



Neuroprotective functions of the APPI domain of amyloid precursor protein (APP): Reducing KLK6-mediated APP cleavage and A β 42 formation and aggregation

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ABSTRACT

Amyloid beta 42 (A β 42) and amyloid precursor protein inhibitor (APPI) are amyloid precursor protein (APP) domains. As fragments, A β 42 is neurotoxic and APPI inhibits kallikrein-related peptidase 6 (KLK6). While KLK6-dependent cleavage of APP695 (an APP isoform that lacks APPI) has been shown to result in A β production, its proteolytic activity on APPI-containing isoforms (APP751 and APP770) and the molecular function of APPI in APP proteolysis have not been explored. In addressing this knowledge gap, we show that APP695 serves as a KLK6 substrate in SH-SY5Y neuroblastoma cells. We also show that APPI, as either a soluble fragment or an APP751 domain, strongly inhibits KLK6 catalytic activity and reduces KLK6-mediated APP695 cleavage in a dose-dependent manner. Moreover, the soluble extracellular APPI fragment reduces A β 42 aggregation and fibril length, thereby favoring the formation of A β 42 oligomers that cannot permeate into cells and thus significantly reducing apoptosis in SH-SY5Y neuroblastoma cells. In a similar way, the expression of both intracellular APPI and A β 42 (vs. A β 42 alone) reduces A β 42 aggregate formation and increases neuronal cell viability. Our results provide an explanation for the presence of alternatively spliced APP isoforms in the brains of patients with Alzheimer's disease (AD) and reveal the roles of APPI in APP cleavage and in the formation, aggregation and toxicity of both extra- and intracellular A β peptide fragments. Our results suggest that APPI may serve as a dual inhibitor, disrupting both KLK6 proteolytic activity and A β 42 aggregation, thereby offering a potential therapeutic agent for reducing amyloid beta toxicity in AD.

1. Introduction

A comprehensive understanding of the etiology underlying Alzheimer's disease (AD) remains as elusive as ever. It may be said, however, that there is universal acceptance of the idea that there will not be one single light-bulb moment, but that the understanding will be built up study by study on different physiological, genetic and environmental aspects of the disease. One of the major current research directions focuses on amyloid precursor protein (APP), which is uniquely associated with the pathology of AD, since the amyloid-beta (A β) peptide sequence that has been implicated in AD is found exclusively in APP [1], and not in the other members of the mammalian APP superfamily, i.e., the APP-like proteins-1 and 2 (APLP1 and APLP2) that share high sequence

similarity with APP [2].

Due to alternative splicing, APP exists in three major isoforms, APP695, APP751, and APP770, with molar masses ranging from 110 to 140 kDa [3]. The extracellular regions of the latter two isoforms – but not of APP695 – include the Kunitz protease inhibitor domain (KPI), also known as protease nexin 2 or amyloid precursor protein inhibitor (APPI) [4,5]. Previous studies have shown that APP695 is predominantly expressed in healthy human brains, while the other two APPI-containing isoforms are widely distributed in other body tissues of healthy individuals and – importantly – in the brains of patients with AD [6,7]; e.g., it has been shown that there is an increase in mRNA expression levels of APP751 and APP770 compared to APP695 in AD brains [8,9].

Despite the well-known expression and distribution profiles of the

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three APP isoforms in healthy human brains and the brains of patients with AD, the significance of APP alternative splicing and the roles played by APPI in the brains of AD patients are not well understood. To address some of the questions that still remain open, we focus on the APP fragment, APPI, and show for the first time that this domain plays a dual role in preventing neurodegeneration, first, by impeding the cleavage of APP that generates the potentially neurotoxic A β 42 peptide and, second, by inhibiting the aggregation of A β 42 into neurotoxic A β 42 oligomers and the subsequent internalization and apoptotic effects of these oligomers.

To date, research on the role in AD of full-length APP in the central nervous system (CNS) [10] has concentrated primarily on its function as a cell surface receptor that promotes cell adhesion and interactions with the extracellular matrix (ECM) through its extracellular domain [11,12]. This function enables A β peptide fragments, cleaved from APP by β - and γ -secretases [13,14], to attach to the neuronal surface, triggering the intracellular signaling that leads to neuronal toxicity [15–17]. APP is abundantly expressed both on plasma membranes and on the membranes of organelles, such as the endoplasmic reticulum, the Golgi apparatus and mitochondria [18,19], and, as such, it is localized both outside and inside neuronal cells [20]. Thus, proteolysis of APP can occur both intracellularly and extracellularly, although numerous studies have shown that most of the intraneuronal A β may be attributed to the uptake of extracellular A β fragments [20,21].

