

Modulating amyloid- β 42 aggregation and neurotoxicity by Kunitz domains and their derived peptides

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ABSTRACT

Alzheimer's disease is associated with the aggregation of amyloid- β 42 (A β 42) into species of varying sizes, with intermediate oligomers being the most neurotoxic. We recently reported that amyloid precursor protein inhibitor (APPI), a Kunitz-type protein, and a cyclic peptide derived from its β -domain reduced A β 42-mediated neurotoxicity, the former by reducing A β 42 aggregation and formation of toxic A β 42 oligomers, and the latter by promoting A β 42 aggregation to form fibrils rather than the neurotoxic A β 42 oligomers. To address the question of whether these two inhibition mechanisms are controlled by the structure or the amino acid sequence of the protein/peptide, we exploited three Kunitz-type proteins, bikunin, bovine pancreatic trypsin inhibitor (BPTI) and tissue factor pathway inhibitor (TFPI) – chosen for their similar β -strand-rich structures but different sequences to one another and to APPI – and also short peptides that mimic their β -domains, in either cyclic or linear conformation. In-vitro studies showed that the formation of A β 42 aggregates was reduced by the three Kunitz-type proteins and by their derived cyclic peptides, but not by the linear counterparts of the cyclic peptides. In SH-SY5Y neuroblastoma cells, the Kunitz-type proteins and the cyclic (but not the linear) peptides reduced the intracellular and extracellular accumulation of A β 42 aggregates, respectively. Both the Kunitz-type proteins and the cyclic peptides inhibited A β 42-induced mitochondrial membrane depolarization and reduced A β 42-mediated apoptosis and cell death. Overall, this study thus reveals the potential of the β -hairpin structure, whether as a segment within the Kunitz-type proteins or isolated as a cyclic peptide, to interact with A β 42, thereby reducing A β 42 aggregation and hence its neurotoxicity.

Introduction

Alzheimer's disease (AD) – currently the most common neurodegenerative disease – is a significant public health concern, affecting millions of people worldwide and increasing with age from < 1% of people younger than 60 years to > 40% of those older than 85 years [1]. Although the etiology of AD remains a subject of intense study, it is widely accepted that AD is associated with the extracellular and intracellular accumulation of aggregations of β -amyloid (A β) peptide fragments of amyloid precursor protein (APP). These fragments are generated by sequential cleavage by β -secretase at the ectodomain of APP and then by γ -secretase at its transmembrane domain [2]. Among

the resultant cleavage fragments, the 42-amino-acid-containing A β fragment (designated A β 42) is a particularly neurotoxic isoform, by virtue of its propensity to misfold and to aggregate into amyloid plaques in the brain, and it is thus a key player in the pathology of AD [3,4].

The nucleation of disordered monomeric A β 42 into neurotoxic oligomeric aggregates and ordered β -sheet-structured fibrils [5] results from the interaction between the two hydrophobic segments within the A β 42 sequence, namely, the central hydrophobic core (residues 16–22) and the C-terminal region (residues 30–42), which fold into a β -hairpin structure [6–8]. Numerous studies have sought to block the aggregation of A β 42 (as a means to prevent/retard the onset of AD) by using a variety of inhibitors, with focus in recent years on two main types of

Abbreviations: A β , amyloid β ; A β 42, amyloid β 1–42; AD, Alzheimer's disease; APP, amyloid precursor protein; APPI, amyloid precursor protein inhibitor; BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; PBS, phosphate-buffered saline; TEM, transmission electron microscope; TFPI, tissue factor pathway inhibitor; ThT, thioflavin T; TMRE, tetramethylrhodamine, ethyl ester; TTR, transthyretin; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-tetrazolium-carboxanilide.

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proteinaceous molecule—sequence-based peptides, i.e., those with hydrophobic sequences that are the same or very similar to the native sequence of A β 42 [9–11], and structure-based peptides, i.e., those with a β -strand structure [12–14]. In an example of the first approach, treatment of A β 42 with the short hydrophobic peptide LPYFD-amide (a derivative of residues 17–21 of the central hydrophobic core of A β 42) reduced A β 42 aggregation and its associated toxicity in neuronal cells and in mice [15,16]. By applying the second approach, a similar inhibitory effect was obtained by a different peptide (LYFGA), also mimicking the hydrophobic core of A β 42 (i.e., residues 17–21) but having a β -strand structure that was generated by chemically cross-linking Tyr at position 2 and Gly at position 4 [17].

In yet another approach that appeared to offer promise for the generation of peptides inhibiting A β 42 aggregation, ring-like peptide structures were designed and tested [18]. A representative example is provided by a 23-residue bicyclic peptide in which three cysteine residues enabled double cyclization to produce a molecule with two central regions, LGIKI and TSVYHA, that bind the C-terminal region of A β 42 at residues 31–36 and 38–42, respectively [19]. Nonetheless, although this bicyclic peptide remodeled A β 42 aggregation, producing shorter, thicker, and non-fibrillar structures, which reduced A β 42-associated toxicity in a *Caenorhabditis elegans* model, its interaction with the monomeric form of A β 42 was weak and transient and showed no inhibitory effect on pre-formed fibrils [19]. In parallel, studies on cyclic peptides that were specifically designed to mimic the β -hairpin motif in A β 42 showed that they were indeed able to reduce A β 42 aggregation and toxicity [20–23]. For example, Yamin et al. synthesized a 13-mer cyclic peptide with hairpin structure that interacts with A β 42 [24]. Although they showed that the cyclic peptide modulated A β 42 aggregation by inhibiting the formation of oligomers and fibrils while promoting the generation of non-fibrillar aggregates [24], the lack of cellular toxicity assays leaves the conclusions of the study somewhat limited. In summary, although cyclic peptides appear to have potential as blockers of A β 42 aggregation, work to date on the effect on A β 42 aggregation of the amino acid sequence or the structure of these peptides has not produced definitive conclusions that may ultimately be leveraged in the design of AD therapeutics.

A starting point for addressing the above issues is an examination of the structure and activity of human APP. This protein has three major isoforms that are produced as a result of alternative splicing, namely, APP695, APP751, and APP770. The latter two isoforms each contain a 58-amino acid extracellular subunit known as a Kunitz protease inhibitor or as an amyloid precursor protein inhibitor (APPI) [25–27]. The two APPI-containing isoforms, APP751 and APP770, are expressed in multiple tissues (e.g., glia cells, skin, and lungs), while the APPI-deficient isoform, APP695, is expressed primarily in neurons [28,29]. Of note, previous studies have reported a significant increase in mRNA and protein levels of the APPI-containing isoforms in AD brains, but the involvement of APPI in AD progression has yet to be elucidated [30,31].

Thus, to date, APPI has been studied predominantly for its function as a serine protease inhibitor that targets proteases, such as mesotrypsin, whose catalytic activity plays roles in various malignancies and in pancreatitis [32–34]. Nonetheless, two recent papers from our laboratory have investigated the role of APPI in the formation of A β 42 aggregates [35,36]. The first showed that a cyclic β -hairpin peptide derived from the β -domain of APPI (but not its linear counterpart) reduced the toxicity of A β 42 and enhanced the formation of mature A β 42 fibrils from intermediate, toxic oligomeric A β 42 species [35]. The second study demonstrated the ability of both extra- and intracellular full-length APPI to reduce A β 42 aggregate formation and hence to decrease the cellular toxicity mediated by both extra- and intracellular A β 42 [36]. These two studies thus revealed an apparent contradiction in that the full-length APPI protein reduced A β 42 aggregation and the formation of toxic A β 42 oligomers, but the cyclic APPI peptide enhanced A β 42 aggregation, forming fibrils rather than neurotoxic A β 42 oligomers and thereby

ameliorating A β 42-mediated neurotoxicity.

To further investigate this issue and in line with previous reports highlighting the potential of cyclic β -hairpin mimetics in AD therapeutics, we sought to address the open question of whether it is a structural element or the specific sequence of the cyclic APPI fragment that interacts with A β 42 to enhance its aggregation and hence to attenuate A β 42 neurotoxicity. To this end, we investigated the putative inhibitory activities of three other Kunitz-type proteins, namely, tissue factor pathway inhibitor (TFPI), bovine pancreatic trypsin inhibitor (BPTI) and bikunin [37], and their derived β -domain peptides in cyclic or linear conformation. The rationale for choosing these three Kunitz-type proteins was that they have similar lengths (58 amino acids) and structures (β -hairpin domain with two antiparallel β -sheets and one helical region) to APPI [investigated in our previous studies [1,36]], but different sequences to one another and to APPI, both in the full-length protein and within the β -hairpin region [37–40].

Herein, we demonstrate that, like the β -domain of APPI [36], the β -domains of the three Kunitz-type proteins interact with A β 42, thereby modulating its aggregation mechanism. Using thioflavin T (ThT) fluorescence assays, circular dichroism (CD) spectroscopy, and transmission electron microscopy (TEM) imaging, we show that both the Kunitz-type proteins, at a molar ratio of 64:1 (A β 42:Kunitz), and the Kunitz-derived cyclic peptides with a β -hairpin structure, at a molar ratio of 1:2 (A β 42:cyclic peptide), reduce the formation of A β 42 aggregates. In contrast, the Kunitz-derived linear peptides do not result in a significant reduction in A β 42 aggregation. Similarly, treatment of SH-SY5Y neuroblastoma cells with the Kunitz-type proteins and their derived cyclic peptides, but not their linear counterparts, reduce the intracellular and extracellular accumulation of A β 42 aggregates, respectively, as assessed by confocal microscopy. Notably, the Kunitz-type proteins and the cyclic peptides prevent A β 42-mediated mitochondrial membrane depolarization and reduce A β 42-mediated apoptosis and cell death.

Taken together, the findings of this study show that the combination of the amino acid sequence of the Kunitz-type proteins and the β -hairpin structure present in both Kunitz-type proteins and cyclic peptides impacts A β 42 aggregation and that the β -hairpin structure is responsible for reducing neurotoxicity—irrespective of the amino acid sequence.

Results

The inhibitory effect of the Kunitz-type proteins on A β 42 aggregation in vitro is similar to that of their derived cyclic peptides

To examine the effect of the Kunitz-type proteins and their derived peptides on A β 42 aggregation, it was first necessary to produce and purify BPTI, TFPI, and bikunin. The three proteins were expressed and purified as described previously [42,43] (see Methods), and for TFPI and bikunin representative results for the different stages of the procedure are given in the Supporting Information (Figures S1 and S2, respectively). Yields of 15 mg, 7.4 mg, and 10 mg per 1 L of yeast culture were obtained for BPTI, TFPI, and bikunin, respectively.

The ability of the Kunitz-type proteins to inhibit aggregation of A β 42 was then investigated by incubating A β 42 for 16 h in the absence or presence of the proteins and monitoring the aggregation levels of untreated or treated A β 42 (Fig. 1A). As expected, the highest aggregation signal was observed for A β 42 alone, consistent with its intrinsic tendency to self-assemble first into oligomers and aggregates and then into fibrils [5], while a decrease in aggregation was observed when A β 42 was incubated with the Kunitz-type proteins.

With the aims to study the effect of the β -domain of Kunitz-type proteins on A β 42 aggregation and to determine whether it is the structure or the sequence of the domains that influences the aggregation, we designed three linear peptides and their three cyclic counterparts that were derived from the β -hairpin domains of the three native Kunitz-type proteins, BPTI, TFPI and bikunin (Figure S3A), as follows: Three 18-mer linear peptides were derived from the full β -hairpin sequences of BPTI,

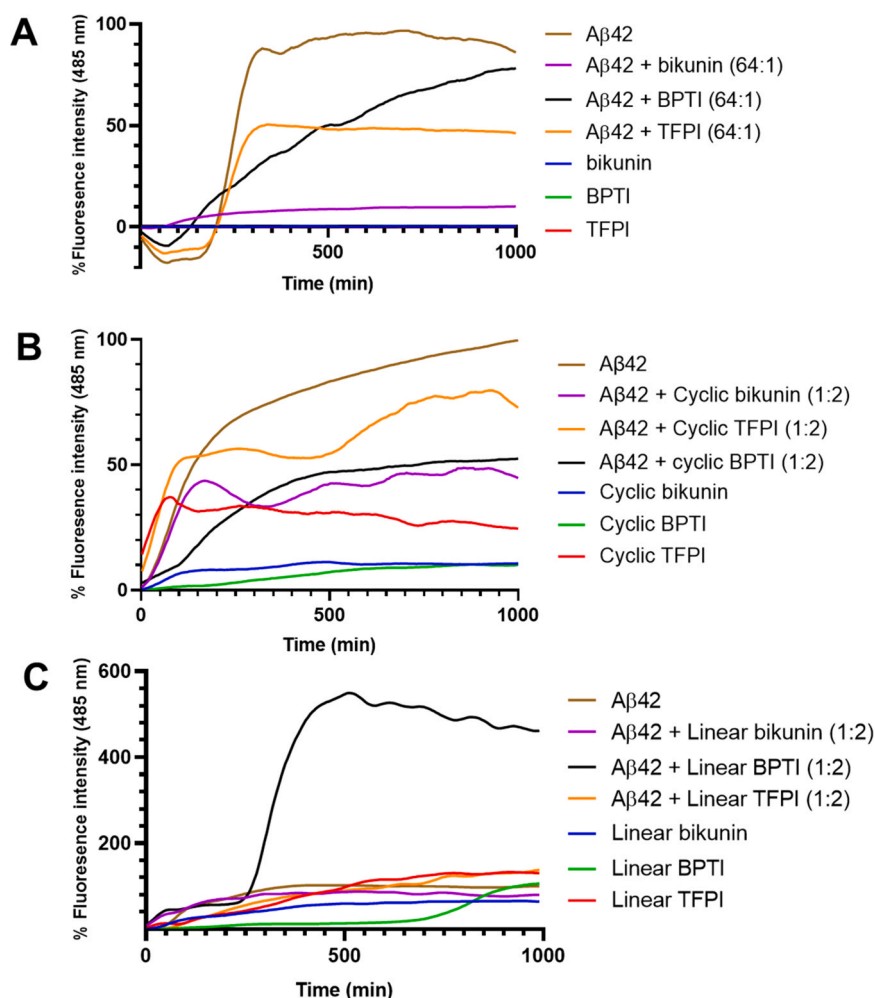


Fig. 1. Ability of the Kunitz-type proteins and their derived cyclic peptides to inhibit Aβ42 aggregation. Aβ42 aggregation was monitored for 16 h by using the ThT assay, as follows. Mixtures of 4 μM Aβ42 with (A) 62.5 nM Kunitz proteins at a molar ratio of 64:1 (Aβ42:protein), (B) 8 μM cyclic peptides at a molar ratio of 1:2 (Aβ42:peptide), or (C) 8 μM linear peptides at a molar ratio of 1:2 (Aβ42:peptide) dissolved in phosphate buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl) were incubated with 10 μM ThT at 37 °C with constant shaking. The fluorescence was read using BioTek Synergy H1 microplate reader (Winooski, Vermont, USA) using excitation and emission wavelengths of 440 and 485 nm, respectively.

