Amyloid Inhibitors

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A Hot-Segment-Based Approach for the Design of Cross-Amyloid Interaction Surface Mimics as Inhibitors of Amyloid Self-Assembly

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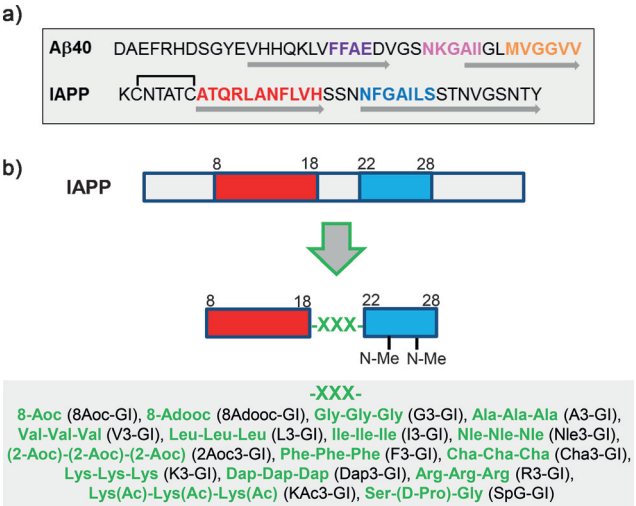
Abstract: *The design of inhibitors of protein–protein interactions mediating amyloid self-assembly is a major challenge mainly due to the dynamic nature of the involved structures and interfaces. Interactions of amyloidogenic polypeptides with other proteins are important modulators of self-assembly. Here we present a hot-segment-linking approach to design a series of mimics of the IAPP cross-amyloid interaction surface with Aβ (ISMs) as nanomolar inhibitors of amyloidogenesis and cytotoxicity of Aβ, IAPP, or both polypeptides. The nature of the linker determines ISM structure and inhibitory function including both potency and target selectivity. Importantly, ISMs effectively suppress both self- and cross-seeded IAPP self-assembly. Our results provide a novel class of highly potent peptide leads for targeting protein aggregation in Alzheimer's disease, type 2 diabetes, or both diseases and a chemical approach to inhibit amyloid self-assembly and pathogenic interactions of other proteins as well.*

The self-assembly of proteins to form amyloid fibrils is linked to numerous devastating cell-degenerative diseases.[1,2] However, the design of inhibitors of this process is a very challenging task mainly due the intrinsically disordered nature of many amyloidogenic polypeptides.[1,3] For instance, amyloidogenesis of β-amyloid peptide (Aβ) and islet amyloid polypeptide (IAPP) in Alzheimer's disease (AD) and type 2 diabetes (T2D), respectively, proceeds via conversion of dynamic nonfibrillar ensembles into transiently populated, poorly characterized, β-sheet-rich cytotoxic assemblies.[1,3–5] So far, none of the reported inhibitors of Aβ or IAPP amyloidogenesis has reached the clinic.[1,3] Moreover, increasing evidence suggests that AD and T2D might be linked to each other.[6–10] Therefore, compounds blocking self-assembly of both Aβ and IAPP might become promising candidates for therapeutics.

Interactions of aggregation-prone polypeptides with other proteins are important modulators of self-assembly.[1,2,7,8,10,14,15] We have shown that non-fibrillar Aβ interacts with high affinity with non-fibrillar IAPP species in vitro resulting in the suppression of the self-assembly of both polypeptides.[16,17] The physiological role of this interaction—if existent in vivo—remains to be elucidated.[7–10,18] Nevertheless, recent findings suggest that it can be used for the design of amyloid inhibitors.[17,19,20] In fact, IAPP-GI and related full-length IAPP analogues block amyloid self-assembly of both Aβ40 and IAPP.[17,19]

The IAPP segments IAPP(8-18) and IAPP(22-28) are "hot segments" in both IAPP self- and its cross-interaction