APPI is a potent inhibitor of the serine protease, kallikrein-related peptidase 6 (KLK6), which is highly expressed in the CNS and the spinal cord [22]. KLK6, in turn – like β - and γ -secretases [13,23] – cleaves APP at the C-terminal to release neurotoxic A β peptide fragments of ~4 kDa, including A β 42 [24–26]. Changes in KLK6 expression levels have been correlated to AD [27,28], with several studies suggesting that KLK6 promotes AD pathogenesis via two simultaneous pathways: (i) by degrading the laminin component of the ECM, thereby preventing laminin from inhibiting A β 42 aggregation and fibril formation, which results in neuronal death [29,30], and (ii) by cleaving extracellular APP, leading to A β formation. The second pathway has indeed been demonstrated experimentally [24], but only in HEK293 cells and not for purified APP.

Whereas A β 42 is generated by the cleavage of APP by KLK6 or sequentially by β - and γ -secretases [13,14,24], APPI is the cleavage product of APP proteolysis by either α - and β -secretases [14,31] or by KLK6 [25]. Numerous reports over the years have demonstrated the inhibitory effect of APPI on serine proteases in general [32,33], including KLK6 [34–36]. Yet, none has shown whether there is a correlation between the high expression levels of APP751 and APP770, both of which contain the APPI domain, and the presence of KLK6 in the brains of AD patients. Specifically, no studies have examined whether APP, via its APPI domain, can inhibit the proteolytic activity of KLK6 and the subsequent formation and accumulation (both extra- and intracellularly) of A β 42.

In addition to its effect on A β 42 formation and accumulation in neuronal cells, it is likely that APPI also affects A β 42 aggregation and the formation of neurotoxic species both extra- and intracellularly. This premise is based on our recent findings showing that a cyclic 20-mer synthetic peptide that mimics a β -hairpin structural component of APPI interacts with A β 42, changing the oligomeric pathway of A β 42 to favor the formation of non-toxic high molar weight A β 42 aggregates (i.e., fibrils) rather than the more neurotoxic low molecular weight A β 42 species [37].

Against the above background, we sought to evaluate the two inhibitory pathways that A β 42 may undergo in concert, the first via inhibition of KLK6 catalytic activity and subsequent A β 42 formation and the second through interference with A β 42 aggregation. Herein, we demonstrate that APP751, specifically via its APPI domain, effectively inhibits APP cleavage by KLK6 (K_i value in the nanomolar range) in SH-SY5Y neuroblastoma cells, whereas APP695, which lacks the APPI domain, undergoes excessive cleavage. In addition, similar to the short

synthetic APPI-derived β -hairpin peptide, the entire isolated APPI domain interacts with A β 42 oligomers in such a way that A β 42 aggregation is reduced, with a molar ratio of APPI:A β 42 of 1:64 giving optimal inhibition. Moreover, treatment of SH-SY5Y cells with either intracellular (overexpressed) or extracellular (purified) APPI reduces the formation of intracellular A β 42 aggregates and prevents the internalization of extracellular A β 42, respectively. Importantly, in both pathways, A β 42-mediated cytotoxicity is reduced.