bikunin and TFPI (Figure S3B); and three 20-mer cyclic peptides were designed such that they had the same sequences as the linear peptides, but with two cysteine residues added at the N- and C-termini to form a disulfide bridge, creating a cyclic structure (Figure S3C) and thereby mimicking the structure of the β-domain in the Kunitz-type proteins [41]. In addition, to prevent potential undesired interactions, the internal cysteine residue in the sequences of the cyclic peptides was replaced with serine, and to better mimic the β-domains in the native Kunitz-type protein sequences, the N-termini of all six peptides were acetylated and the C-termini were amidated.

To confirm both the formation of the disulfide bridge within the cyclic peptides and the correct molar weights of the linear and cyclic peptides, all six peptides were analyzed by mass spectrometry (Figure S4). The spectra showed good agreement between theoretical and experimental values; for example, the calculated molar weight of the cyclic BPTI peptide, taking into consideration the formation of the disulfide bridge and a consequent reduction of 2 Da, was 2399.81 Da, which matched the experimentally obtained molar weight of 2399.78 Da (Figure S4B).

Next, the ability of the cyclic and linear peptides to inhibit the aggregation of Aβ42 was investigated by incubating Aβ42 for 16 h in the absence or presence of the peptides. Monitoring of the aggregation levels of untreated and treated Aβ42 revealed a decrease in aggregation upon incubation of Aβ42 with the cyclic peptides but not the linear peptides

(Fig. 1B,C). Of note, while the cyclic BPTI peptide was a potent inhibitor of Aβ42 aggregation, the linear BPTI peptide was the only linear peptide that enhanced Aβ42 aggregation. Moreover, neither the three Kunitz-type proteins per se nor their derived peptides gave any significant aggregation signals (cyclic TFPI gave a low signal), indicating that these proteins/peptides do not undergo self-aggregation (Fig. 1).

The ability of the Kunitz-type proteins to reduce the β-sheet and fibrillar contents of Aβ42 is retained by the cyclic peptides

We then aimed to explore – at the molecular and macroscopic levels – the inhibition of Aβ42 aggregation by the Kunitz-type proteins and their derived peptides. To this end, we first recorded the CD spectra of samples of Aβ42 (50 μM) post 18 h of incubation in the absence and presence of the Kunitz-type proteins (781.25 nM) at a molar ratio of 64:1 (Aβ42:protein) or the peptides (100 μM) at a molar ratio of 1:2 (Aβ42:peptides). The CD spectrum of Aβ42 revealed a negative peak at 216 nm and a positive peak at 195 nm—both characteristic of a β-sheet secondary structure [42], which is commonly regarded as the signature of Aβ42 aggregates [43,44]. Reductions in β-sheet structures and increases in random secondary structures were observed upon incubation of Aβ42 with the cyclic peptides and to a lesser extent with the linear peptides and the Kunitz-type proteins (Fig. 2). The CD spectra of control cyclic and linear peptides and Kunitz-type proteins revealed predominantly

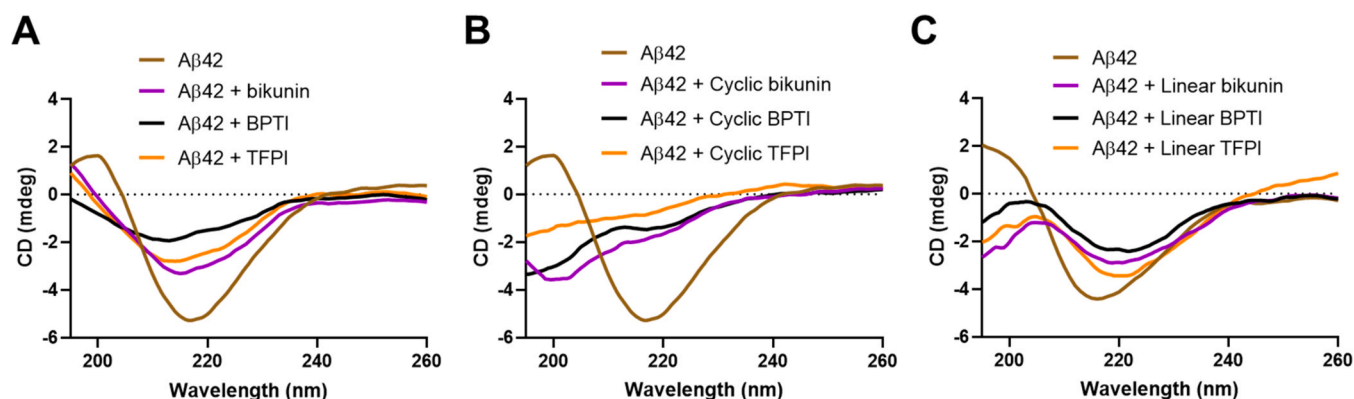


Fig. 2. Kunitz-type proteins and their derived peptides reduce β -sheet content in A β 42. (A) Kunitz-type proteins, 781.25 nM, (B) cyclic peptides, 100 μ M, or (C) linear peptides, 100 μ M, at a molar ratio of 1:2 (A β 42:peptide) were incubated with A β 42, 50 μ M, for 18 h. CD spectra were acquired over the range 195–260 nm.

random β -sheet structures at insignificant levels (Figure S5).

To complement the molecular level structural results, samples taken from the CD experiment (18 h post incubation) were analyzed by TEM. Consistent with the high ThT signal and β -sheet content of A β 42 and with the findings of previous studies [45,46], the TEM images showed that untreated A β 42 formed elongated and branched fibrils (Fig. 3A). The TEM images of A β 42 treated with Kunitz-type proteins revealed a decrease in both the abundance and the branching of the fibrils, with a tendency toward shorter and unbranched fibrils (Fig. 3B). TEM images of A β 42 samples incubated with the cyclic peptides revealed a dramatic reduction in the quantity of aggregates, with the fibrils being shorter and less entangled (Fig. 3C). In contrast, in the presence of the linear peptides, only a slight decrease in the quantity of A β 42 aggregates was observed, with the species appearing more densely packed and tangled (Fig. 3D). The above findings were confirmed by dot blot analysis with an anti-amyloid fibril antibody, which showed a strong reduction in the quantity of A β 42 fibrils upon 18 h of incubation with the proteins and peptides, in the order: cyclic peptides > Kunitz-type proteins > linear peptides (Fig. 4).

The Kunitz-type proteins reduce intracellular A β 42 aggregate formation in SH-SY5Y Cells

To determine whether bikunin, BPTI, and TFPI can reduce the formation of A β 42 aggregates in cells, SH-SY5Y cells were co-transfected with either the A β 42-GFP genetic construct and an empty plasmid or with A β 42-GFP together with each Kunitz-type protein gene (*bikunin*, *BPTI*, or *TFPI*). Confocal microscopy imaging performed 48 h post-transfection revealed a high accumulation of green fluorescence protein (GFP) in inclusion bodies in the absence of a Kunitz-type protein, indicating the presence of A β 42 aggregates [47]. In contrast, when Kunitz-type proteins were co-expressed with A β 42-GFP, the observed GFP signal was spread throughout the cells (Fig. 5A). In addition, western blot analysis confirmed that intracellular A β 42-GFP expression levels did not change upon co-expression with the Kunitz-type proteins (Fig. 5B).

Extracellular cyclic peptides inhibit the internalization and accumulation of A β 42 in SH-SY5Y cells

Studies have shown that extracellular A β 42 interacts with the cell membrane and is internalized into the cell via multiple uptake mechanisms [48,49]. To follow the effect of extracellular cyclic and linear peptides on the internalization and accumulation of A β 42 in cells, SH-SY5Y cells were treated with HiLyte™ Fluor 488-labeled A β 42 (designated A β 42-488) in the presence or absence of cyclic or linear peptides and visualized using confocal microscopy. The cells treated with A β 42-488 alone exhibited strong internal fluorescence signals,

indicating internalization of the extracellular A β 42 (Fig. 6A). Upon exposure of the cells to a mixture of A β 42-488 and each of the cyclic peptides at molar ratio of 1:2 (A β 42:peptides), internalization and accumulation of A β 42 in the cells was reduced (Fig. 6A). In contrast, confocal microscopy images of the cells following treatment with mixtures of A β 42-488 and linear peptides revealed the presence of inclusion bodies within the cells, which indicated that the accumulation of A β 42 had not been affected by the presence of the linear peptides (Fig. 6B). Based on the inhibitory activity observed for the Kunitz-type proteins and the cyclic peptides but not the linear peptides, our subsequent cellular assays were performed only with the Kunitz-type proteins and the cyclic peptides.

The Kunitz-type proteins and cyclic peptides enhance the viability of SH-SY5Y cells exposed to extracellular A β 42

To further investigate the ability of Kunitz-type proteins and cyclic peptides to reduce extracellular A β 42-mediated cytotoxicity in SH-SY5Y cells, we performed cell-based assays to assess cell viability and apoptosis. The XTT viability assay revealed an average of ~30% reduction in viability vs. control cells for SH-SY5Y cells exposed for 48 h to pre-formed A β 42 aggregates (Fig. 7A). However, SH-SY5Y cells treated with mixtures of A β 42 and Kunitz-type proteins (pre-incubated together for 18 h at 37 °C before exposure to the cells) exhibited improved viability, reaching complete recovery (normalized to control cells) for bikunin and BPTI treatments and ~90% for the TFPI treatment (Fig. 7A). Similarly, treating SH-SY5Y cells with a mixture of A β 42 and cyclic peptides (pre-incubated for 18 h at 37 °C) improved cell viability compared to control cells, reaching 86% for cyclic bikunin and complete recovery for both cyclic BPTI and cyclic TFPI (Fig. 7B). Notably, exposure of the cells to the Kunitz-type proteins and the cyclic peptides alone did not significantly affect cell viability (Fig. 7C,D).