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8-Aoc (8Aoc-GI), 8-Adooc (8Adooc-GI), Gly-Gly-Gly (G3-GI), Ala-Ala-Ala (A3-GI), Val-Val-Val (V3-GI), Leu-Leu-Leu (L3-GI), Ile-Ile-Ile (I3-GI), Nle-Nle-Nle (Nle3-GI), (2-Aoc)-(2-Aoc)-(2-Aoc) (2Aoc3-GI), Phe-Phe-Phe (F3-GI), Cha-Cha-Cha (Cha3-GI), Lys-Lys-Lys (K3-GI), Dap-Dap-Dap (Dap3-GI), Arg-Arg-Arg (R3-GI), Lys(Ac)-Lys(Ac)-Lys(Ac) (KAc3-GI), Ser-(D-Pro)-Gly (SpG-GI)

Scheme 1. Design concept, linkers, and ISMs. a) Sequences of Aβ40 and IAPP; hot segments of the Aβ40/IAPP interaction interface are colored.[11] Gray arrows: segments involved in amyloid β-sheet formation.[12,13] b) Design concept and linkers (green) (abbreviations: 2- or 8-Aoc: 2-or 8-aminooctanoic acid; 8-Adooc: 8-amino-3,6-dioxaoctanoic acid; Cha: cyclohexylalanine; Dap: 2,3 diaminopropionic acid; Lys(Ac): lysine with acetylated N^{ε}-amino group). Short names of ISMs (C-terminal amides) are in parentheses.

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interface with Aβ (Scheme 1a).[5,11,13] IAPP(8-28) forms thus major parts of the surfaces involved in the two processes. These two surfaces, an amyloid-promoting and an amyloid-suppressing one, might share similarities.[11,15] However, neither IAPP(8-28), IAPP(8-28)-GI, nor each of the hot segments alone inhibit Aβ40 amyloidogenesis.[21] This finding suggests that specific, yet unknown structural/topological features of the hot segments are required for inhibitory function.[21]

Here we present a hot-segment-linking approach to design a series of IAPP cross-amyloid interaction surface mimics (ISMs) as highly potent inhibitors of amyloid self-assembly of Aβ, IAPP, or both polypeptides (Scheme 1b). We hypothesized that linking the hot segments IAPP(8-18) and IAPP(22-28) to each other through different structurally biased linkers should result in ISMs exhibiting different structures and, thus, inhibitory effects. IAPP(22-28) was used in its N-methylated form, IAPP(22-28)-GI, to block inherent amyloidogenicity.[22] Based on the polymorphism of amyloid surfaces, we envisioned that some of the linkers would stabilize alternative, amyloid-like β-sheet folds relative to the non-inhibitory fold of IAPP(8-28).[1,13] Some of these structures should exhibit favorable preorganization of surface residues and maximized hydrophobic interactions and H-bond-forming ability across the interface resulting in inhibitory function.[1,14] Additionally, their strong self-assembly propensities and expected lack of amyloidogenicity should contribute to sequestering Aβ or IAPP into non-fibrillar/non-toxic hetero-assemblies.[16,17,19]

To systematically evaluate the concept, we designed and studied 16 ISMs (Scheme 1b, Table S1 in the Supporting Information). Linkers were tripeptide segments, except for the first two ISMs, and consisted of identical amino acids (Scheme 1b). According to the concept, some of the linkers containing hydrophobic β-sheet-structure-propagating residues should yield inhibitory ISMs, while linkers containing small or polar residues, as in IAPP(19-21) and in β-turns/β-arcs, should rather result in non-inhibitory ISMs.[23,24]

First, we tested whether a specific structural preorganization of the hot segments is necessary for inhibitory function.[21] To this aim, 8Aoc-GI and 8Adooc-GI containing the flexible linkers 8-Aoc (hydrophobic) and 8-Adooc (hydrophilic) were synthesized (Scheme 1b). Neither ISMs affected the self-assembly of Aβ40 or IAPP according to the thioflavin T (ThT) binding and 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assays in rat pheochromocytoma (PC12) and rat insulinoma (RIN5fm) cells (Figure S1). Next, G3-GI, A3-GI, V3-GI, and L3-GI were synthesized (Scheme 1b, Figure 1). L3-GI and V3-GI strongly suppressed Aβ40 fibrillogenesis and cytotoxicity (1/1), whereas G3-GI, A3-GI, and IAPP(8-28)-GI did not inhibit (Figure 1a–c). The CD spectra of L3-GI and V3-GI suggested large amounts of β-sheet/β-turn structure (Figure 1d).[25] By contrast, the spectra of A3-GI, G3-GI, and IAPP(8-28)-GI suggested significant amounts of unor-