2. Results

2.1. APP695 is a natural substrate for KLK6 in SH-SY5Y and HEK293 cells

Two previous observations motivated us to determine whether KLK6 has differential proteolytic capabilities for the two APP isoforms, APP695 and APP751, namely, (i) elevated levels of APP751 and APP770 isoforms (which contain the APPI domain) were observed in the brains of AD patients, whereas in healthy brains the predominant isoform expressed was APP695 (which lacks the APPI domain) [8,9,38], and (ii) the levels of KLK6 that had diffused from the CSF into the blood of AD patients were higher than those in healthy individuals [27,28]. For this part of the study, we used SH-SY5Y neuroblastoma cells [a well-characterized model for neuronal disorders [39]] transfected with genes encoding for secreted KLK6 and for APP695 fused at its N-terminal to secreted embryonic alkaline phosphatase (SEAP). The rationale for fusion of the APP695 extracellular region to SEAP was that it allowed us to monitor the cleavage of APP695 by KLK6 spectrophotometrically (Fig. 1A). In this experiment, the cell medium was collected 48 h post-transfection and incubated with 4-nitrophenyl phosphate (pNPP), which is a substrate for alkaline phosphatase and hence a reporter for SEAP release and cleavage of APP695. Cells transfected with SEAP-APP695 exhibited moderate background SEAP activity (i.e., APP695 cleavage or activity when fused to APP695), as indicated by an increase in absorbance values throughout the experiment (Fig. 1A). However, the highest absorbance values were observed for cells expressing both SEAP-APP695 and KLK6 (Fig. 1A), which may be attributed to the elevated level of free SEAP (i.e., cleaved from APP695). This observation suggests that APP695 served as a specific substrate for KLK6.

As mentioned above, a distinguishing characteristic of the APP695 isoform is the absence of APPI, which is a natural serine protease inhibitor with a high affinity for KLK6 (K_i = 2.24 $\pm$ 0.11 nM) [34,40]. Therefore, to confirm the specificity of KLK6 for cleaving APP695, we examined whether exposure to purified soluble APPI of cells co-transfected with SEAP-APP695 and KLK6 would reduce the concentration of free SEAP (i.e., of cleaved APP695) in the culture medium. Pure APPI was prepared as described in Materials and Methods (SI Appendix, Fig. S1 A–C). Figs. 1A and B show a dose-dependent effect on KLK6 inhibition in response to addition to the cell culture medium of different concentrations (1, 5 and 10 μ M) of soluble APPI. The decreased quantities of free SEAP indicate specific cleavage of SEAP-APP695 by KLK6 and inhibition of KLK6 by APPI.

To visualize the cleavage products of APP695 in cells, SH-SY5Y cells were transiently transfected with either APP695 or both APP695- and KLK6-containing vectors. The APP695 gene, fused to two fluorescent proteins, mCherry at the N-terminus and green fluorescent protein (GFP) at the C-terminus (i.e., to extracellular and intracellular regions of APP695, respectively) was inserted into a pcDNA 3.1 vector. The KLK6 gene, which has a secretion factor at the N-terminus for extracellular expression, was inserted into a pHAGE vector. Live cell imaging at 48 h post transfection confirmed co-localization of the two fluorescent proteins on the surface of cells overexpressing APP695 alone (Fig. 1C, upper panel). However, in cells overexpressing both APP695 and KLK6, only the green fluorescence signal was detected on the cell surface, suggesting cleavage of the extracellular region of APP695 by KLK6 (Fig. 1C, lower panel). This cleavage was confirmed in a kinetic experiment