We also sought to investigate the effect of our proteins/cyclic peptides on A β 42-induced apoptosis, since previous studies have reported that most of the accumulated intraneuronal A β may be attributed to the uptake of extracellular A β fragments, which induce a neuronal apoptosis cascade [50,51] by promoting uncontrolled elevation of cytosolic Ca²⁺ levels [52] and impairing mitochondrial redox activity [53]. Thus, to evaluate the ability of the Kunitz-type proteins and the cyclic peptides to reduce A β 42-induced apoptosis in SH-SY5Y cells, treated and untreated cells were stained with Annexin-V APC and SYTOX Green [54,55], and the apoptotic stages were analyzed by flow cytometry. Fig. 8A shows that 98% of the control cells were healthy (negative for both Annexin-V APC and SYTOX Green) and 42.2% of cells exposed to extracellular A β 42 aggregates for 24 h were in early-stage apoptosis (positive for Annexin-V APC and negative for SYTOX Green), whereas cells treated with mixtures of A β 42 and Kunitz-type proteins (pre-incubated together) revealed a reduction in cell toxicity, with only 17.8%, 31.1%, and 29.5% of cells in

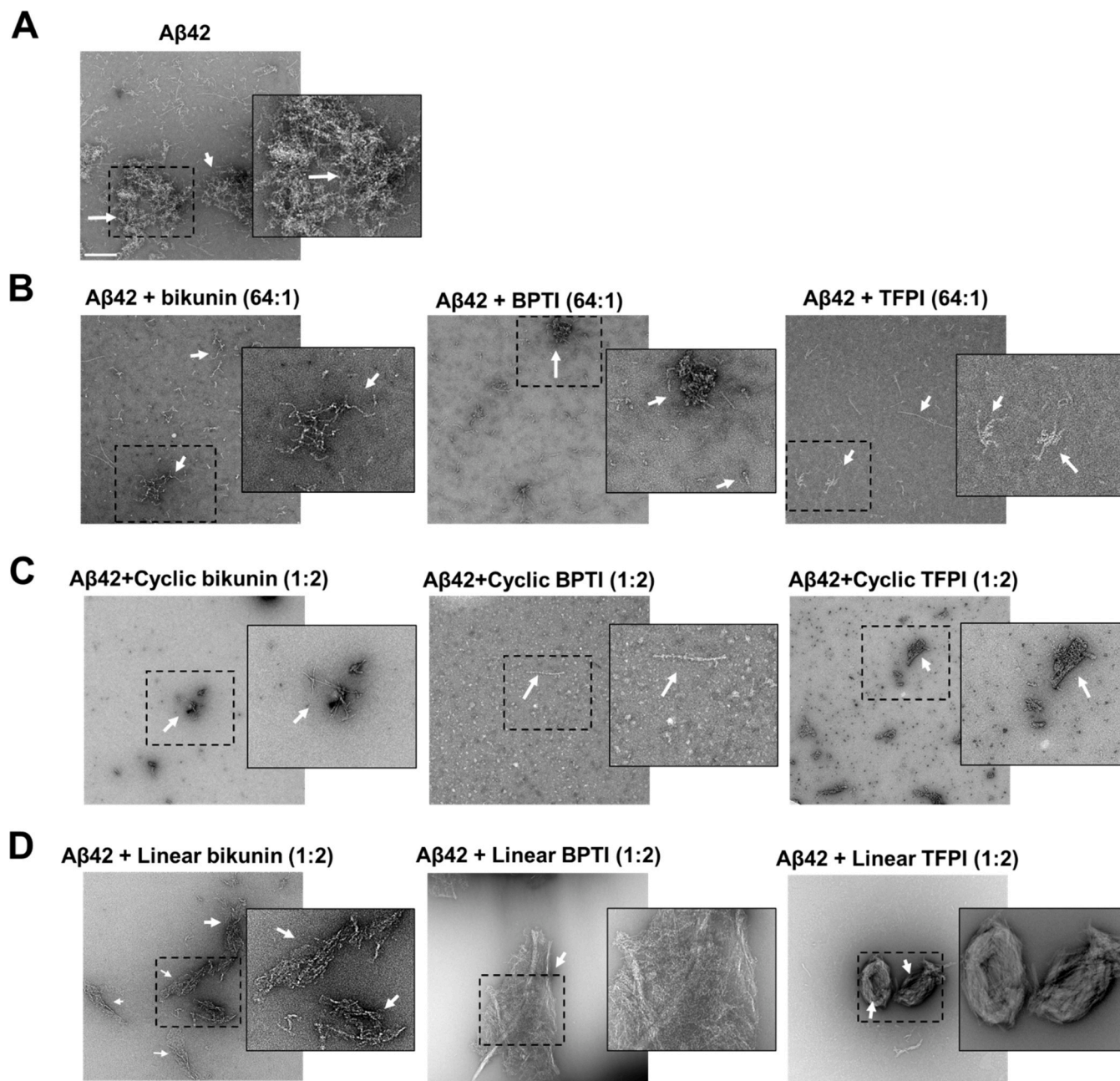


Fig. 3. The Kunitz-type proteins and their derived cyclic peptides reduce the formation of A β 42 aggregates. TEM images of samples of A β 42 (50 μ M) incubated for 18 h in the absence of proteins or peptides (A) or in the presence of (B) the Kunitz-type proteins (781.25 nM), (C) the cyclic peptides (100 μ M), or (D) the linear peptides (100 μ M). White arrows indicate A β 42 fibrils. Insets show magnified views of the boxed regions. Scale bar, 500 nm.

the early apoptotic stage following treatment with A β 42 together with bikunin, BPTI, or TFPI, respectively (Fig. 8B, left panels). Treatment with Kunitz-type proteins alone, as control groups, showed that approximately 85% of the cells were healthy living cells, with less than 1% in a late apoptosis stage and less than 15% of cells in an early apoptosis stage (Fig. 8B, right panels). A more marked reduction in A β 42-induced apoptosis was obtained for the cyclic peptides, with only 8.86%, 9.47%, and 7.59% of cells in early-stage apoptosis for cyclic bikunin, cyclic BPTI, or cyclic TFPI, respectively (Fig. 8C, left panels). These results indicate an approximately 30% decrease in early apoptotic cells compared to those treated with extracellular A β 42 aggregates (Fig. 8A, right panel). Notably, approximately 85% of the control SH-SY5Y cells treated with cyclic peptides alone were healthy, and < 9% were in an early apoptotic stage (Fig. 8C, right panels).

The Kunitz-type proteins and cyclic peptides suppress the mitochondrial membrane depolarization potential mediated by A β 42 in SH-SY5Y cells

Previous studies have shown that A β 42 impairs mitochondrial function by disrupting the membrane potential and the electron transport chain, thereby generating reactive oxygen species and damaging mitochondrial DNA and proteins and ultimately leading to neuronal apoptosis [56–58]. Thus, following the viability and apoptosis assays showing that both the Kunitz-type proteins and the cyclic peptides suppressed A β 42-mediated apoptosis and cell death in SH-SY5Y cells, we sought to examine the effect of those proteins and peptides on the mitochondrial membrane potential of intact SH-SY5Y cells. Mitochondrial damage was assessed by measuring the changes in mitochondrial polarization resulting from exposure to A β 42 aggregates, pre-formed in the presence or absence of Kunitz-type proteins and cyclic peptides. As

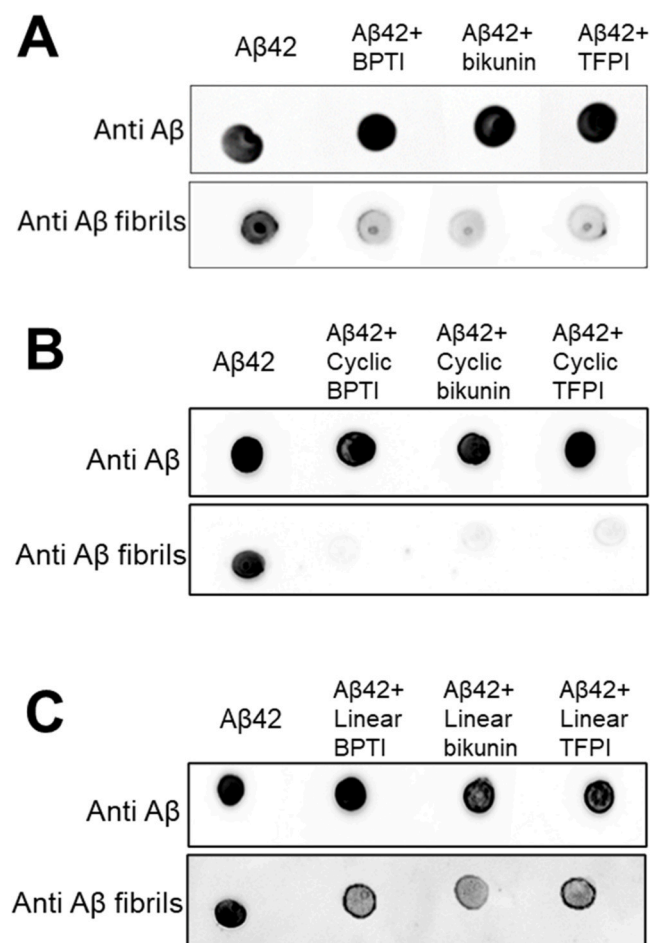


Fig. 4. The Kunitz-type proteins and their derived peptides reduce the quantity of Aβ42 fibrils. Dot blot analysis of samples of Aβ42, 50 μM, incubated for 18 h in the absence or in the presence of (A) the Kunitz-type proteins, 781.25 nM, (B) the cyclic peptides, 100 μM, or (C) the linear peptides, 100 μM. The quantity of Aβ42 fibrils was assessed using anti-amyloid fibril antibody. The total quantity of Aβ42 in each sample was detected by blotting with anti-Aβ42 antibody.

shown in Fig. 9, cells that were exposed to pre-formed Aβ42 aggregates for 48 h exhibited an average of ~40% reduction in mitochondrial membrane potential compared to untreated cells. The addition of the Kunitz-type proteins improved mitochondrial function and reduced the changes in mitochondrial membrane potential, which reached complete recovery in SH-SY5Y cells treated with mixtures of Aβ42 and bikunin or TFPI, and 97% in cells treated with Aβ42 and BPTI (Fig. 9A). Similarly, the reduction in mitochondrial membrane potential was almost abolished in cells treated with Aβ42 and cyclic peptides, with improvements of 95%, 98%, and 96% in cells treated with Aβ42 and cyclic bikunin, cyclic BPTI, and cyclic TFPI, respectively, compared to untreated cells (Fig. 9B). We observed that both Kunitz-type proteins and cyclic peptides did not induce significant changes in mitochondrial membrane potential (Fig. 9C,D).

Discussion

In recent years, the development of innovative anti-amyloid strategies has expanded significantly beyond traditional small molecules to include advanced therapeutic and diagnostic modalities. Notably, nanomaterials for modulating the aggregation of β-amyloid peptides have emerged as powerful platforms due to their unique structural properties and biocompatibility [59]. Within this framework, photo-thermal and photodynamic strategies for diagnosis and therapy of AD by modulating amyloid-β aggregation have gained traction, offering non-invasive, spatiotemporally controlled intervention mechanisms that simultaneously disrupt aggregates and enable theranostic imaging [60]. A prominent example of this dual-functional biomimetic approach includes porphyrin-substituted phenylalanine-phenylalanine (Phe-Phe) nanoparticles for modulating β-amyloid aggregation, which combine the strong self-assembling capabilities of core peptide motifs with the photo-excited singlet oxygen generation of porphyrins to remodel toxic oligomers [61]. While these nanostructures and photofunctional systems offer excellent therapeutic efficacy, they often face challenges regarding complex in vivo delivery, potential systemic toxicity, and large molecular footprints. To address these limitations, precisely engineered short peptide mimics represent a highly promising alternative. In this work, we present a novel class of rationally designed Kunitz domains and their derived peptides that directly target the amyloidogenic cascade, offering a minimal, highly stable structural framework to

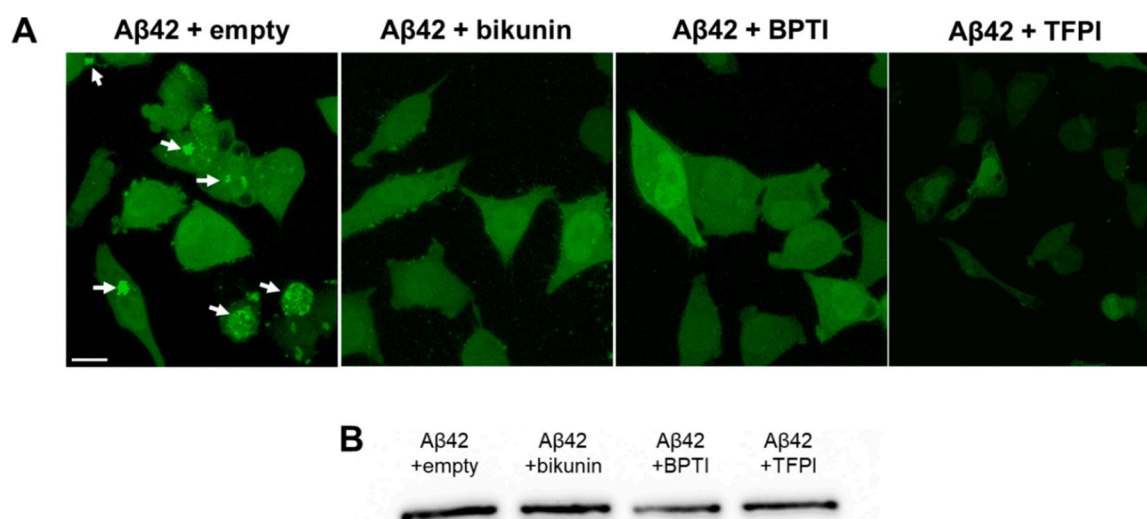


Fig. 5. The Kunitz-type proteins reduce intracellular Aβ42 aggregate formation in SH-SY5Y cells. (A) Confocal microscopy images of SH-SY5Y cells expressing Aβ42-GFP or coexpressing Aβ42-GFP and the Kunitz-type proteins. Images were acquired with an Olympus FV1000 confocal microscope with a ×60 objective. White arrows indicate Aβ42 aggregates. Scale bar, 50 μm. (B) Lysates of cells transiently expressing Aβ42-GFP or both Aβ42-GFP and a Kunitz-type protein, followed by western blot analysis using an anti-Aβ42 antibody.