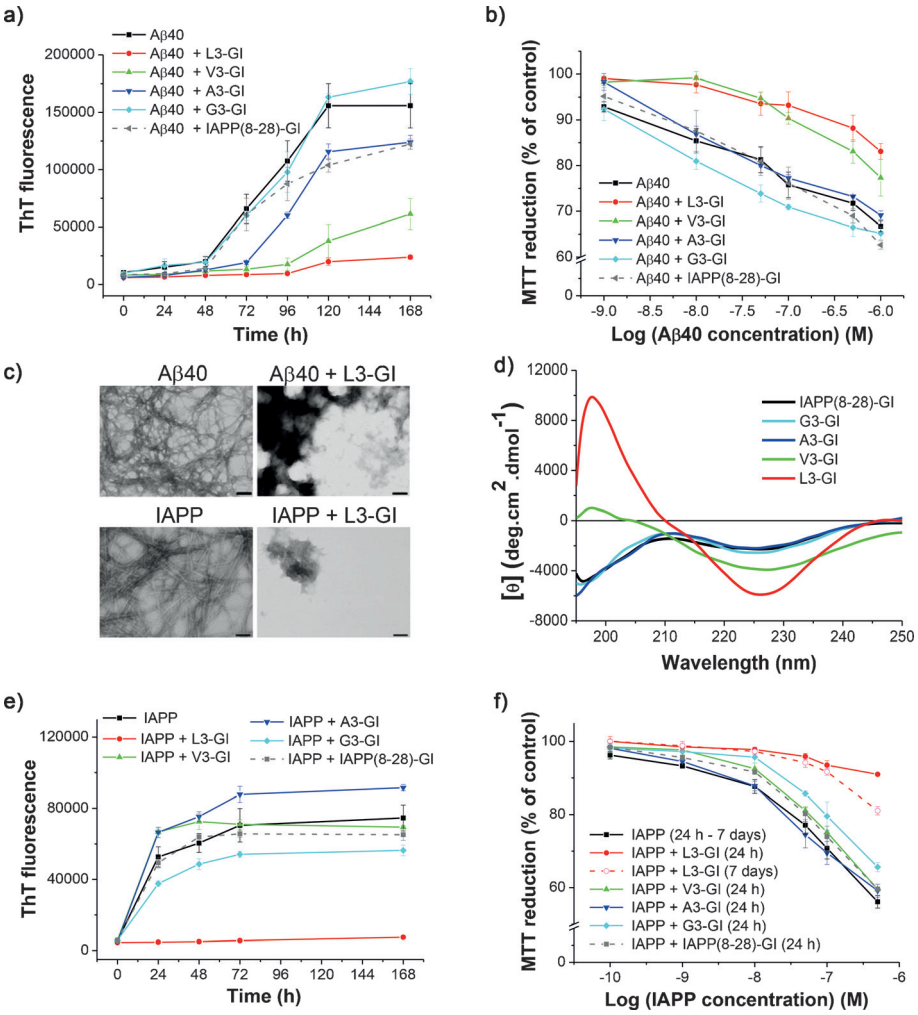

Figure 1. Effects of G3-GI, A3-GI, V3-GI, and L3-GI versus IAPP(8-28)-GI on Aβ40 or IAPP fibrillogenesis and cytotoxicity and conformations of ISMs. a) Fibrillogenesis of Aβ40 (16.5 μM) alone or with the peptides (1/1) determined by ThT binding (means (±SEM), three assays). b) Effects on Aβ40 cytotoxicity. Solutions (from a) aged 7 days were added to PC12 cells. Cell damage assessed by MTT reduction (means (±SEM), 3 assays ($n=3$ each)). c) TEM images of Aβ40 aged 7 days, its mixture with L3-GI (from 1a) and of IAPP aged 7 days and its mixture with L3-GI (from 1e) (bars 100 nm). d) CD spectra of ISMs and IAPP(8-28)-GI (5 μM, pH 7.4). e) Fibrillogenesis of IAPP (16.5 μM) alone or with peptides (1/2) assessed by ThT binding (means (±SEM), 3 assays). f) Effects on IAPP cytotoxicity. Solutions of 1e (aged 1 or 7 days) were added to RIN5fm cells. Cell damage assessed as in (b) (means (±SEM), 3 assays ($n=3$ each)).

dered structure as well (Figure 1d).[21] L3-GI (twofold) also blocked IAPP fibrillogenesis and cytotoxicity, whereas all other ISMs were ineffective (Figure 1c,e,f). These results provided a first proof-of-principle of the concept.

Next, the five hydrophobic-linker-containing I3-GI, Nle3-GI, 2Aoc3-GI, F3-GI, and Cha3-GI and the four polar-linker-containing K3-GI, Dap3-GI, R3-GI, and KAc3-GI were synthesized (Scheme 1b). In addition, SpG-GI containing the β-hairpin-stabilizing (D-Pro)Gly and expected to adopt a similar conformation as IAPP(8-28)-GI was synthesized (negative control).