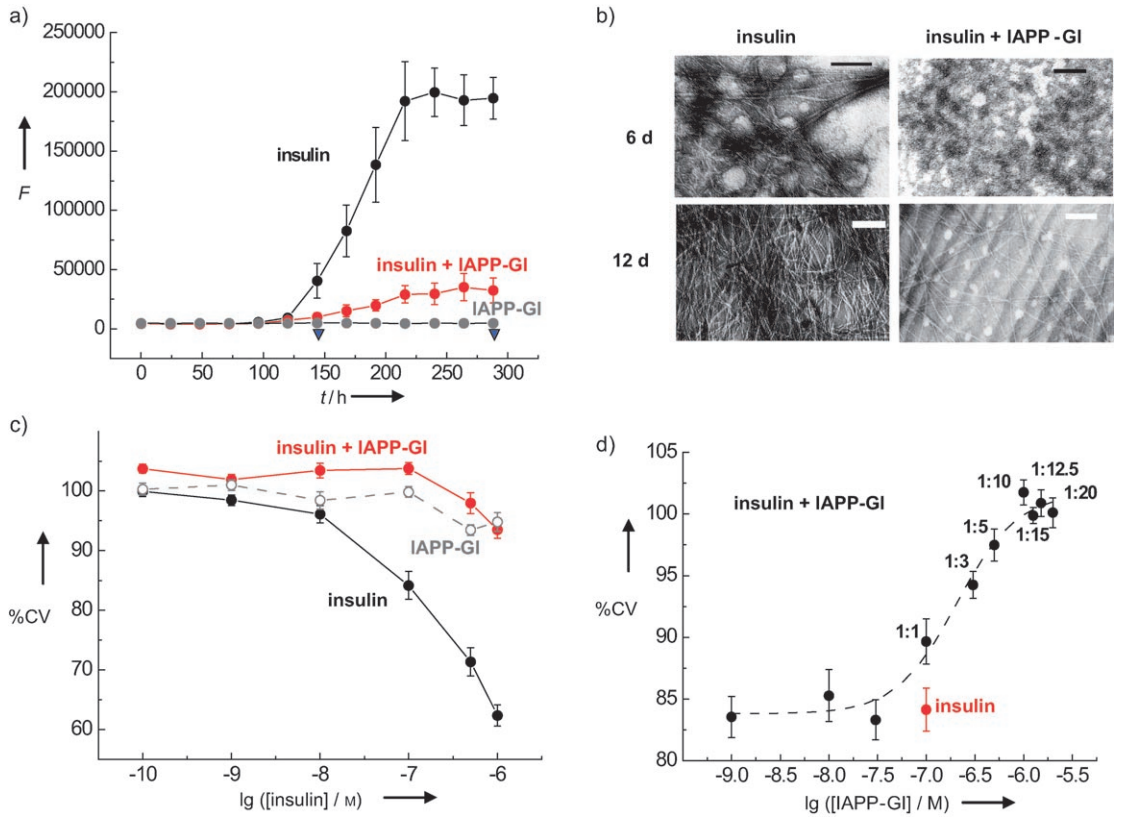

Figure 2. Inhibition of insulin fibrillogenesis and cytotoxic self-assembly by IAPP-GI. a) Fibrillogenesis of insulin, of an insulin/IAPP-GI mixture, and of IAPP-GI alone as assessed by the ThT assay (*F*: fluorescence). Data are means (± SEM) from three assays (SEM = standard error of the middle value). Arrowheads indicate time points of TEM analysis (Figure 2b). b) TEM image of insulin alone and of an insulin/IAPP-GI mixture (incubations of Figure 2a) at the indicated time points (scale bars: 100 nm). c) Effects of 24 h aged insulin, an insulin/IAPP-GI mixture, and IAPP-GI alone on RIN5fm cell viability (%CV: cell viability (% of control)) as assessed by the MTT reduction assay. Data are means (± SEM) from three assays ($n = 3$ each). d) Determination of the IC_{50} value of IAPP-GI on formation of cytotoxic insulin assemblies by the MTT assay. Data are means (± SEM) from three assays ($n = 3$ each).