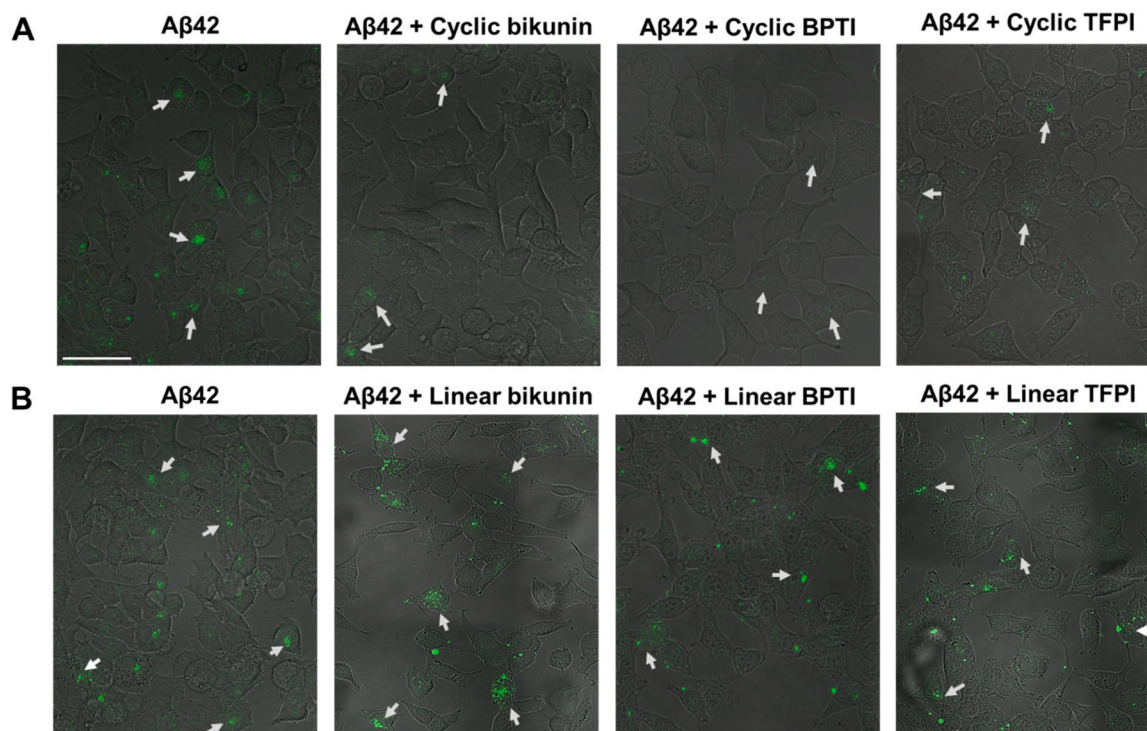


Fig. 6. Inhibition of extracellular A β 42 internalization and accumulation by the cyclic peptides. SH-SY5Y cells were treated with samples of 1 μ M A β 42 labeled with HyLyte Fluor 488 that had been pre-incubated for 18 h in the absence or presence of 2 μ M (A) cyclic peptides and (B) linear peptides. Confocal microscopy images of the cells were acquired with a Zeiss LSM 880 confocal microscope. Arrows indicate inclusion bodies. Scale bar, 50 μ m.

inhibit β -amyloid aggregation without the prerequisite of external photo-activation or complex nanoparticle formulation.

Studies have shown Kunitz-type proteins to be implicated in the etiology of AD, although open questions remain regarding their roles in the development of AD and the generation of A β aggregates. For example, it was shown that TFPI, which is abundant in the brains of AD patients [62], is elevated in the frontal cortex and blood plasma of AD patients and, notably, that TFPI is localized in some A β plaques in AD brains [63,64]. Less was known about another Kunitz-type protein, APPI, until our recent studies of the protein and of a peptide sequence taken from its β -hairpin domain demonstrated that both are potent inhibitors of A β 42-induced neuronal cell toxicity, but act via different mechanisms: the full-length protein reduces A β 42 aggregation, whereas its β -hairpin domain enhances aggregation [35,36]. To investigate these differences in modes of inhibition and to elucidate whether the inhibition mechanism depends on the structure or on the sequence of the peptides – or perhaps on both – we extended our work on APPI [35,36] to other Kunitz-type proteins, namely, TFPI, BPTI and bikunin, and the short cyclic peptides that mimic their β -hairpin domains and their unstructured linear peptide counterparts.

Integrating the findings of the current study with those of our previous study on APPI reveals that a β -hairpin motif of the interacting cyclic peptide is a prerequisite for conferring inhibition (cyclic TFPI, BPTI and bikunin peptides) or enhancement (the cyclic APPI peptide) of A β 42 aggregation, whereas the lack of a β -hairpin loop in the interacting linear peptide may either not affect (linear APPI, TFPI and bikunin) or enhance (linear BPTI) A β 42 aggregation. Moreover, for any particular β -hairpin sequence in a peptide, the specific amino acid composition may finetune the level of inhibition of A β 42 aggregation. An analysis of the three studies together also shows that all four different Kunitz-type protein family members, which have a similar fold (i.e., an α/β fold with two antiparallel β -sheets and one helical region) but different sequences (with only ~25% similarity) [32,37,65], inhibit the aggregation of A β 42.

An examination of the mechanism of A β 42 inhibition indicates that

the β -sheet domain of the α/β fold is the segment that interacts with A β 42 to modulate its aggregation pathway, probably through hydrophobic interactions with A β 42, as observed for other peptide-based β -sheet breakers designed to inhibit or reverse the misfolding of A β 42 into β -sheet-rich structures [66–68], which is a key pathological event in neurodegenerative diseases, such as AD [7,69]. By binding to the central hydrophobic core of A β 42 aggregates, probably via their β -hairpin peptide domain, the Kunitz proteins disrupt the stability of the aggregated A β 42, preventing further fibril formation and thereby reducing neurotoxicity. It is possible that – like APPI – bikunin, BPTI, and TFPI proteins inhibit the A β 42 'on-pathway', in which unordered monomeric A β 42 self-assembles to form toxic oligomeric intermediates that promote further elongation of A β 42 into mature amyloid fibrils [70,71].

The potential of β -hairpin-like cyclic peptides to inhibit 'on-pathway' A β 42 aggregation has been examined in previous studies using synthetic peptides (not related to the A β 42 sequence) [23,24] or peptides comprising the self-assembly region of A β 42 [72]. However, in view of the lack of a comprehensive study, the role played by the cyclic conformation in these inhibitory activities of the peptides remains unclear. Consistent with our premise that the β -sheet domain interacts with A β 42, Costa et al. demonstrated that A β 42 aggregation and cytotoxicity can be mitigated by the β -hairpin domain of transthyretin (TTR), either as a segment within TTR [73,74] or as an isolated cyclic peptide mimetic derived from the A β -TTR binding interface [75]. Similar to our current study, the above authors also showed that the linear versions of the peptides exhibited only minor inhibitory activity, whereas significant inhibition of A β 42-induced toxicity was achieved only with the cyclic peptides and at concentrations comparable to those observed for the Kunitz-derived cyclic peptides (20–40 μ M).

In the current study, an examination of the inhibition of A β 42 aggregation by the linear peptides revealed that the effects of these peptides differed from those of the cyclic peptides: In particular, linear BPTI markedly increased aggregation, while both linear TFPI and linear bikunin exerted only a minor effect on A β 42 aggregation. The reduction in the β -sheet content of A β 42 aggregates treated with the linear

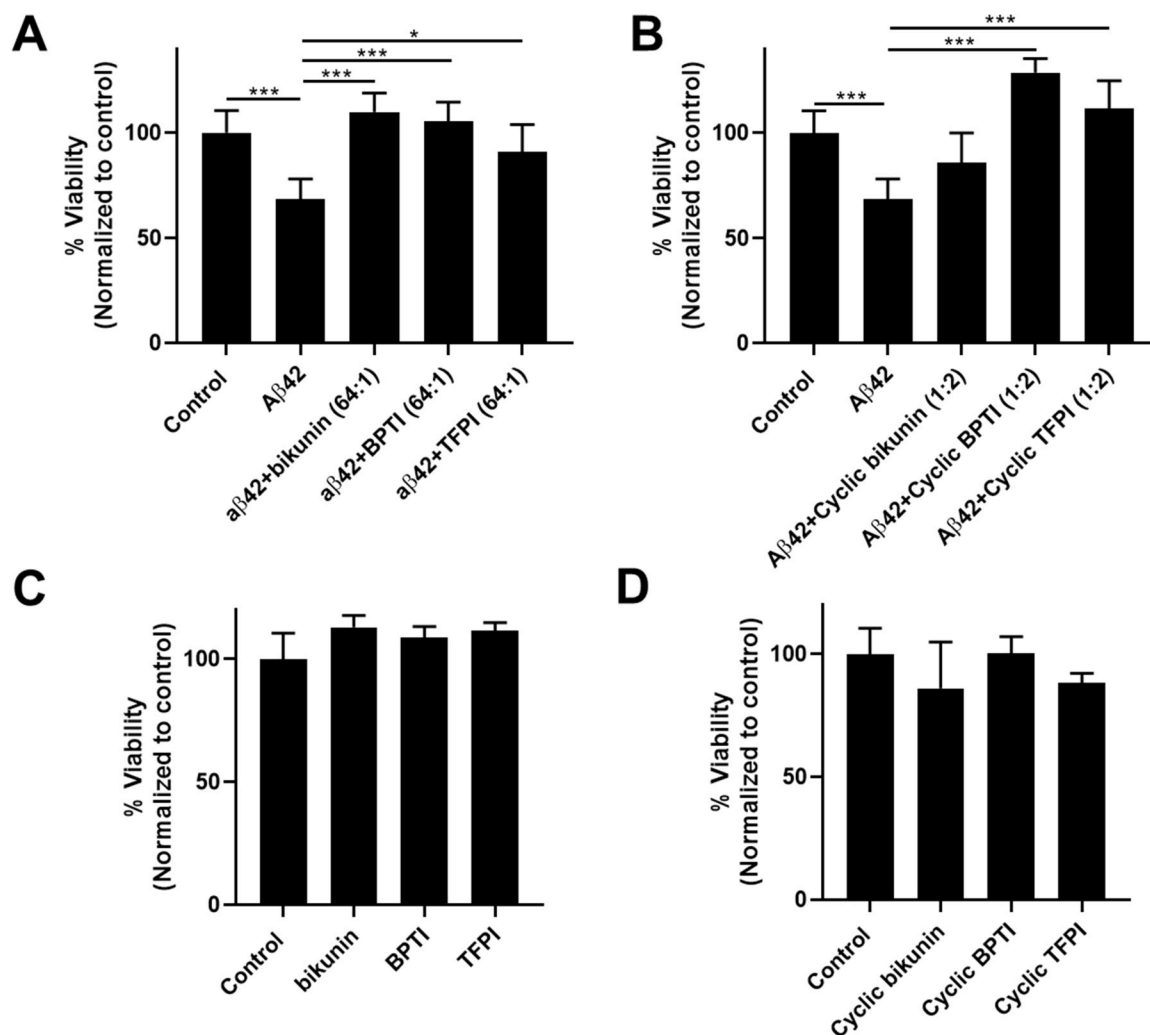


Fig. 7. The Kunitz-type proteins and their derived cyclic peptides reduce Aβ42 toxicity in SH-SY5Y cells. XTT cell viability assay of SH-SY5Y cells after 48 h of treatment with: (A,B) 10 μM Aβ42 that had been pre-incubated for 18 h at 37 °C in the absence or presence of 156.25 nM Kunitz-type proteins or 20 μM cyclic peptides, respectively, or (C,D) 156.25 nM Kunitz-type proteins or 20 μM cyclic peptides, respectively. Statistical analysis (n = 3) was performed by an unpaired Student's *t*-test. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

peptides, as shown by the CD spectra, may be a result of the formation of large insoluble fibrils [76,77]. Indeed, TEM images of Aβ42 treated with linear peptides showed the formation of large aggregates with a morphology different from that of the aggregates of untreated Aβ42, with the former species appearing to be more densely packed and tangled than in untreated Aβ42. A comparison between cyclic peptides and their linear versions has also been undertaken in other studies [17, 78]. For example, Jha et al., who tested a 10-mer cyclic peptide and its linear counterpart, showed that the cyclic peptide reduced both aggregation and β-sheet content of Aβ42 in vitro and mitigated Aβ42-induced toxicity in SH-SY5Y cells, whereas the linear peptide had no effect on Aβ42 aggregation in vitro and or in cells [79].

Reinforcing the findings that the three Kunitz-type proteins (i.e., bikunin, BPTI, and TFPI) and their derived cyclic peptides – but not their linear counterparts – inhibited Aβ42 aggregation in vitro, our results in cells indicate that treatment with the Kunitz-type proteins and their derived cyclic peptides also led to a reduction in the β-sheet content of Aβ42; namely, a reduction in the formation of Aβ42 aggregates (as shown in the ThT assay and TEM images) was visualized using confocal microscopy of SH-SY5Y cells, where the internalization of extracellular Aβ42 aggregates was reduced upon interaction with the cyclic peptides but not with the linear peptides. In contrast, as shown in one of our previous studies, the interaction of the cyclic APPI peptide with Aβ42

enhanced Aβ42 aggregation, thereby leading to the formation of more fibrils and less toxic Aβ42 oligomers [35]. The explanation for the above differences may lie in the ability of the cyclic peptides (whether cyclic APPI or the cyclic bikunin, BPTI, and TFPI peptides) to interfere with the interaction of Aβ42 with the cell membrane due to structural changes induced in Aβ42 upon interaction with the peptides [80]. These structural changes, in turn, led to reduced Aβ42-induced cytotoxicity in SH-SY5Y cells, as reflected in a reduction of both apoptotic processes and mitochondrial dysfunction. Taken together, these findings led us to posit that, like the Kunitz-type proteins, all four cyclic peptides inhibited the Aβ42 'on-pathway' mechanism, thereby reducing the quantity of toxic oligomers that subsequently nucleate to form Aβ fibrils.