[26] I3-GI, Nle3-GI, F3-GI, Cha3-GI, and R3-GI blocked Aβ40 fibrillogenesis and cytotoxicity (1/1) and TEM revealed amorphous aggregates as major species (Figure 2a,b, Figure S2). By contrast, all other ISMs did not affect Aβ40 self-assembly (Figure S3). Notably, inhibitory ISMs suppressed Aβ40 self-assembly when added (1/1) both before and after nucleation (Figure S4). Most importantly, titrations of cytotoxic Aβ40 with inhibitory ISMs yielded nanomolar IC_{50} values (Table 1). Nle3-GI, Cha3-GI, and 2Aoc3-GI also blocked IAPP fibrillogenesis and cytotoxicity (2/1) and TEM revealed amorphous aggregates as main species (Figure 2c,d, Figure S2). By contrast, all other ISMs were ineffective (data not shown). Of note, ISMs suppressed IAPP fibrillogenesis and cytotoxicity when added both at pre- and at postnucleation stages (Figure S4). Most importantly, nanomolar IC_{50} values were obtained (Table 1). Of note, MTT assay results were confirmed by apoptosis assessment (Figure S5).[16,17]

The above results identified three ISMs (L3-GI, Nle3-GI, Cha3-GI) as inhibitors of self-assembly of both Aβ40 and IAPP, four ISMs (V3-GI, I3-GI, F3-GI, R3-GI) as Aβ40-selective inhibitors, and one ISM (2Aoc3-GI) as an IAPP-selective inhibitor. Of note, target-selectivities (Aβ40 versus IAPP) were maintained up to an at least 10-fold ISM excess (Table 1; data not shown).[16,17] Also, ISMs did not affect amyloidogenesis of lysozyme, an unrelated amyloidogenic protein (Figure S6). As expected based on the design concept, ISMs oligomerized in the low micro- to submicromolar concentration range but were non-amyloidogenic and non-