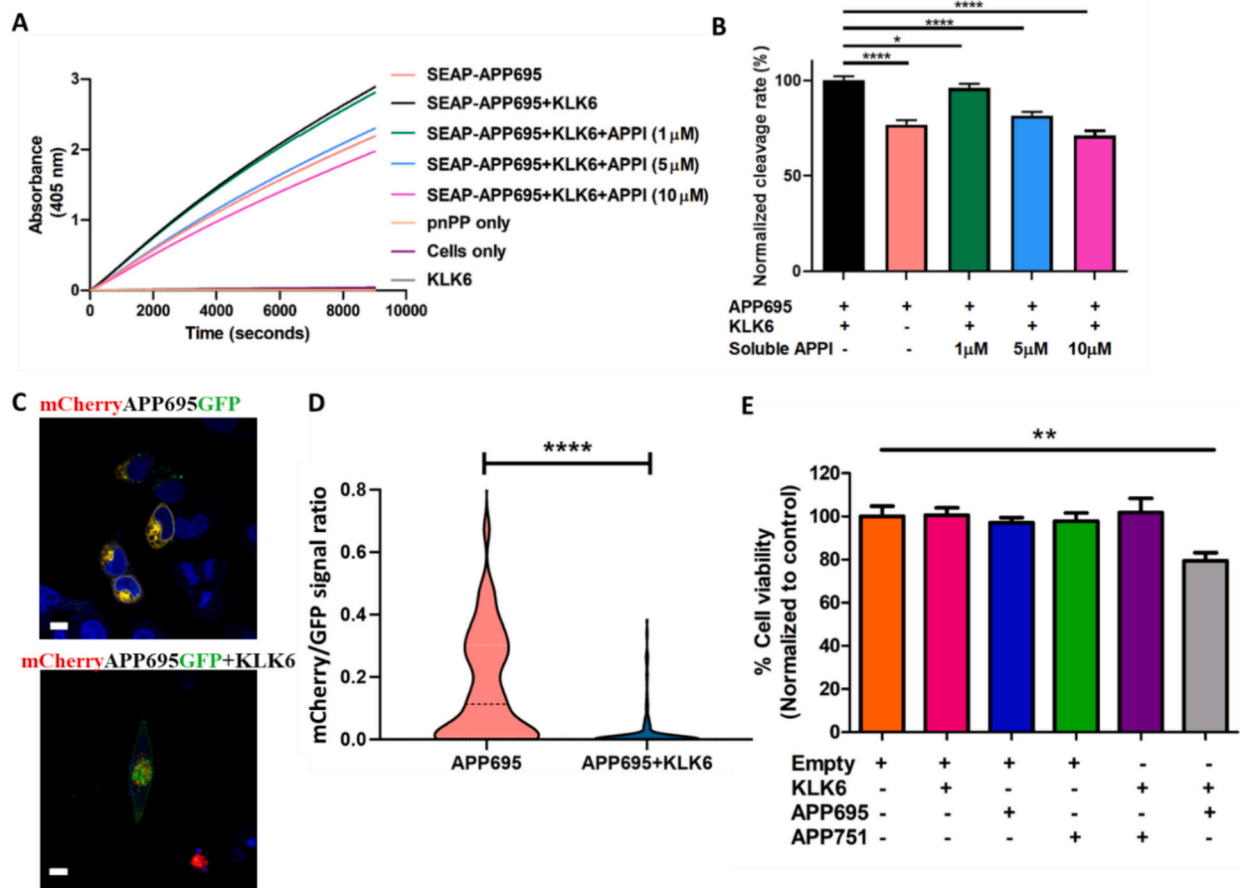


Fig. 1. Both APPI and APP751 reduce cleavage of APP695 by KLK6 and inhibit cell cytotoxicity. (A) Cleavage of the alkaline phosphatase substrate, 4-nitrophenyl phosphate (pnPP), was monitored over 2 h in media collected from cells transfected with different plasmids (containing the SEAP-APP695 or KLK6 gene) and treated with different concentrations of purified APPI. Cells transfected with an empty plasmid served as the control for intrinsic background alkaline phosphatase activity. (B) pnPP cleavage rates (obtained from A) normalized to pnPP cleavage rates of cells overexpressing SEAP-APP695 and KLK6. (C) Confocal microscopy imaging of SH-SY5Y cells with membrane-expressed mCherry-APP695-GFP in the absence or presence of KLK6 overexpression (top and bottom panels, respectively). Scale bar - 10 μm. (D) Violin charts of the ratio of the mCherry fluorescence signal to the GFP fluorescence signal in cells overexpressing either APP695 or both APP695 and KLK6. The Wilcoxon paired test was performed to compare the mCherry/GFP ratios for the APP695 vs. APP695 + KLK6 samples; **** $P \leq 0.0001$. (E) Percent viability (crystal violet staining) of HEK293 cells transiently transfected with different plasmids, as shown on the figure. Control cells were transfected with an empty plasmid. For panels B and E ($n = 5$); data are presented as means $\pm$ SD and statistical analysis was performed using Student's *t*-test, * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$.

monitoring SEAP release (Fig. 1A). In addition, we determined the mCherry/GFP signal ratio, since the mCherry protein is located in the extracellular region of APP695, and its signal is thus expected to decrease as a result of cleavage by KLK6. Indeed, the mCherry/GFP signal ratio was significantly reduced in cells co-transfected with APP695 and KLK6, as indicated in the violin chart in Fig. 1D.