The study's collective results confirm that both the specific amino acid sequence and the β-hairpin structure found in Kunitz-type proteins and cyclic peptides are critical for altering Aβ42 aggregation but the only the β-hairpin structure is essential for mitigating neurotoxicity. Nevertheless, the possibility of non-specific hydrophobic effects which may also affect Kunitz- Aβ42 interaction and Aβ42 toxicity, cannot be excluded. This study also reveals the potential of members of the Kunitz-type protein family and their derived cyclic peptides to reduce the neurotoxicity of Aβ42 aggregates either by decreasing formation of toxic oligomers or by enhancing the formation of Aβ42 fibrils, both resulting in disruption of the 'on-pathway' mechanism of Aβ42 oligomerization

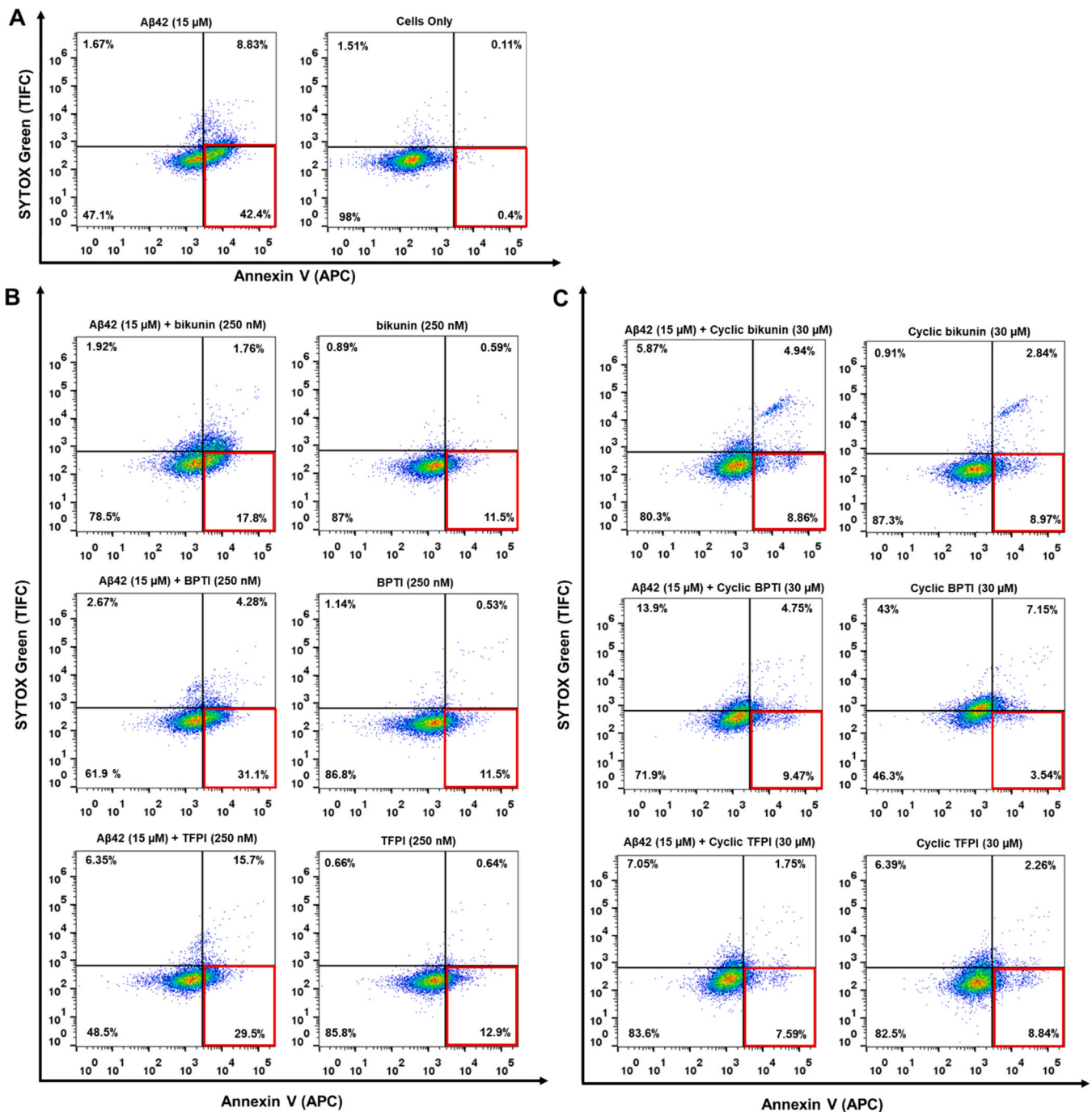


Fig. 8. Apoptotic processes induced by extracellular Aβ42 aggregates were reduced by the Kunitz-type proteins and cyclic peptides. Flow cytometry analysis of Annexin-V- and SYTOX-Green-stained SH-SY5Y cells after 24 h of incubation with Aβ42 (15 μM) that had been pre-incubated for 18 h at 37 °C in the absence (A) or presence of (B) Kunitz-type proteins (250 nM) or (C) cyclic peptides (30 μM). Untreated cells served as a negative control.

[81]. We emphasize that it is the alterations to the aggregation pathway of Aβ42 – whether enhancing or impeding aggregation – controlled by the β-hairpin domain (either as a sub-domain within a Kunitz-type protein or as an isolated cyclic peptide) and its amino acid sequence, that lead to a reduction in the quantity of the toxic 'on-pathway' oligomers, which subsequently elongate and nucleate to form Aβ fibrils.

Methods

BPTI, Bikunin and TFPI expression and purification

BPTI, bikunin and TFPI genes were separately cloned into pPIC9K

plasmids as described previously [82,83]. The plasmids were transformed into *Pichia pastoris* strain GS115 and then inoculated into and allowed to stand overnight in 5 ml of BMGY medium (1% yeast extract, 2% peptone, 0.23% potassium phosphate monobasic, 1.18% potassium phosphate dibasic, 1.34% yeast nitrogen base, 4×10^{-5} % biotin and 1% glycerol). Thereafter, the cells were transferred to 50 ml of BMGY, followed by scaling up to 500 ml of BMGY. For protein expression, the cells were resuspended in 500 ml of BMMY (same as BMGY, but with 0.5% methanol instead of glycerol) and grown for three days, with 2% methanol being added every 24 h to maintain induction. Following four days of induction, the cultures were centrifuged at $4700 \times g$ for 20 min, and the supernatants containing the secreted proteins were adjusted to

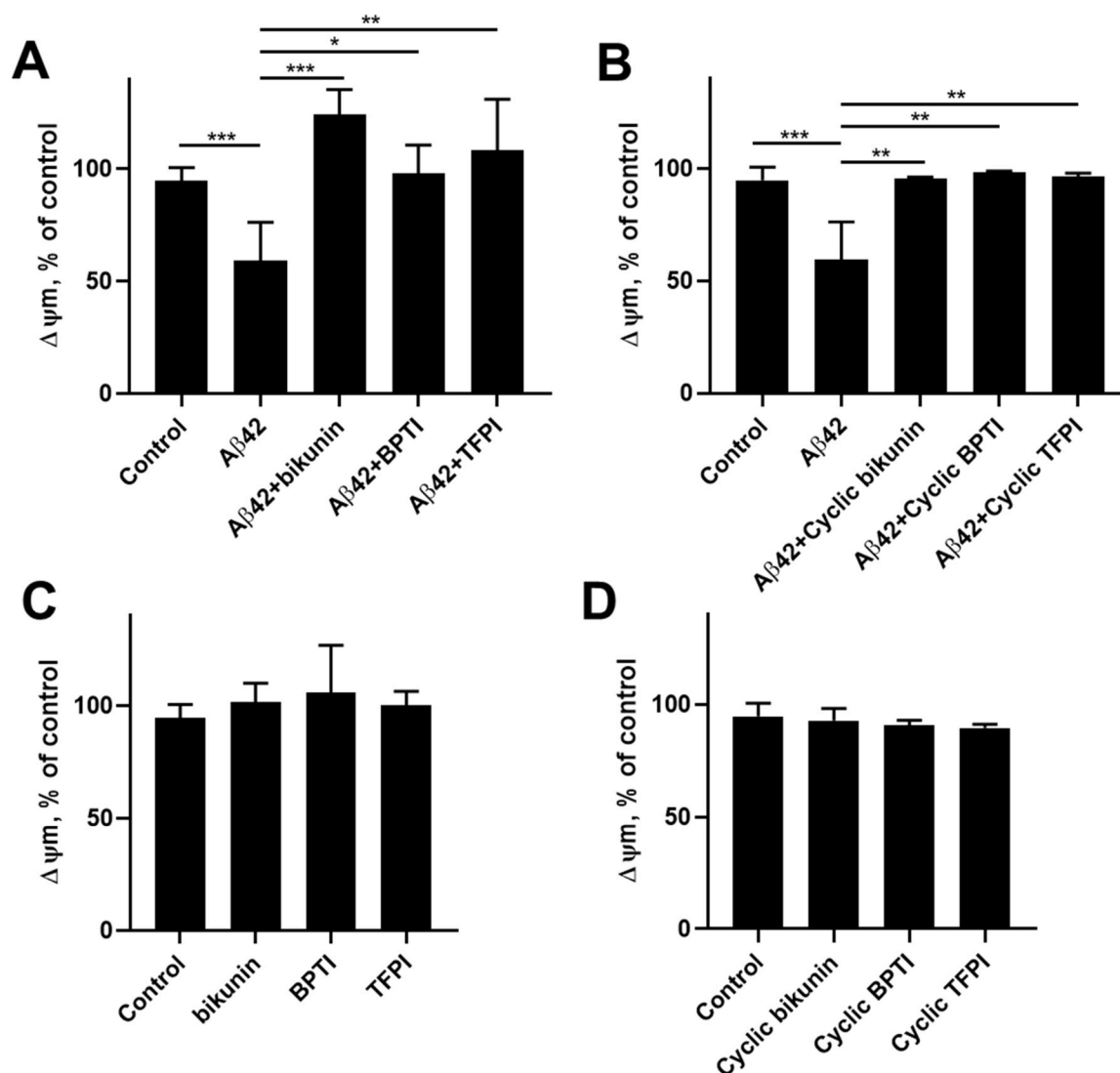


Fig. 9. Suppression of the A β 42-mediated mitochondrial membrane depolarization potential ($\Delta\psi_m$) by the Kunitz-type proteins and the cyclic peptides in SH-SY5Y cells treated with: (A, B) 10 μ M A β 42 that had been pre-incubated for 18 h at 37 °C in the absence or presence of 156.25 nM Kunitz-type proteins or 20 μ M cyclic peptides, respectively; or (C,D) 156.25 nM of each Kunitz-type protein or 20 μ M of each cyclic peptide, respectively. Changes in $\Delta\psi_m$ were assessed after 48 h by using tetramethylrhodamine ethyl ester (TMRE), which is an indicator of mitochondrial function in living cells. Statistical analysis (n = 3) was performed by an unpaired Student's *t*-test. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

10 mM imidazole and 0.5 M NaCl at pH 8.0, followed by incubation for 1 h at 4 °C and then centrifugation at 4700 $\times$ g for 20 min. Next, the supernatants were filtered through 0.22- μ m Stericup bottle-top filters (Millipore, MA, USA). The filtered supernatants were loaded onto 5-ml HisTrap columns (GE Healthcare, Piscataway, NJ, USA) at a flow rate of 0.9 ml/min for 16 h, washed with a washing buffer (20 mM sodium phosphate, 0.5 M NaCl, and 10 mM imidazole; pH 8.0), and eluted with an elution buffer (as for washing buffer but with 0.5 M imidazole) using ÄKTA™ start (GE Healthcare). As the final step for BPTI, bikunin and TFPI purification, SEC was performed on Superdex 75 16/600 columns (GE Healthcare) equilibrated with PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄; pH 7.4), at a flow rate of 1 ml/min on ÄKTA start. The purity of the proteins was confirmed by 15% SDS-PAGE gel under non-reducing conditions. The concentrations of the proteins were determined with a BCA Protein Assay Kit (Thermo Fisher, MA, USA) according to manufacturer's instructions. The molar weights of the pure proteins, validated using a MALDI-TOF (Bruker, Billerica, MA, USA) mass spectrometer (The Ilse Katz Institute for Nanoscale Science and Technology, BGU), were 9.68, 9.31, and 9.71 kDa for BPTI, bikunin, and TFPI, respectively.