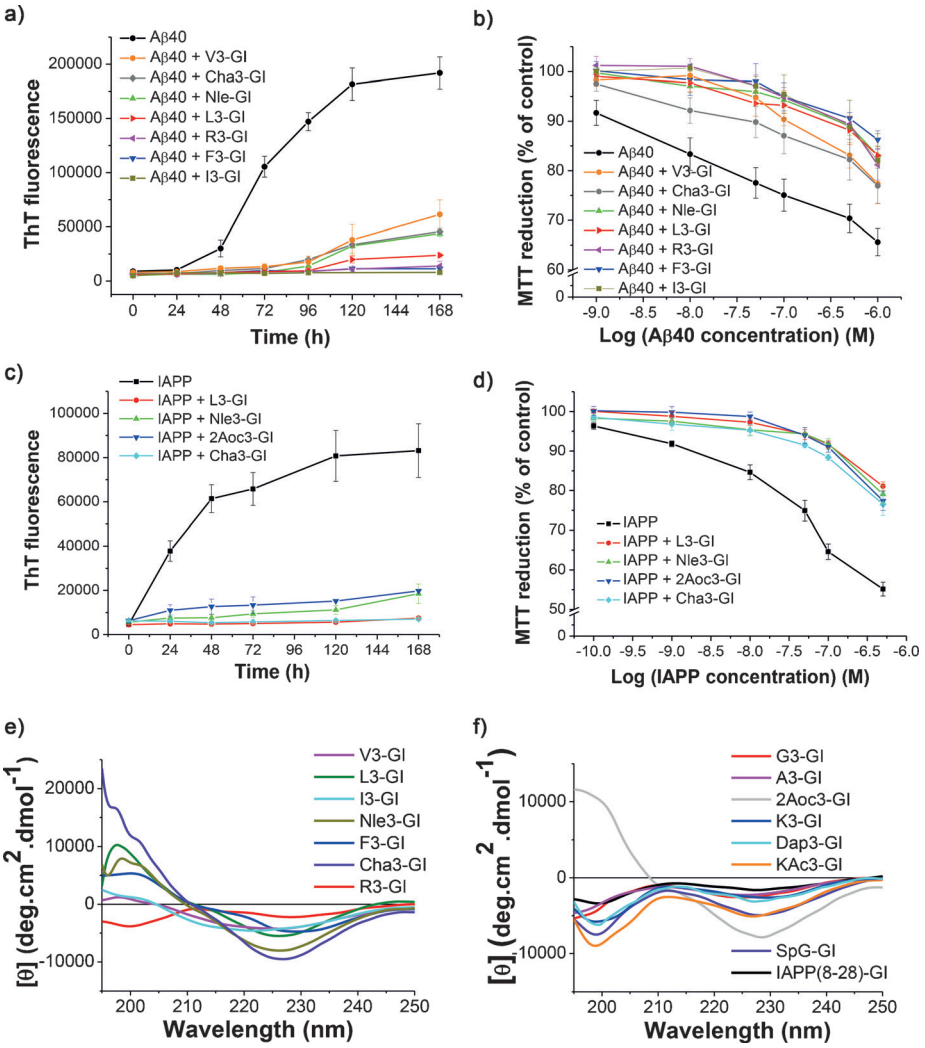

Figure 2. Inhibition of amyloidogenesis and cytotoxicity of Aβ40 or IAPP by various ISMs and ISM conformations. a) Aβ40 fibrillogenesis alone or with ISMs (16.5 μM; 1/1) determined by ThT binding (means (±SEM), 3 assays). b) Cytotoxicity of Aβ40 aged 7 days or its mixtures with ISMs (from a) (PC12 cells) assessed by MTT reduction (means (±SEM), 3 assays ($n=3$ each)). c) IAPP fibrillogenesis alone or with ISMs (16.5 μM; IAPP/ISM, 1/2) determined by ThT binding (means (±SEM), 3 assays). d) Cytotoxicity of solutions of IAPP aged 7 days alone or its mixtures with ISMs (from c) assessed by MTT reduction (RIN5fm cells) (means (±SEM), 3 assays ($n=3$ each)). e,f) CD spectra of Aβ40 inhibitors (e) and non-inhibitors (f) (5 μM, pH 7.4).

Table 1: IC_{50} of inhibitory effects of ISMs on cytotoxic self-assembly of Aβ40 or IAPP.