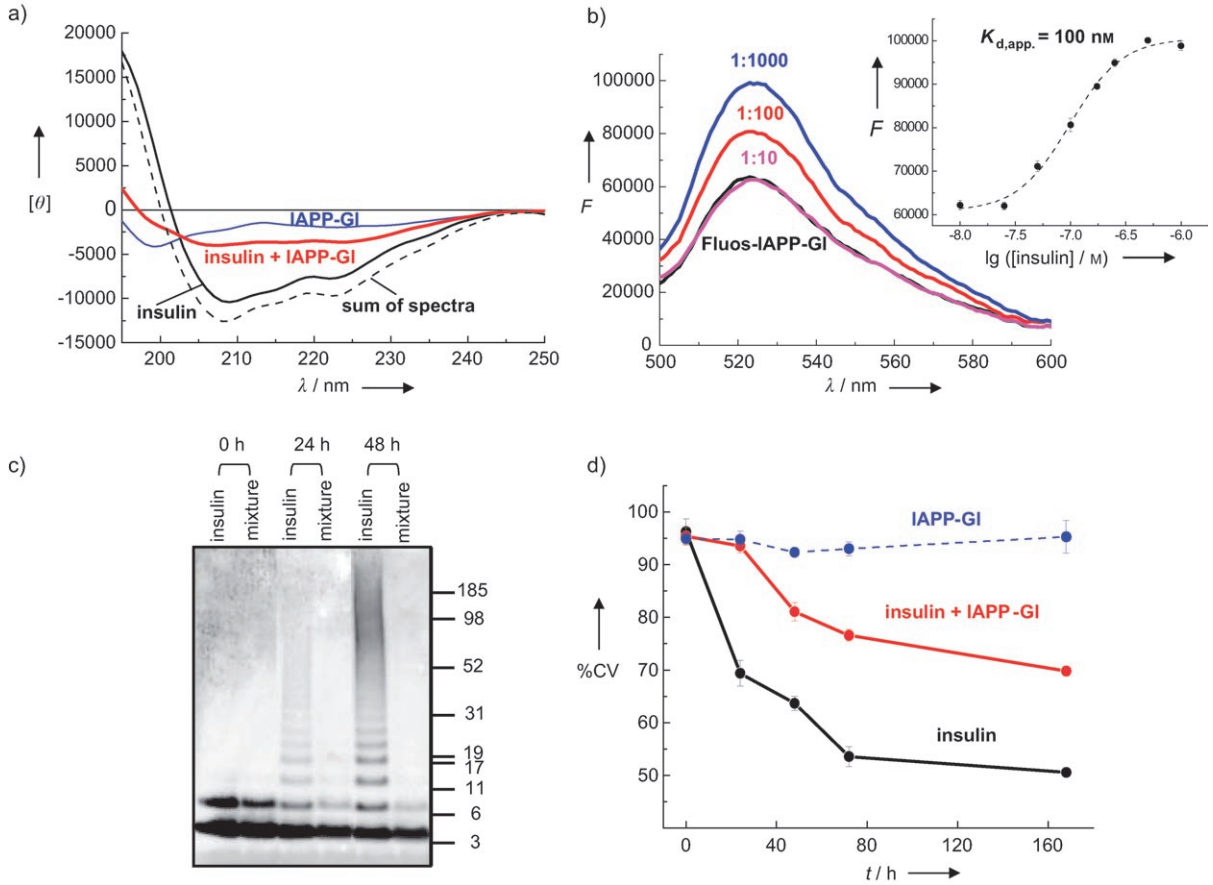

Figure 3. Characterization of the insulin/IAPP-GI interaction and its effects on insulin oligomerization and formation of cytotoxic species. a) CD spectra (mean residue ellipticity [θ], deg cm^2 dmol^{-1}) of insulin, the insulin/IAPP-GI mixture, and IAPP-GI. The sum of the spectra of insulin and IAPP-GI is also shown. b) Fluorescence emission spectra of Fluos-IAPP-GI alone and following titration with insulin (at the indicated Fluos-IAPP-GI/insulin molar ratios). In the inset, the binding curve is presented. Data are means (±SEM) from three binding curves. c) Kinetics of insulin oligomerization in the absence or presence of IAPP-GI by NuPAGE and Western blot with anti-insulin antibody. Blots are representative of three assays. d) Kinetics of formation of cytotoxic insulin species (1 μM) in the presence or absence of IAPP-GI as assessed by MTT reduction assays. Insulin, insulin/IAPP-GI, and IAPP-GI were incubated and added to RIN5fm cells at the indicated incubation time points. Data are means (±SEM) from three assays (n = 3 each).

major species are monomers and dimers of natively folded insulin generated by dissociation of natively folded hexamers. At higher temperatures, dimer dissociation and partial unfolding occur and result in misfolded monomers, oligo- and multimers, and fibrils. A misfolded monomer is believed to be the required precursor species of non-native insulin aggregation.[14,15] We followed the kinetics of insulin oligomerization by non-reducing NuPAGE and Western blot. Insulin or mixtures of insulin with IAPP-GI (1:5) were incubated (pH 2, 60 °C) and analyzed at various time points (Figure 3c). The insulin solution at 0 h consisted mainly of monomers and few dimers, whereas significant amounts of oligo- and multimers appeared at 24 and 48 h. In the insulin/IAPP-GI mixture, however, the main species were mono- and dimers. Thus, IAPP-GI strongly suppressed insulin oligo- and multimerization.