Synthetic peptides

Synthetic A β [1–42] (A β 42) was purchased from AnaSpec (Fremont, CA, USA) and from GL Biochem (Shanghai, China). Fluorescently labeled A β 42-Hylight 488 (A β 42–488) was purchased from AnaSpec (Fremont, CA, USA). The unlabeled A β 42 from AnaSpec was dissolved in hexafluoroisopropanol (HFIP; Sigma-Aldrich, Israel) to a final concentration of 221 μ M. The required quantity of synthetic A β 42 peptide from AnaSpec was taken, followed by evaporation of the HFIP for 10 h. The synthetic A β 42 peptide from GL Biochem was dissolved in 10 mM NaOH, and the concentration was determined by NanoDrop spectrophotometer (Thermo Fisher, MA, USA) using an extinction coefficient of 1490 M^{−1} cm^{−1}. A β 42–488 was dissolved in 10 mM NaOH to final concentration of 205 μ M. The synthetic peptides, cyclic TFPI (CMKRFFFNIFTRQSEEFYIC), linear TFPI (MKRFFFNIFTRQSEEFYI), cyclic BPTI (CHRYFYNAKAGLSQTFVYC), linear BPTI (IIR-YFYNAKAGLSQTFVY), cyclic bikunin (CIQLWAFDAVKGKSVLFYPC), and linear bikunin (IQLWAFDAVKGKSVLFY), were synthesized and divided into 1-mg aliquots by GL Biochem. For all experiments, except the CD measurements, the linear and cyclic peptides were dissolved in

1 ml of DMSO. The concentrations of the stock solutions were determined according to the Mw of the synthetic peptides (Figure S4).

Pre-incubation of the peptides

For all experiments (except ThT assays and CD measurements), A β 42 was dissolved in phosphate buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl) in the presence or absence of the peptides or Kunitz-type proteins and incubated in a Thermo Shaker incubator for 18 h (300 rpm, orbital shaking) at 37 °C.

Cell Culture

SH-SY5Y neuroblastoma cells were grown at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Sartorius, Göttingen, Germany) supplemented with 10% tetracycline-free fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 2 mM L-glutamine (Gibco), and penicillin (100 units/ml)/streptomycin (0.1 mg/ml) (Gibco).

Detection of A β 42 aggregation by using the ThT assay

The aggregation of A β 42 was determined using the fluorescent dye ThT, as follows. A β 42, 4 μ M, and ThT (Sigma-Aldrich, Israel), 10 μ M, were incubated in the absence or presence of 62.5 nM Kunitz-type proteins (BPTI, TFPI or bikunin) or 8 μ M linear or cyclic peptides in 350 μ l of phosphate buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.4). Aliquots of 100 μ l of reaction mixture were added to each well of a black 96-well plate (Greiner Bio-One, Germany), and the fluorescence intensity was determined at 5-min intervals, for 16 h at 37 °C, with constant orbital shaking at 205 rpm. The fluorescence signals were detected using BioTek Synergy H1 microplate reader (Winooski, Vermont, USA) with excitation and emission wavelengths of 440 and 485 nm, respectively. Each experiment was performed in triplicate.

CD Spectra

The linear and cyclic peptides were dissolved in phosphate buffer and mixed with 50% acetonitrile to a concentration of 2000 μ M. Samples of 50 μ M A β 42 in the absence or presence of Kunitz-type proteins (781.25 nM) or linear or cyclic peptides (100 μ M) were added to 200 μ l of diluted phosphate buffer (5 mM sodium phosphate, 37.5 mM NaCl, pH 7.4) in order to maintain an appropriate high-tension (HT) voltage of the instrument. The samples were incubated in a Thermo Shaker Incubator for 18 h (500 rpm, orbital shaking) at 37 °C. The pre-incubated peptide samples were scanned using a Jasco J-715 spectropolarimeter (Jasco, Japan). The spectrum of each sample was recorded three times at 25 °C in a range between 195–260 nm using a quartz cuvette with a path length of 1 mm, a scanning speed of 50 nm/min, and a data interval of 0.5 nm. The spectral scans were averaged to smooth the data curves. For baseline correction, protein-free phosphate buffer was used.

TEM Imaging of A β 42 Aggregates

Samples of A β 42 with Kunitz-type proteins, cyclic peptides, or linear peptides taken from the above CD assay post 18 h of incubation were investigated using TEM. For this purpose, 2.5 μ l of each sample was deposited on a carbon-coated copper 300-mesh grid. After 1 min, excess liquid was carefully removed with filter paper. Then, 2% uranyl acetate in doubly distilled water was added to each sample, and after 1 min any excess of uranyl acetate solution was carefully removed using a filter paper. Images were acquired using a Tecnai G2 12 BioTWIN (FEI, ThermoFisher Scientific, Paisley, UK) TEM with an acceleration voltage of 120 kV (The Ilse Katz Institute for Nanoscale Science and Technology, BGU). Different magnifications were used for visualization, depending on the size of the aggregates, and multiple fields were analyzed in each grid.

Dot Blot Analysis

Samples from the CD measurements of A β 42 with Kunitz-type proteins, cyclic peptides, or linear peptides were analyzed. Each sample (5 μ l) was loaded onto two nitrocellulose membranes (Bio-Rad, Hercules, California, USA), which were blocked with 5% skim milk (Sigma-Aldrich, Israel) in Tris-buffered saline with 0.1% Tween (TBST; Biolab, Israel), and then further washed with TBST three times for 5 min each. The first membrane was incubated overnight with rabbit amyloid fibril polyclonal antibody (Thermo Fisher, Israel) diluted 1:1000 in PBS. The second membrane was incubated overnight with mouse anti-A β 42 antibody (Abcam, Cambridge, UK) diluted 1:1000 in PBS to confirm that the same amount of A β 42 had been loaded in each sample. After washing, the membranes were incubated for 1 h with HRP-linked anti-mouse secondary antibody (Cell Signaling, Danvers, MA, USA) and anti-rabbit secondary antibody (Cell Signaling), respectively. The chemiluminescence signals were detected using a Fusion FX imaging system (Vilber, Germany). The experiments were performed in triplicate.

Mammalian expression plasmids

For expression in SH-SY5Y cells, A β 42-GFP, BPTI, bikunin, and TFPI were cloned into a pPHAGE2 vector under the control of the human cytomegalovirus promoter. A β 42-GFP was cloned as described previously [84]. BPTI, bikunin, and TFPI genes were cloned into the pPHAGE2 plasmid using *NotI* and *BamHI* restriction enzymes (New England Biolabs, MA, USA) as described previously [36]. The pPHAGE2 plasmid was used as a mammalian vector for expressing BPTI, bikunin, and TFPI linked to blue fluorescent protein via an internal ribosome entry site in SH-SY5Y cells under the control of the human cytomegalovirus promoter.

Confocal microscopy imaging

In all experiments, SH-SY5Y cells were seeded at a density of 10⁴ cells per well in 1-cm micro-slides (ibidi, Gräffelfing, Germany). For intracellular expression of Kunitz-type proteins (i.e., BPTI, bikunin, and TFPI) and A β 42-GFP, the cells were transfected one day after seeding with either 1 μ g of a mixture of A β 42-GFP plasmid and empty plasmids or a mixture of A β 42-GFP and Kunitz-type gene containing pPHAGE plasmids by using the jetOPTIMUS transfection reagent (Polyplus, Illkirch, France), according to the manufacturer's manual. After 48 h of incubation, the cells were washed with PBS and fixed by incubation with 4% paraformaldehyde (PFA) in PBS for 30 min. Cell imaging was performed using Olympus FV1000 confocal microscope with a long-working distance $\times$ 60/1.35 numerical aperture oil-immersion objective [The National Institute for Biotechnology in the Negev (NIBN), BGU].

For extracellular treatments with A β 42 and cyclic or linear peptides, the cells were treated with pre-incubated samples of 1 μ M A β 42–488 in the absence or presence of 2 μ M cyclic or linear peptides. After the incubation, the cells were washed with PBS and fixed by incubation with 4% PFA in PBS for 30 min. Cell imaging was performed using a Zeiss LSM 880 confocal microscope with a $\times$ 40 objective (The Ilse Katz Institute for Nanoscale Science and Technology, BGU).

Detection of A β 42-GFP Expression in SH-SY5Y Cells

SH-SY5Y cells (3 $\times$ 10⁵ cells per well) were seeded in a six-well tissue culture plate (Greiner, Sigma-Aldrich, Israel). For transient expression of A β 42-GFP in the presence or absence of the Kunitz-type proteins, the cells were transiently transfected on the following day using the jetOPTIMUS transfection reagent (Polyplus) according to the manufacturer's manual. Each well was transfected with 1 μ g of pPHAGE-A β 42-GFP and 1 μ g of pPHAGE2-empty, pPHAGE2-BPTI, pPHAGE2-bikunin, or pPHAGE2-TFPI. After 48 h, the medium was removed, and the cells were

incubated for 20 min with RIPA buffer (EMD Millipore Corp., Billerica, MA, USA) and protease inhibitor cocktail (APE×BIO, Boston, MA, USA) for cell lysis. The cell cultures were centrifuged, and protein concentrations were determined in the supernatants by the BCA assay (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Each lysate (50 µg) was loaded onto a 15% SDS-PAGE and then transferred to a nitrocellulose membrane for western blot analysis. The membrane was blocked with 5% skim milk and incubated overnight with mouse anti-Aβ42 antibody diluted 1:1000 in PBS (Abcam). After washing, the membrane was incubated for 1 h with HRP-linked anti-mouse secondary antibody (Cell Signaling, Danvers, MA, USA), and chemiluminescence signals were detected.

XTT cell viability assay

SH-SY5Y cells (10⁴ cells per well) were seeded in 96-well tissue culture plate (Greiner, Sigma-Aldrich, Israel). After 24 h, the cells were treated with pre-incubated samples (see details in 'pre-incubation of the peptides' subsection) of 10 µM Aβ42 in the presence or absence of the Kunitz-type proteins (156.25 nM) or the cyclic peptides (8 µM). The viability of the cells was assessed 48 h post-treatment by using an XTT-based kit (Cell Signaling) according to the manufacturer's protocol. The absorbance was read at a wavelength of 450 nm using a BioTek Synergy H1 microplate reader. Three repetitions were performed for each treatment. The values were normalized to the control group (untreated cells).

Mitochondrial membrane depolarization potential measurements

To measure the changes in the mitochondrial membrane potential in SH-SY5Y cells, 10⁴ cells/well were seeded in a black-clear bottomed 96-well tissue culture plate (Greiner, Sigma-Aldrich, Israel). The cells were treated for 48 h with pre-incubated samples (see details in 'pre-incubation of the peptides' subsection) of 10 µM Aβ42 in the absence or presence of the Kunitz-type proteins (156.25 nM) or the cyclic peptides (8 µM). The changes in the mitochondrial membrane potential were assessed using TMRE-Mitochondrial Membrane Potential Assay Kit (Abcam), according to the manufacturer's protocol. The fluorescence intensity, which indicated mitochondrial potential, was detected using TECAN infinite M200 microplate reader (Männedorf, Switzerland) with excitation and emission wavelengths of 549 and 575 nm, respectively. Each experiment was performed in triplicate. The values were normalized to the control group (untreated cells).

Flow cytometry analysis of apoptotic cells

SH-SY5Y cells (3 × 10⁴ cells per well) were seeded in a 24-well plate and incubated for 18 h with 15 µM pre-formed Aβ42 aggregates in the absence or presence of 250 nM Kunitz-type proteins (BPTI, TFPI and bikunin) or 30 µM cyclic peptides (see details in the 'pre-incubation of the peptides' subsection). Untreated cells served as a negative control. After 24 h of incubation, the cells were harvested, washed, and stained with Annexin V-APC and SYTOX Green using an Apoptosis Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The cells were analyzed using a BD FACS Canto II (BD Biosciences, Erembodegem, Belgium). The following cell populations were analyzed: intact cells (Annexin V-APC negative and SYTOX Green negative), early apoptotic cells (Annexin V-APC positive and SYTOX Green negative), late apoptotic cells (annexin V-APC and SYTOX Green positive), and necrotic cells (annexin V-APC negative and SYTOX Green positive).

Statistical analysis

All data were analyzed statistically with GraphPad Prism, version 8.00, for Windows (La Jolla, CA, USA). Data are shown as means ± SD. Statistical significance between the control group and different

treatments was determined by an unpaired Student's *t*-test, with statistical significance **p* < 0.05; ***p* < 0.01; ****p* < 0.001. Ethics Declaration

This study does not require ethics approval.

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CRedit authorship contribution statement

Maya Rabinovich: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Shiran Lacham-Hartman:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Niv Papo:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2026.08.001.

Data availability

Data will be made available on request.

References

- [1] Beydoun MA, Beydoun HA, Gamaldo AA, Teel A, Zonderman AB, Wang Y. Epidemiologic studies of modifiable factors associated with cognition and dementia: systematic review and meta-analysis. *BMC Public Health* 2014 Dec 24; 14(1):643. <https://doi.org/10.1186/1471-2458-14-643>.
- [2] Hampel H, Hardy J, Blennow K, Chen C, Perry G, Kim SH, et al. The Amyloid-β Pathway in Alzheimer's Disease. *Mol Psychiatry* 2021 Oct 30;26(10):5481–503. <https://doi.org/10.1038/s41380-021-01249-0>.
- [3] Azargoonjahromi A. The duality of amyloid-β: its role in normal and Alzheimer's disease states. *Mol Brain* 2024 Jul 17;17(1):44. <https://doi.org/10.1186/s13041-024-01118-1>.
- [4] De S, Wirthensohn DC, Flagmeier P, Hughes C, Aprile FA, Ruggeri FS, et al. Different soluble aggregates of Aβ42 can give rise to cellular toxicity through different mechanisms. *Nat Commun* 2019 Apr 4;10(1):1541. <https://doi.org/10.1038/s41467-019-09477-3>.
- [5] Im D, Kim S, Yoon G, Hyun DG, Eom YG, Lee YE, et al. Decoding the Roles of Amyloid-β (1–42)'s Key Oligomerization Domains toward Designing Epitope-Specific Aggregation Inhibitors. *JACS Au* 2023 Apr 24;3(4):1065–75. <https://doi.org/10.1021/jacsau.2c00668>.
- [6] Abelein A, Abrahams JP, Danielsson J, Gräslund A, Jarvet J, Luo J, et al. The hairpin conformation of the amyloid β peptide is an important structural motif along the aggregation pathway. *JBIC J Biol Inorg Chem* 2014 Jun 16;19(4–5): 623–34. <https://doi.org/10.1007/s00775-014-1131-8>.
- [7] Khaled M, Rönnbäck I, Ilag LL, Gräslund A, Strodel B, Österlund N. A Hairpin Motif in the Amyloid-β Peptide Is Important for Formation of Disease-Related Oligomers. *J Am Chem Soc* 2023 Aug 23;145(33):18340–54. <https://doi.org/10.1021/jacs.3c03980>.