ISM	IC_{50} (±SEM) [nM] Aβ40 inhibition[a]	IC_{50} (±SEM) [nM] IAPP inhibition[a][b]
V3-GI	186 (±17)	n.d.
I3-GI	267 (±7)	n.d.
L3-GI	81 (±18)	55.4 (±9.6)
Nle3-GI	189 (±32)	40.5 (±3.1)
F3-GI	123 (±24)	n.d.
Cha3-GI	117 (±29)	38.3 (±8.8)
R3-GI	116 (±11)	n.d.
2Aoc3-GI	n.d.	18.3 (±1.4)

[a] IC_{50}s means (±SEM) from three titration assays ($n=3$ each) (Aβ40, 500 nM; IAPP, 100 nM).[16,17] [b] n.d., not determined (non-inhibitory ISMs).

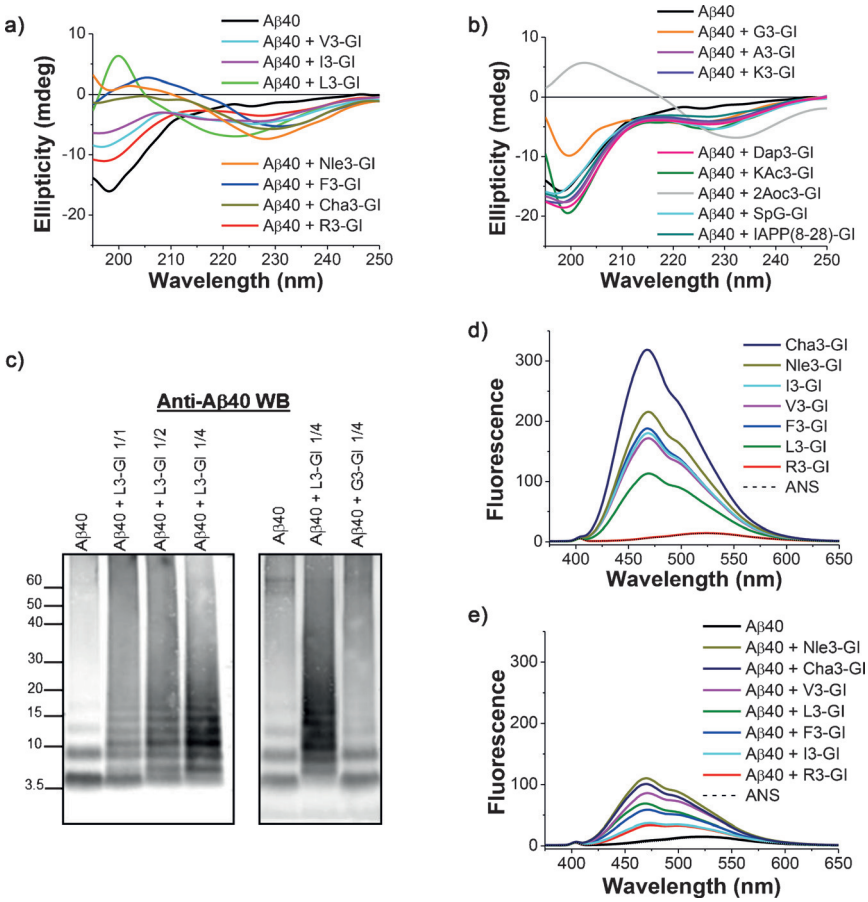

Figure 3. Characterization of the interactions of ISMs with Aβ40. a,b) CD spectra of Aβ40 and mixtures (1/1) of Aβ40 with inhibitory (a) or non-inhibitory ISMs (b) (5 μM, pH 7.4). c) Characterization of hetero-assemblies of Aβ40 with L3-GI or G3-GI via cross-linking with glutaraldehyde, NuPAGE, and Western blot (WB) with anti-Aβ40 antibody. d,e) Determination of surface hydrophobicities via anilinonaphthalene 8-sulfonate (ANS) binding: Fluorescence spectra of ANS alone and ANS mixtures with inhibitory ISMs (d) or mixtures of inhibitory ISMs with Aβ40 (1/1) (e) (10 μM, pH 7.4).

cytotoxic up to concentrations at least 100-fold higher than their IC_{50} values (Table S2, Figure S6).[16] Notably, in contrast to full-length IAPP mimics, ISMs were devoid of IAPP-like bioactivity by design due to the lack of sequence parts required for bioactivity (Figure S6 and text in the Supporting Information).[16]