Next, the evolution of cell-damaging species in initially nontoxic insulin solutions in the absence or presence of IAPP-GI (1:10) was studied (pH 2, 60 °C; Figure 3d and Figure S3a in the Supporting Information).[26] The results suggested that IAPP-GI binds early nonfibrillar and nontoxic insulin species and attenuates their conversion into cytotoxic ones. The specificity of this interaction was confirmed by studies with parathormone (see Figure S4 in the Supporting Information).

Because IAPP-GI represents a non-amyloidogenic IAPP conformer, our findings indicated that there might be at least one nonfibrillar and nontoxic IAPP conformer which might intervene with insulin self-assembly.[22] In fact, our studies showed that nonfibrillar IAPP binds insulin with a $K_{d,app.}$ value of (142 ± 34) nM and that it has a clear but significantly weaker inhibitory effect on insulin self-assembly than IAPP-GI (see Figure S5 and the Supporting Information). Most importantly, these studies suggested that a conformationally specific interaction of early prefibrillar and nontoxic IAPP and insulin species—most likely monomer and dimers—attenuates self-assembly and fibrillization of both insulin and IAPP (see Figures S6 and S7 and the Supporting Information).

Finally, we addressed the crucial question whether the interaction between IAPP-GI (or IAPP) and insulin might

intervene with insulin function. Insulin bioactivity is mediated through the binding of a monomeric insulin conformer to the insulin receptor (IR).[28,29] Physiological signaling occurs then through a cascade of protein interactions and phosphorylation processes.[28–30] The earliest phosphorylation step following the binding of insulin to the IR is autophosphorylation of the IR β subunit (IRβ).[29,30] We therefore investigated whether insulin-induced IR autophosphorylation was affected by the presence of IAPP-GI (or IAPP) using cultured human breast cancer cells.[31,32] The cells were stimulated with insulin, or mixtures of insulin with IAPP-GI or IAPP (1:10). The same amounts of phosphorylated IRβ were found in insulin-stimulated cells and in cells stimulated by mixtures of insulin with IAPP-GI or IAPP (Figure 4 and other data not shown). These findings were confirmed by studying the activation of Akt (see Figure S8 and the Supporting Information).[30] Thus, the interaction of IAPP-GI (or IAPP) with insulin did not affect insulin function.

In summary, we have successfully tested a novel chemical strategy to inhibit non-native aggregation of bioactive or therapeutic polypeptides without affecting their function by mimicking aggregation suppressing native cross-amyloid peptide interactions. We have exemplified the validity of this approach by demonstrating that IAPP-GI, a designed peptide mimic of a non-amyloidogenic conformation of IAPP, is a nanomolar-affinity inhibitor of non-native aggregation of insulin without affecting its function (Figure 5 a). Our studies also provide evidence for a novel function of the insulin–IAPP interaction which has been so far known only as an

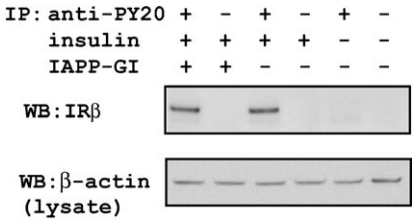

Figure 4. The insulin/IAPP-GI interaction does not affect physiological signaling of insulin as assessed by studying IR activation and signal transduction in human breast cancer cells. Cells were treated with insulin alone, with mixtures of insulin with IAPP-GI, or with buffer alone. Phosphorylated proteins were immunoprecipitated (IP) with antiphosphotyrosine antibody (anti-PY20) and IRβ was revealed by WB with anti-insulin receptor β-subunit antibody (anti-IRβ). Equal amounts of cellular proteins were used (see β-actin WBs). Blots are representative of three experiments.