We then went on to examine whether – and how – the proteolytic cleavage by KLK6 of the APP isoform APP695 (lacking APPI) differed from that of APP751 (containing APPI), as manifested in the viability of HEK293 cells. These cells were used for this part of the study, because HEK293 cells overexpressing KLK6 and APP695 were previously reported to generate A β fragments [24]. As shown in Fig. 1E, cells co-transfected with plasmids containing the KLK6 gene or the SEAP-APP695 gene showed a reduction of ~20 % in cell viability 48 h post-transfection compared to control cells (transfected with an empty plasmid). However, cells transfected with both KLK6 and SEAP-APP751 showed similar viability to the control cells, indicating that APP751 had a protective effect against APP cleavage by KLK6. Notably, cells overexpressing KLK6, SEAP-APP695, or SEAP-APP751 showed the same percent viability as the control cells. These observations led us to conclude that KLK6 was involved directly in extracellular APP695 cleavage, and its proteolytic products affected cell viability, whereas cell

viability was preserved by APP751, probably due to the presence of its APPI domain, which inhibits KLK6 activity.

2.2. APPI and APP751 inhibit the proteolytic activity of KLK6

Before determining the effect of the proteolytic activity of KLK6 on the different APP isoforms in the presence of APPI or APP751, it was necessary to prepare purified extracellular domains of APP695 and APP751 protein isoforms, both containing A β 42. To this end, we designed gene constructs for both APP695 and APP751 that contained a 6 $\times$ His tag in the N-terminus but lacked the 17-amino-acid signal peptide (SI Appendix, Fig. S2). The rationale for the removal of the signal peptide derived from a previous study showing not only that removal of the 17-amino-acid signal peptide increased the solubility of APP isoforms but also that such signal peptides self-assemble into cytotoxic aggregates and enhance A β 42 aggregation [41]. The two recombinant APP proteins were then purified by nickel affinity chromatography (SI Appendix, Fig. S3A). SDS-PAGE analysis revealed apparent bands at 71.6 and 80.0 kDa for recombinant APP695 and APP751, respectively, corresponding to their correct theoretical molecular weights (SI Appendix, Fig. S3B), where the difference in the molecular weights was due to the presence of the APPI domain in the APP751 isoform. However,

additional bands ranging between 35 and 60 kDa were also detected. Therefore, for further purification of the proteins, they were subjected to heparin affinity chromatography, taking advantage of the fact that APP isoforms contain numerous heparin-binding domains [12] (*SI Appendix*, Fig. S3B). As shown by SDS-PAGE, the second peak in each heparin chromatogram represented isolated intact APP695 and APP751 proteins (upper and lower graphs, respectively, in *SI Appendix*, Fig. S3C). Purification of the entire sequences of the recombinant proteins was confirmed by western blot analysis using both anti-6 × His tag and anti A β 42 antibodies (*SI Appendix*, Fig. S3D).

With the purified recombinant APP695 and APP751 proteins in hand, our next step was to validate the proteolytic activity of purified recombinant KLK6, for which we used an in-gel zymography assay [42] (*SI Appendix*, Fig. S4). Thereafter, we examined the ability of APPI to inhibit the cleavage of APP695 by KLK6 by incubating APP695 with different concentrations of soluble APPI. While SDS-PAGE analysis of APP695 treated with KLK6 showed several bands attributed to APP695 cleavage by KLK6 (Fig. 2A, lane 2), the addition of soluble APPI prevented APP695 cleavage by KLK6 (Fig. 2A, lanes 3 and 4). Thereafter,

we tested whether APP695 (lacking the APPI domain, an inhibitor of KLK6) and APP751 (containing the APPI domain) were cleaved by KLK6 to different extents. Incubation of the recombinant APP695 or APP751 in the presence or absence of soluble KLK6 and APPI showed that APP695 was indeed hydrolyzed by KLK6, whereas APP751 remained intact, as indicated by an intense band at ~80 kDa (Fig. 2B). This finding suggests that the APPI domain of APP751 conferred an inhibitory effect on proteolytic cleavage of APP751 by KLK6. This conclusion was further strengthened by our findings that the inhibitory effect was conserved when APP751 was treated with KLK6 plus soluble APPI (Fig. 2C).