The CD spectra of all inhibitory ISMs suggested large amounts of β-sheet/β-turn structure except for R3-GI (Figure 2e,f for 2Aoc3-GI).[25] In fact, estimation of secondary-structure contents by deconvolution of CD spectra suggested 80–100% β-sheet/β-turn contents for all inhibitory ISMs except for R3-GI (46% β-sheet/β-turn and 54% random coil) (Table S3).[27,28] NMR studies on R3-GI supported the presence of β-hairpin-like populations (Figure S7 and text in the Supporting Information). CD spectra of non-inhibitors suggested significant amounts of unordered structure in addition to β-sheet/β-turn structure (Figure 2 f, Table S3).

Next, we studied interactions of ISMs with Aβ40 or IAPP (see text in the Supporting Information). We found that mostly nanomolar affinity interactions of inhibitory ISMs with prefibrillar Aβ40 resulted in non-fibrillar hetero-oligomers exhibiting large amounts of β-sheet/β-turn structure and marked surface hydrophobicity (Figure 3, Table S4, and Figures S8 and S9). By contrast, most non-inhibitory ISMs bound Aβ40 with high affinity but hetero-assemblies were mainly disordered and did not show surface hydrophobicity (except for 2Aoc3-GI) (Figure 3b,d, Table S4, and Figures S9–S11). Similar results were obtained for ISM interactions with IAPP (Table S4, Figures S12 and S13). Inhibitory effects are thus likely mediated by the high-affinity binding of β-sheet-structured ISMs of high surface hydrophobicity to prefibrillar Aβ40 or IAPP species, resulting in their sequestration from amyloidogenesis in form of amorphous and non-toxic hetero-assemblies.[1]

Cross-seeding of IAPP amyloidogenesis by Aβ fibrils might link AD to T2D.[7,8] As ISMs that inhibited IAPP self-assembly also bind Aβ40, we asked whether they might interfere with cross-seeding. In fact, they effectively suppressed the Aβ40-cross-seeded self-assembly of IAPP fibrils (Figure 4a,b, Figure S14). Importantly, all ISMs that inhibited Aβ40 self-assembly also bound with low nanomolar affinity to Aβ42, a key player in AD, and blocked (1/1) its fibrillogenesis and cytotoxicity (Figure 4c,d, Table S5, Figure S15, and text in the Supporting Information).[11] Finally, electrophysiological studies in brain slices ex vivo showed that R3-GI ameliorated synaptic long-term potentiation (LTP) mediated by both Aβ40 and Aβ42, which suggested a physiological relevance of the in vitro results (Figure 4e, Figure S16).[29]

In conclusion, we have presented a novel, segment-linking approach to design peptide-based mimics of the IAPP cross-amyloid interaction surface with Aβ as highly potent inhibitors of cytotoxic self-assembly of Aβ40(42), IAPP, or both polypeptides. A unique feature of the approach is that the nature of the linker determines ISM structure and inhibitory function, including both potency and target selectivity. We obtained three nanomolar inhibitors of cytotoxic self-assembly of both Aβ40 and IAPP, four nanomolar inhibitors of Aβ40 cytotoxic self-assembly, and one nanomolar IAPP inhibitor. These ISMs belong thus to the most potent inhibitors of cytotoxic self-assembly of Aβ40 or IAPP.[3] In addition, so far the only reported nanomolar inhibitors of both Aβ40 and IAPP are IAPP-GI and related full-length IAPP analogues.[16,17,19] In comparison to them, ISMs are roughly half as long and lack IAPP-like bioactivity, the latter being a prerequisite for amyloid inhibitors in AD. Furthermore, IAPP inhibitory ISMs are the first reported inhibitors