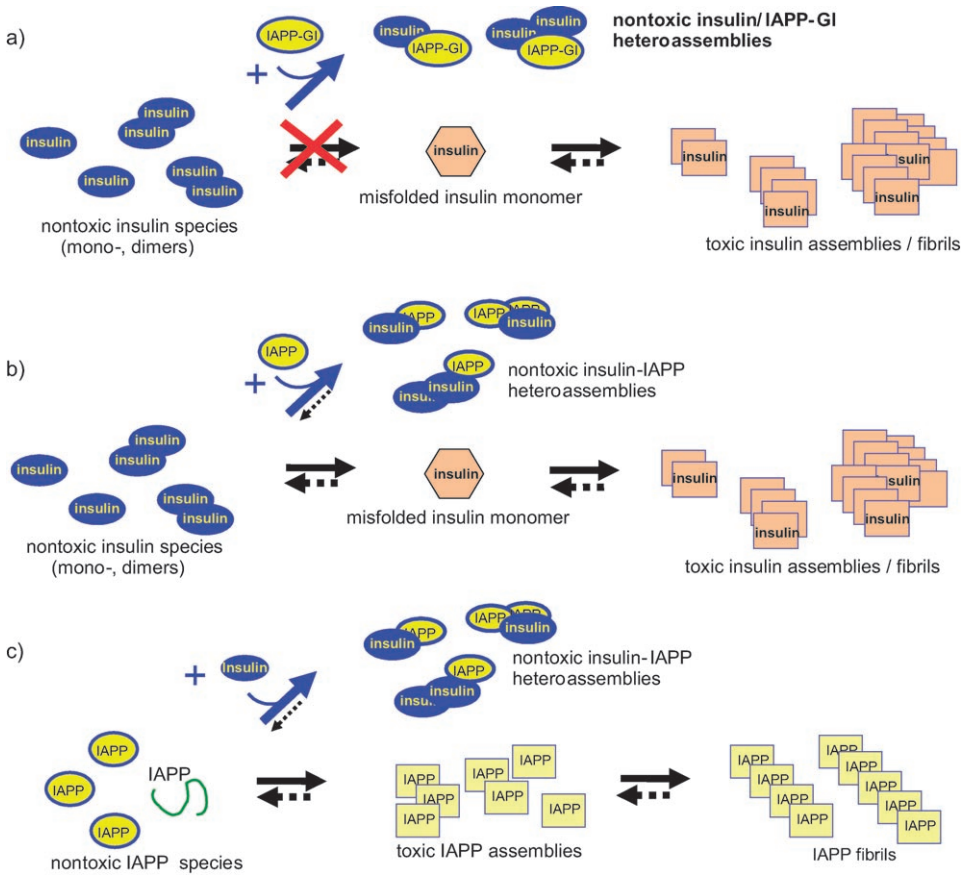

Figure 5. Proposed mechanism of interaction of insulin with IAPP-GI and a nontoxic IAPP conformer. a) IAPP-GI is proposed to bind with high affinity early, nonfibrillar, and nontoxic insulin species—likely natively folded monomers and/or dimers—and thus to block insulin misfolding and self-assembly. b) A nonfibrillar and nontoxic IAPP conformer—likely a monomer—is proposed to bind with high affinity early, nonfibrillar, and nontoxic insulin species—likely natively folded monomers and/or dimers—and thus to attenuate insulin misfolding and aggregation. c) Interaction of early nonfibrillar and nontoxic insulin and IAPP species—likely monomers and/or dimers—is suggested to attenuate cytotoxic self-assembly and fibrillogenesis of IAPP.

IAPP fibrillogenesis inhibitory interaction (Figure 5b,c).[5,6] The proposed high-affinity self-association-suppressing insulin–IAPP heteroassociation is reminiscent of the interaction between the β-amyloid peptide (Aβ) of Alzheimer's disease (AD) and IAPP.[7] Our findings support the view that cross-amyloid interactions might "protect" all three amyloidogenic polypeptides—and possibly other amyloid polypeptides as well—from pathogenic self-association in vivo.[7] Such "functional" cross-amyloid interactions, if existent in vivo, would be molecular links between AD and T2D.[7,33]

In addition to its function as an insulin-aggregation inhibitor, IAPP-GI is also a nanomolar inhibitor of IAPP self-assembly and a soluble and nontoxic IAPP analogue and receptor agonist.[22] Of note, the use of soluble IAPP analogues and receptor agonists as insulin adjuncts in treatment of T2D has been recently established.[34,35] IAPP-GI or similar compounds would be thus expected to combine good solubility and IAPP-like favorable effects on glycemic control with potent inhibitory effects on both in vivo IAPP amyloidogenesis in T2D and in vitro insulin aggregation without affecting insulin function.[11,22,34] The potential use of such multifunctional compounds in diabetes treatment will have to be addressed by future in-depth studies.

Furthermore, IAPP-GI has been found to block the cytotoxic self-association of Aβ40 with nanomolar activity; this makes it a promising candidate for the development of an AD therapeutic compound.[7] In fact, as IAPP is able to enter the blood–brain barrier, IAPP-GI would be also expected to cross it, allowing thus for intervention with Aβ40 aggregation both in and outside the central nervous system.[36,37]

IAPP-GI is so far the only known peptide that binds with nanomolar affinity and inhibits cytotoxic self-assembly and fibrillogenesis of three different amyloidogenic polypeptides. Importantly, Aβ40, IAPP, and insulin are key amyloidogenic polypeptides of AD and T2D, and common molecular and pathological processes appear to underly the two diseases.[33,38,39] As the incidence of both diseases increases with age and T2D patients have an increased risk of AD and vice versa, our results support the suggestion that IAPP-GI could become a unique lead compound for development of novel drugs and therapeutic concepts targeting both AD and T2D.[33,38,39] Moreover, our approach to generate inhibitors of non-native protein aggregation that do not affect protein function may be applicable to other aggregation-prone polypeptides and proteins.

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