2.3. Soluble APP751 and APPI inhibit Boc-FSR-AMC cleavage by KLK6

To examine the differences in the ability of the two purified APP isoforms (APP695 and APP751) and APPI to inhibit the enzymatic activity of KLK6, we performed kinetic experiments to follow the cleavage by KLK6 of the well-known fluorogenic substrate, Boc-FSR-AMC [25]. This assay allowed us to quantitatively compare KLK6 inhibition potencies of the two purified APP isoforms and APPI. Monitoring (for 2.5 h) of the

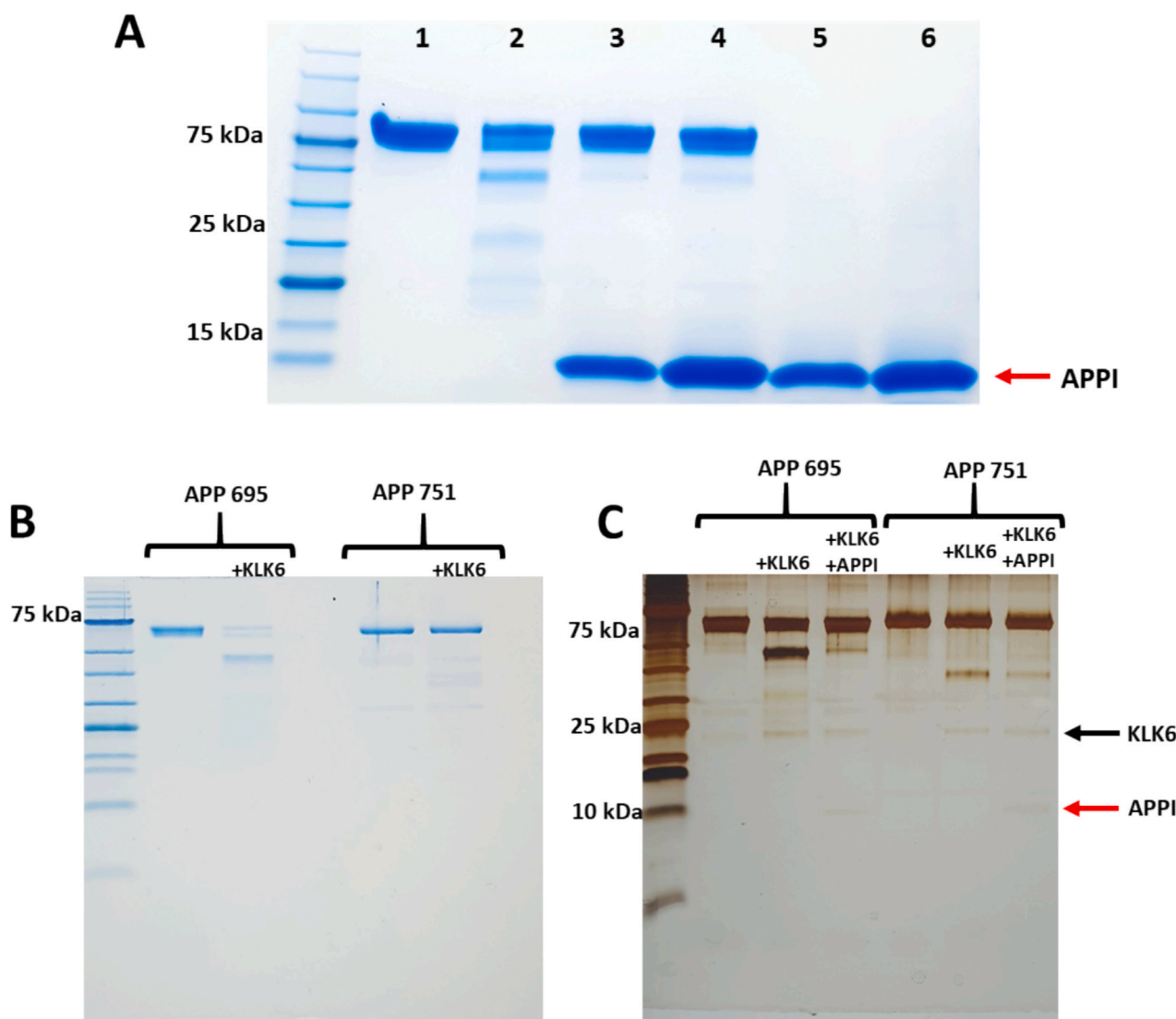


Fig. 2. Cleavage of APP isoforms, APP695 and APP751, by KLK6. (A) 12.5 % SDS-PAGE analysis of different purified protein samples followed by InstantBlue staining. Lanes 1 and 2, respectively: APP695 (3 μ M) was incubated in the absence or presence of KLK6 (0.3 μ M) for 20 h at 37 $^{\circ}$ C. Lanes 3 and 4, respectively: An APP695:KLK6 mixture (3 μ M:0.3 μ M) was incubated with 30 μ M or 60 μ M of APPI. Lanes 5 and 6 represent the amount of APPI that was used in lanes 3 and 4, respectively. All samples were incubated for 20 h at 37 $^{\circ}$ C. (B&C) 12.5 % SDS-PAGE, followed by (B) InstantBlue staining and (C) silver staining of APP695 (4.7 μ M) and APP751 (4.7 μ M) incubated for 16 h at 37 $^{\circ}$ C in the absence or presence of KLK6 or KLK6 + soluble APPI, as shown on the figure. In panel C, black arrow indicates addition of KLK6 (0.47 μ M), and red arrow indicates addition of soluble APPI (4.7 μ M).

changes in the fluorescence intensity as a result of substrate cleavage by KLK6 in the absence of the APP isoforms (i.e., negative control, no substrate cleavage) or in the presence of APP695, APP751, or APP695 + APPI showed that APP695 did not confer a protective effect, whereas both APP751 and APPI significantly reduced the proteolytic effect of KLK6 on the substrate (Fig. 3A and B). These results indicate that the APPI domain within the APP751 sequence retains its inhibitory activity towards KLK6. This observation was verified by determining the exact affinity constants (K_i) of APPI and APP751 for KLK6: K_i values of 4.38 ± 0.12 nM and 70.75 ± 0.94 nM were obtained for APPI and APP751, respectively (Fig. 3C and D).

2.4. The APPI domain reduces the formation of A β 42 aggregates in vitro

The next stage of the study was designed to determine whether APP – in addition to its ability to inhibit A β 42 formation (by inhibiting KLK6 activity) – can also inhibit A β 42 aggregation (which could enhance its potential as a treatment for AD due to a dual inhibition effect). The premise that APPI inhibits A β 42 aggregation was based on our recent work, in which we showed that a beta-hairpin region derived from the APPI sequence