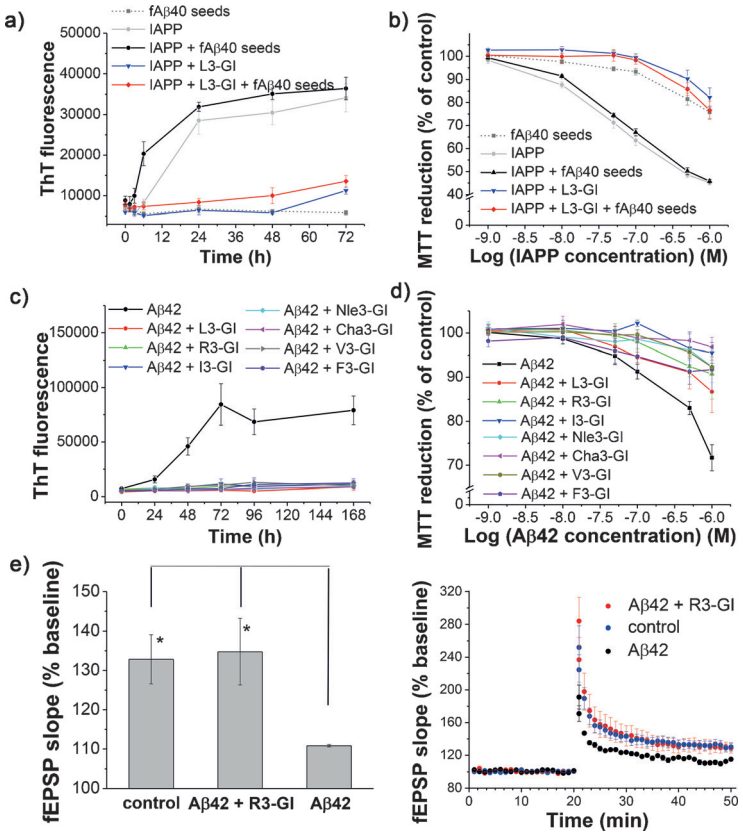

Figure 4. Inhibitory effects of ISMs on IAPP cross-seeding by Aβ40 fibrils (fAβ40) and on Aβ42 fibrillogenesis and cytotoxicity. a,b) Inhibition of fAβ40 (10%) cross-seeding of IAPP fibrillogenesis (12 μM) (a) and cytotoxicity (b) by L3-GI (fivefold) assessed by ThT binding (means (±SEM), 4 assays) (a) and MTT reduction assays (b) (24 h aged solutions from a) (RIN5fm cells) (means (±SEM), 4 assays ($n=3$ each)). c,d) Inhibitory effects of ISMs (1/1) on Aβ42 fibrillogenesis (16.5 μM) determined by ThT binding (means (±SEM), 3 assays) (c) and Aβ42 cytotoxicity (d) (solutions from c aged 7 days) via MTT reduction (PC12 cells) (means (±SEM), 3–4 assays ($n=2$–3 each)). e) Amelioration of Aβ42-mediated LTP impairment in murine acute hippocampal slices by R3-GI. Left: LTP in the presence of Aβ42 (50 nM), Aβ42 + R3-GI (1/10) or R3-GI alone (500 nM) (control) (average from the last 5 min of recordings) (means (±SEM), $n=6$). Significant effects (*) for Aβ42 versus mixture or control ($p<0.05$, $n=6$) (unpaired t-test). Right: Time course of synaptic transmission (means (±SEM), $n=6$).

of cross-seeded IAPP self-assembly, while all seven Aβ40 inhibitory ISMs block cytotoxic self-assembly of Aβ42 as well. Thus, these ISMs might become promising leads for compounds targeting protein aggregation in AD and/or T2D.[1,3,19] Most importantly, our results offer a simple chemical strategy to control structure and confer potent self- and/or cross-amyloid inhibitory function to an initially non-inhibitory (cross-)amyloid surface. As formation of β-sheet interaction interfaces is an early step in amyloid self-assembly and other protein–protein interactions and similar surfaces are often used in both homo- and hetero-association, our approach should find application in devising potent inhibitors of amyloid self-assembly and pathogenic interactions of other proteins as well.[1,13–15]

Acknowledgements

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Keywords: Alzheimer's disease · amyloid inhibitors · islet amyloid polypeptide · protein–protein interactions · β-amyloid peptide

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