- [8] Finder VH, Glockshuber R. Amyloid- β aggregation. *Neurodegener Dis* 2007;13:27–48. <https://doi.org/10.1159/000100355>. PubMed PMID: 17429215.
- [9] Goyal D, Shuaib S, Mann S, Goyal B. Rationally Designed Peptides and Peptidomimetics as Inhibitors of Amyloid- β (A β) Aggregation: Potential Therapeutics of Alzheimer's Disease. *ACS Comb Sci* 2017 Feb 13;19(2):55–80. <https://doi.org/10.1021/acscmb.5b00116>. PubMed PMID: 28045249.
- [10] Wang F, Zhou XL, Yang QG, Xu WH, Wang F, Chen YP, et al. A Peptide That Binds Specifically to the β -Amyloid of Alzheimer's Disease: Selection and Assessment of Anti- β -Amyloid Neurotoxic Effects. *PLoS One* 2011 Nov 10;6(11):e27649. <https://doi.org/10.1371/journal.pone.0027649>.
- [11] Taylor M, Moore S, Mayes J, Parkin E, Beeg M, Canovi M, et al. Development of a Proteolytically Stable Retro-Inverso Peptide Inhibitor of β -Amyloid Oligomerization as a Potential Novel Treatment for Alzheimer's Disease. *Biochemistry* 2010 Apr 20;49(15):3261–72. <https://doi.org/10.1021/bi100144m>.
- [12] Arai T, Sasaki D, Araya T, Sato T, Sohna Y, Kanai M. A Cyclic KLVFF-Derived Peptide Aggregation Inhibitor Induces the Formation of Less-Toxic Off-Pathway Amyloid- β Oligomers. *ChemBioChem* 2014 Nov 24;15(17):2577–83. <https://doi.org/10.1002/cbic.201402430>.
- [13] Eichler J. Peptides as protein binding site mimetics. *Curr Opin Chem Biol* 2008 Dec;12(6):707–13. <https://doi.org/10.1016/j.cbpa.2008.09.023>.
- [14] Pelay-Gimeno M, Glas A, Koch O, Grossmann TN. Structure-Based Design of Inhibitors of Protein-Protein Interactions: Mimicking Peptide Binding Epitopes. *Angew Chem Int Ed* 2015 Jul 27;54(31):8896–927. <https://doi.org/10.1002/anie.201412070>.
- [15] Datki Z, Papp R, Zádori D, Soós K, Fülöp L, Juhász A, et al. *in vitro* model of neurotoxicity of A β 1–42 and neuroprotection by a pentapeptide: Irreversible events during the first hour. *Neurobiol Dis* 2004 Dec;17(3):507–15. <https://doi.org/10.1016/j.nbd.2004.08.007>. PubMed PMID: 15571986.
- [16] Granic I, Masman MF, (Kees) Mulder C, Nijholt IM, Naude PJW, de Haan A, et al. LPYFDa Neutralizes Amyloid- β -Induced Memory Impairment and Toxicity. *J Alzheimer's Dis* 2010 Jan 28;19(3):991–1005. <https://doi.org/10.3233/JAD-2010-1297>.
- [17] Tanaka F, Shibata K, Monobe Y, Akagi K ichi, Masuda Y. Design and synthesis of β -strand-fixed peptides inhibiting aggregation of amyloid β -protein. *Bioorg Med Chem* 2020 Sep;28(18):115676. <https://doi.org/10.1016/j.bmc.2020.115676>.
- [18] Khairnar BD, Jha A, Rajwade JM. Rationally designed cyclic peptides and nanomaterials as 'next-generation' anti-amyloid therapeutics. *J Mater Sci* 2023 Jun 9;58(24):9834–60. <https://doi.org/10.1007/s10853-023-08654-6>.
- [19] Ikenoue T, Aprile FA, Sormanni P, Ruggeri FS, Perni M, Heller GT, et al. A rationally designed bicyclic peptide remodels A β 42 aggregation *in vitro* and reduces its toxicity in a worm model of Alzheimer's disease. *Sci Rep* 2020 Sep 17;10(1):15280. <https://doi.org/10.1038/s41598-020-69626-3>.
- [20] Kreutzer AG, Guaglianone G, Yoo S, Parrocha CMT, Ruttenberg SM, Malonis RJ, et al. Probing differences among A β oligomers with two triangular trimers derived from A β . *Proc Natl Acad Sci* 2023 May 30;120(22). <https://doi.org/10.1073/pnas.2219216120>.
- [21] Lu J, Cao Q, Wang C, Zheng J, Luo F, Xie J, et al. Structure-Based Peptide Inhibitor Design of Amyloid- β Aggregation. *Front Mol Neurosci* 2019 Mar 4;12. <https://doi.org/10.3389/fnmol.2019.00054>.
- [22] Luo J, Abrahams JP. Cyclic Peptides as Inhibitors of Amyloid Fibrillation. *Chem A Eur J* 2014 Feb 24;20(9):2410–9. <https://doi.org/10.1002/chem.201304253>.
- [23] Richman M, Wilk S, Chmerovskiy M, Wärmländer SKTS, Wahlström A, Gräslund A, et al. *in vitro* and mechanistic studies of an anti-amyloidogenic self-assembled cyclic D,L- α -peptide architecture. *J Am Chem Soc* 2013 Mar 6;135(9):3474–84. <https://doi.org/10.1021/ja310064v>. PubMed PMID: 23360549.
- [24] Yamin G, Ruchala P, Teplow DB. A peptide hairpin inhibitor of amyloid β -protein oligomerization and fibrillogenesis. *Biochemistry* 2009 Nov;48(48):11329–31. <https://doi.org/10.1021/bi901325g>. PubMed PMID: 19877710.
- [25] Sisodia S, Koo E, Hoffman P, Perry G, Price D. Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system. *J Neurosci* 1993 Jul 1;13(7):3136–42. <https://doi.org/10.1523/JNEUROSCI.13-07-03136.1993>.
- [26] Tanaka S, Shiojiri S, Takahashi Y, Kitaguchi N, Ito H, Kameyama M, et al. Tissue-specific expression of three types of β -protein precursor mRNA: Enhancement of protease inhibitor-harboring types in Alzheimer's disease brain. *Biochem Biophys Res Commun* 1989 Dec;165(3):1406–14. [https://doi.org/10.1016/0006-291X\(89\)92760-5](https://doi.org/10.1016/0006-291X(89)92760-5).
- [27] Puig KL, Combs CK. Expression and function of APP and its metabolites outside the central nervous system. *Exp Gerontol* 2013 Jul;48(7):608–11. <https://doi.org/10.1016/j.exger.2012.07.009>.
- [28] Guo Y, Wang Q, Chen S, Xu C. Functions of amyloid precursor protein in metabolic diseases. *Metabolism* 2021 Feb;115:154454. <https://doi.org/10.1016/j.metabol.2020.154454>.
- [29] Cho Y, Bae HG, Okun E, Arumugam TV, Jo DG. Physiology and pharmacology of amyloid precursor protein. *Pharmacology and Therapeutics*. Elsevier Inc; 2022. <https://doi.org/10.1016/j.pharmthera.2022.108122>. PubMed PMID: 35114285.
- [30] Kang J, Müller-Hill B. Differential splicing of Alzheimer's disease amyloid A4 precursor RNA in rat tissues: PreA4695 mRNA is predominantly produced in rat and human brain. *Biochem Biophys Res Commun* 1990 Feb;166(3):1192–200. [https://doi.org/10.1016/0006-291X\(90\)90992-V](https://doi.org/10.1016/0006-291X(90)90992-V).
- [31] Matsui T, Ingelsson M, Fukumoto H, Ramasamy K, Kowa H, Frosch MP, et al. Expression of APP pathway mRNAs and proteins in Alzheimer's disease. *Brain Res* 2007 Aug;1161:116–23. <https://doi.org/10.1016/j.brainres.2007.05.050>.
- [32] Cohen I, Kayode O, Hockla A, Sankaran B, Radisky DC, Radisky ES, et al. Combinatorial protein engineering of proteolytically resistant mesotrypsin inhibitors as candidates for cancer therapy. *Biochem J* 2016 May 15;473(10):1329–41. <https://doi.org/10.1042/BJ20151410>. PubMed PMID: 26957636.
- [33] Salameh MA, Robinson JL, Navaneetham D, Sinha D, Madden BJ, Walsh PN, et al. The Amyloid Precursor Protein/Protease Nexin 2 Kunitz Inhibitor Domain Is a Highly Specific Substrate of Mesotrypsin. *J Biol Chem* 2010 Jan;285(3):1939–49. <https://doi.org/10.1074/jbc.M109.057216>.
- [34] Jiang G, Cao F, Ren G, Gao D, Bhakta V, Zhang Y, et al. PRSS3 promotes tumour growth and metastasis of human pancreatic cancer. *Gut* 2010 Nov 1;59(11):1535–44. <https://doi.org/10.1136/gut.2009.200105>.
- [35] Lacham-Hartman S, Moshe R, Ben-Zichri S, Shmidov Y, Bitton R, Jelinek R, et al. APP-Derived Cyclic Peptide Enhances A β 42 Aggregation and Reduces A β 42-Mediated Membrane Destabilization and Cytotoxicity. *ACS Chem Neurosci* 2023 Nov. <https://doi.org/10.1021/acscchemneuro.3c00208>. PubMed PMID: 37579500.
- [36] Lacham-Hartman S, Oren O, Rabinovitch M, Papo N. Neuroprotective functions of the APP domain of amyloid precursor protein (APP): Reducing KLK6-mediated APP cleavage and A β 42 formation and aggregation. *Int J Biol Macromol* 2025 Nov; 320. <https://doi.org/10.1016/j.ijbiomac.2025.146066>.
- [37] Pendlebury D, Wang R, Henin RD, Hockla A, Soares AS, Madden BJ, et al. Sequence and conformational specificity in substrate recognition: several human Kunitz protease inhibitor domains are specific substrates of mesotrypsin. *J Biol Chem* 2014 Nov 21;289(47):32783–97. <https://doi.org/10.1074/jbc.M114.609560>. PubMed PMID: 25301953.
- [38] Xu Y., Carr P.D., Guss J.M., Ollis D.L. The Crystal Structure of Bikunin from the Inter- α -a-Inhibitor Complex: A Serine Protease Inhibitor with Two Kunitz Domains. 1998.
- [39] Chang J.Y., Ballatore A. The structure of denatured bovine pancreatic trypsin inhibitor (BPTI).
- [40] Schmidt AE, Chand HS, Cascio D, Kisiel W, Bajaj SP. Crystal Structure of Kunitz Domain 1 (KD1) of Tissue Factor Pathway Inhibitor-2 in Complex with Trypsin. *J Biol Chem* 2005 Jul;280(30):27832–8. <https://doi.org/10.1074/jbc.M504105200>.
- [41] Cho PY, Joshi G, Boersma MD, Johnson JA, Murphy RM. A cyclic peptide mimic of the β -amyloid binding domain on transthyretin. *ACS Chem Neurosci* 2015 May 20; 6(5):778–89. <https://doi.org/10.1021/cn500272a>. PubMed PMID: 25713928.
- [42] Manning MC, Illangasekare M, W. Woody R. Circular dichroism studies of distorted α -helices, twisted β -sheets, and β -turns. *Biophys Chem* 1988 Aug 1;31(1–2):77–86. [https://doi.org/10.1016/0301-4622\(88\)80011-5](https://doi.org/10.1016/0301-4622(88)80011-5). PubMed PMID: 3233294.
- [43] Barrow CJ, Yasuda A, Kenny PTM, Zagorski MG. Solution conformations and aggregational properties of synthetic amyloid β -peptides of Alzheimer's disease. *J Mol Biol* 1992 Jun;225(4):1075–93. [https://doi.org/10.1016/0022-2836\(92\)90106-T](https://doi.org/10.1016/0022-2836(92)90106-T).
- [44] Salomon AR, Marciniowski KJ, Friedland RP, Zagorski MG. Nicotine Inhibits Amyloid Formation by the β -Peptide. *Biochemistry* 1996 Jan 1;35(42):13568–78. <https://doi.org/10.1021/bi9617264>.
- [45] Wang H, Duo L, Hsu F, Xue C, Lee YK, Guo Z. Polymorphic A β 42 fibrils adopt similar secondary structure but differ in cross-strand side chain stacking interactions within the same β -sheet. *Sci Rep* 2020 Mar 31;10(1):5720. <https://doi.org/10.1038/s41598-020-62181-x>.
- [46] Qiang W, Yau WM, Lu JX, Collinge J, Tycko R. Structural variation in amyloid- β fibrils from Alzheimer's disease clinical subtypes. *Nature* 2017 Jan 4;541(7636):217–21. <https://doi.org/10.1038/nature20814>.
- [47] Ochiishi T, Doi M, Yamasaki K, Hirose K, Kitamura A, Urabe T, et al. Development of new fusion proteins for visualizing amyloid- β oligomers *in vivo*. *Sci Rep* 2016 Mar 16;6(1):22712. <https://doi.org/10.1038/srep22712>.
- [48] Bu G, Cam J, Zerbinatti C. LRP in amyloid- β production and metabolism. *Annals of the New York Academy of Sciences*. Blackwell Publishing Inc; 2006. p. 35–53. <https://doi.org/10.1196/annals.1377.005>. PubMed PMID: 17185504.
- [49] Nagele R.G., D'andrea M.R., Anderson W.J., Wang H.Y. INTRACELLULAR ACCUMULATION OF L-AMYLOID 1A42 IN NEURONS IS FACILITATED BY THE K7 NICOTINIC ACETYLCHOLINE RECEPTOR IN ALZHEIMER'S DISEASE [Internet]. Available from: www.neuroscience-ibro.com.
- [50] Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW. Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proc Natl Acad Sci* 1993 Sep;90(17):7951–5. <https://doi.org/10.1073/pnas.90.17.7951>.
- [51] LaFerla FM, Green KN, Oddo S. Intracellular amyloid- β in Alzheimer's disease. *Nat Rev Neurosci* 2007;499–509. <https://doi.org/10.1038/nrn2168>. PubMed PMID: 17551515.
- [52] Demuro A, Parker I. Cytotoxicity of Intracellular A β 42 Amyloid Oligomers Involves Ca²⁺ Release from the Endoplasmic Reticulum by Stimulated Production of Inositol Triphosphate. *J Neurosci* 2013 Feb 27;33(9):3824–33. <https://doi.org/10.1523/JNEUROSCI.4367-12.2013>.
- [53] Awasthi A, Matsunaga Y, Yamada T. Amyloid-beta causes apoptosis of neuronal cells via caspase cascade, which can be prevented by amyloid-beta-derived short peptides. *Exp Neurol* 2005 Dec 1;196(2):282–9. <https://doi.org/10.1016/j.expneurol.2005.08.001>. PubMed PMID: 16137679.
- [54] van Engeland M, Nieland LJW, Ramaekers FCS, Schutte B, Reutelingsperger CPM. Annexin V-Affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 1998 Jan 1;31(1):1–9. [https://doi.org/10.1002/\(SICI\)1097-0320\(19980101\)31:1<1::AID-CYTO1>3.0.CO;2-R](https://doi.org/10.1002/(SICI)1097-0320(19980101)31:1<1::AID-CYTO1>3.0.CO;2-R).
- [55] Thakur S, Cattoni DI, Nöllmann M. The fluorescence properties and binding mechanism of SYTOX green, a bright, low photo-damage DNA intercalating agent. *Eur Biophys J* 2015 Jul 31;44(5):337–48. <https://doi.org/10.1007/s00249-015-1027-8>.