is a potent modulator of A β 42 aggregation and reduces A β 42-dependent neuronal toxicity [37]. To evaluate the ability of APPI to serve as an inhibitor of A β 42 aggregation, we first conducted a thioflavin T (ThT) fluorescence assay that monitors A β 42 aggregation, where ThT is a dye that selectively stains A β 42 fibrils [43]. Samples of synthetic A β 42 (4 μ M) were incubated for 10.5 h without and with APPI at concentrations ranging between 6 and 125 nM (Fig. 4A). As expected, A β 42 alone showed typical self-assembly behavior, as indicated by increased intensity of the ThT signal (Fig. 4A). However, treating A β 42 with APPI resulted in a dose-dependent reduction in the fluorescence signal, with the highest inhibition effect on aggregate formation being observed for 62.5 nM APPI (APPI:A β 42 at a molar ratio of 1:64). A high

concentration of APPI (1 μ M), used as a control, did not show any binding to ThT molecules, indicating that APPI did not undergo self-aggregation and that APPI fibrils were not formed during the 10.5 h period of the experiment (Fig. 4A). To confirm these observations and to determine the morphology of A β 42 fibrils in the presence and absence of APPI, we imaged the samples of the ThT assay by transmission electron microscopy (TEM). As expected and in keeping with previous studies [44,45], TEM images revealed that untreated A β 42 formed elongated, unbranched fibrils (Fig. 4B, left panel). However, in the presence of APPI, the quantity of A β 42 aggregates was markedly reduced, and aggregates of shorter species were detected on the micrographs (Fig. 4B, middle and right panels). In addition, TEM images confirmed the dose-dependent inhibition of A β 42 aggregation observed in the ThT assay. Consistent with these findings, an effective molar ratio of APPI:A β 42 of 1:64 was used for further experiments.

2.5. APPI reduces the β -sheet content and toxicity of A β 42 aggregates

To further explore the basis for inhibition of A β 42 aggregation (and fibril formation) by APPI and its effect on A β 42 cellular toxicity, we recorded the circular dichroism (CD) spectra of samples of A β 42 (20 μ M) incubated for 16 h in the presence or absence of APPI (312.5 nM). The signature CD spectrum of untreated A β 42 was consistent with that in previous studies [45], namely, it exhibited the typical peak at 216 nm that represents the well-ordered β -sheet secondary structure of A β 42 aggregates (Fig. 5A). The spectrum of a mixture of A β 42 with APPI confirmed that treatment with APPI reduced the β -sheet content in the A β 42 structure vs. untreated A β 42, whereas APPI alone did not give a detectable β -sheet signal (Fig. 5A).