- [56] Han XJ, Hu YY, Yang ZJ, Jiang LP, Shi SL, Li YR, et al. Amyloid β -42 induces neuronal apoptosis by targeting mitochondria. *Mol Med Rep* 2017 Oct;16(4): 4521–8. <https://doi.org/10.3892/mmr.2017.7203>.
- [57] Manczak M, Anekonda TS, Henson E, Park BS, Quinn J, Reddy PH. Mitochondria are a direct site of A β accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. *Hum Mol Genet* 2006 May 1;15(9):1437–49. <https://doi.org/10.1093/hmg/ddl066>.
- [58] Reddy PH. Mitochondrial Dysfunction in Aging and Alzheimer's Disease: Strategies to Protect Neurons. *Antioxid Redox Signal* 2007 Oct;9(10):1647–58. <https://doi.org/10.1089/ars.2007.1754>.
- [59] Gao F, Hou Y, Wang Y, Liu L, Yi X, Xia N. Photothermal and Photodynamic Strategies for Diagnosis and Therapy of Alzheimer's Disease by Modulating Amyloid- β Aggregation. *Biosens (Basel)* 2025 Jul 24;15(8). <https://doi.org/10.3390/bios15080480>. PubMed PMID: 40862941.
- [60] Wang X, Zhang W, Hou L, Geng W, Wang J, Kong Y, et al. A Biomimetic Upconversion Nanobait-Based Near Infrared Light Guided Photodynamic Therapy Alleviates Alzheimer's Disease by Inhibiting β -Amyloid Aggregation. *Adv Healthc Mater* 2024 Apr;13(9):e2303278. <https://doi.org/10.1002/adhm.202303278>. PubMed PMID: 38112336.
- [61] Xie S, Liang Y, Song Y, Li T, Jia J. Repurposing Anidulafungin for Alzheimer's Disease via Fragment-Based Drug Discovery. *ACS Chem Neurosci* 2024 Aug 21;15(16):2995–3008. <https://doi.org/10.1021/acschemneuro.4c00150>.
- [62] Sisodia S, Koo E, Hoffman P, Perry G, Price D. Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system. *J Neurosci* 1993 Jul 1;13(7):3136–42. <https://doi.org/10.1523/JNEUROSCI.13-07-03136.1993>.
- [63] Piazza F, Galimberti G, Conti E, Isella V, Perlangeli MV, Speranza T, et al. Increased tissue factor pathway inhibitor and homocysteine in Alzheimer's disease. *Neurobiol Aging* 2012 Feb;33(2):226–33. <https://doi.org/10.1016/j.neurobiolaging.2010.02.016>.
- [64] Hollister RD, Kiesel W, Hyman BT. Immunohistochemical localization of tissue factor pathway inhibitor-1 (TFPI-1), a Kunitz proteinase inhibitor, in Alzheimer's disease. *Brain Res* 1996 Jul;728(1):13–9. [https://doi.org/10.1016/0006-8993\(96\)00384-8](https://doi.org/10.1016/0006-8993(96)00384-8).
- [65] Favre I, Moss GWJ, Goldenberg DP, Otlewski J, Moczydlowski E. Structure-activity relationships for the interaction of bovine pancreatic trypsin inhibitor with an intracellular site on a large conductance Ca $^{2+}$ -activated K $^{+}$ channel. *Biochemistry* 2000 Feb 29;39(8):2001–12. <https://doi.org/10.1021/bi992140v>. PubMed PMID: 10684650.
- [66] Shuaib S, Narang SS, Goyal D, Goyal B. Computational design and evaluation of β -sheet breaker peptides for destabilizing Alzheimer's amyloid- β 42 protofibrils. *J Cell Biochem* 2019 Oct 1;120(10):17935–50. <https://doi.org/10.1002/jcb.29061>. PubMed PMID: 31162715.
- [67] Roy R, Khan J, Pradhan K, Nayak P, Sarkar A, Nandi S, et al. Short Peptoid Evolved from the Key Hydrophobic Stretch of Amyloid- β 42 Peptide Serves as a Potent Therapeutic Lead of Alzheimer's Disease. *ACS Chem Neurosci* 2023 Jan 18;14(2): 246–60. <https://doi.org/10.1021/acschemneuro.2c00549>. PubMed PMID: 36583718.
- [68] Taylor M, Moore S, Mayes J, Parkin E, Beeg M, Canovi M, et al. Development of a Proteolytically Stable Retro-Inverso Peptide Inhibitor of β -Amyloid Oligomerization as a Potential Novel Treatment for Alzheimer's Disease. *Biochemistry* 2010 Apr 20;49(15):3261–72. <https://doi.org/10.1021/bi100144m>.
- [69] Koszla O, Solek P. Misfolding and aggregation in neurodegenerative diseases: protein quality control machinery as potential therapeutic clearance pathways. *Cell Commun Signal* 2024 Aug 30;22(1):421. <https://doi.org/10.1186/s12964-024-01791-8>.
- [70] Muschol M, Hoyer W. Amyloid oligomers as on-pathway precursors or off-pathway competitors of fibrils. *Front Mol Biosci* 2023 Feb 9;10. <https://doi.org/10.3389/fmolb.2023.1120416>.
- [71] Dear AJ, Meisl G, Šarić A, Michaels TCT, Kjaergaard M, Linse S, et al. Identification of on- and off-pathway oligomers in amyloid fibril formation. *Chem Sci* 2020;11(24):6236–47. <https://doi.org/10.1039/C9SC06501F>.
- [72] Arai T, Sasaki D, Araya T, Sato T, Sohma Y, Kanai M. A cyclic KLVFF-derived peptide aggregation inhibitor induces the formation of less-toxic off-pathway amyloid- β oligomers. *ChemBioChem* 2014 Nov 24;15(17):2577–83. <https://doi.org/10.1002/cbic.201402430>. PubMed PMID: 25262917.
- [73] Costa R, Ferreira-da-Silva F, Saraiva MJ, Cardoso I. Transthyretin protects against A-beta peptide toxicity by proteolytic cleavage of the peptide: A mechanism sensitive to the kunitz protease inhibitor. *PLoS One* 2008 Aug 6;3(8). <https://doi.org/10.1371/journal.pone.0002899>. PubMed PMID: 18682830.
- [74] Costa R, Gonçalves A, Saraiva MJ, Cardoso I. Transthyretin binding to A-Beta peptide – Impact on A-Beta fibrillogenesis and toxicity. *FEBS Lett* 2008 Mar 19;582(6):936–42. <https://doi.org/10.1016/J.FEBSLET.2008.02.034>. PubMed PMID: 18295603.
- [75] Cho PY, Joshi G, Boersma MD, Johnson JA, Murphy RM. A cyclic peptide mimic of the β -amyloid binding domain on transthyretin. *ACS Chem Neurosci* 2015 May 20; 6(5):778–89. <https://doi.org/10.1021/cn500272a>. PubMed PMID: 25713928.
- [76] Chen G fang, Xu T hai, Yan Y, Zhou Y ren, Jiang Y, Melcher K, et al. Amyloid beta: structure, biology and structure-based therapeutic development. *Acta Pharmacol Sin* 2017 Sep 17;38(9):1205–35. <https://doi.org/10.1038/aps.2017.28>.
- [77] Bartolini M, Naldi M, Fiori J, Valle F, Biscarini F, Nicolau DV, et al. Kinetic characterization of amyloid-beta 1–42 aggregation with a multimethodological approach. *Anal Biochem* 2011 Jul 15;414(2):215–25. <https://doi.org/10.1016/J.AB.2011.03.020>. PubMed PMID: 21435333.
- [78] Scharfmann E, Schemmert S, Niemietz N, Honold D, Ziehmer T, Tusche M, et al. Vitro Potency and Preclinical Pharmacokinetic Comparison of All-D-Enantiomeric Peptides Developed for the Treatment of Alzheimer's Disease. *J Alzheimer's Dis* 2018 Jul 3;64(3):859–73. <https://doi.org/10.3233/JAD-180165>.
- [79] Jha A, Kumar MG, Gopi HN, Paknikar KM. Inhibition of β -Amyloid Aggregation through a Designed β -Hairpin Peptide. *Langmuir* 2018 Jan 30;34(4):1591–600. <https://doi.org/10.1021/acs.langmuir.7b03617>. PubMed PMID: 29284085.
- [80] Niu Z, Zhang Z, Zhao W, Yang J. Interactions between amyloid β peptide and lipid membranes. *Biochim Et Biophys Acta (BBA) - Biomembr* 2018 Sep;1860(9): 1663–9. <https://doi.org/10.1016/j.bbamem.2018.04.004>.
- [81] Oren O, Taube R, Papo N. Amyloid β structural polymorphism, associated toxicity and therapeutic strategies. *Cellular and Molecular Life Sciences*. Springer Science and Business Media Deutschland GmbH; 2021. p. 7185–98. <https://doi.org/10.1007/s00018-021-03954-z>. PubMed PMID: 34643743.
- [82] Cohen I, Coban M, Shahar A, Sankaran B, Hockla A, Lacham S, et al. Disulfide engineering of human Kunitz-type serine protease inhibitors enhances proteolytic stability and target affinity toward mesotrypsin. *J Biol Chem* 2019 Mar;294(13): 5105–20. <https://doi.org/10.1074/jbc.RA118.007292>.
- [83] Heyne M, Shirian J, Cohen I, Peleg Y, Radisky ES, Papo N, et al. Climbing up and down binding landscapes through deep mutational scanning of three homologous protein-protein complexes. *J Am Chem Soc* 2021 Oct 20;143(41):17261–75. <https://doi.org/10.1021/jacs.1c08707>. PubMed PMID: 34609866.
- [84] Oren O, Banerjee V, Taube R, Papo N. An A β 42 variant that inhibits intra- and extracellular amyloid aggregation and enhances cell viability. *Biochem J* 2018 Oct 15;475(19):3087–103. <https://doi.org/10.1042/BJC20180247>.