Using light scattering spectroscopy, we also followed the formation of A β 42 oligomers, as reflected by changes in the absorbance signal at 400 nm in the presence or absence of APPI. Fig. 5B shows the turbidity

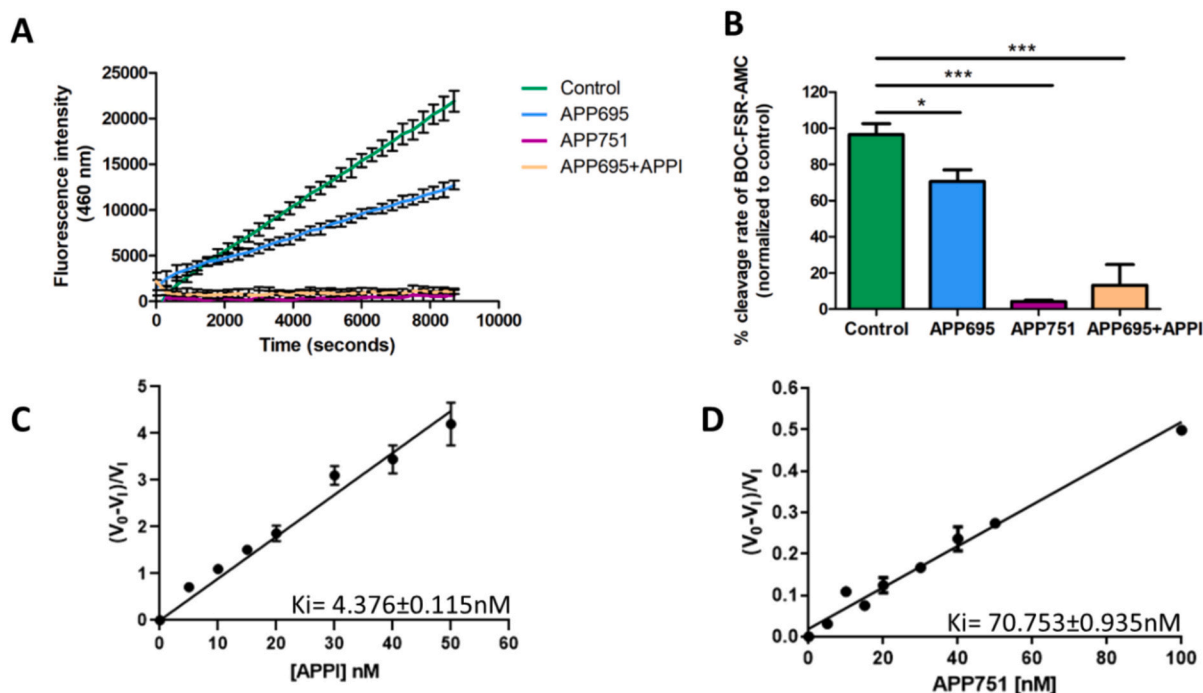


Fig. 3. Purified APPI and APP isoforms have different effects on BOC-FSR-AMC cleavage by KLK6. (A) Cleavage of Boc-FSR-AMC (50 μ M) by KLK6. KLK6 (1 nM) was pre-incubated for 1 h in the absence (control, light green) or presence of different APP isoforms: APP695 (300 nM; light blue) APP751 (300 nM; purple) and APP695 + APPI (300 nM each; light orange). All samples were then monitored for 2.5 h at 37 °C using a Synergy2 microplate spectrophotometer (BioTek). (B) Normalized rates of BOC-FSR-AMC cleavage by KLK6. Data ($n = 3$) are presented as means $\pm$ SD, and statistical analysis was performed using an unpaired Student's t -test; $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. (C and D) Slow tight-binding inhibition of KLK6 catalytic activity by (C) APPI and (D) APP751. K_i values were calculated by using Eq. 1 (see Materials and Methods section). V_0 represents the Boc-FSR-AMC cleavage rate in the absence of KLK6, and V_1 represents the rate in the presence of either APPI or APP751 inhibitors.

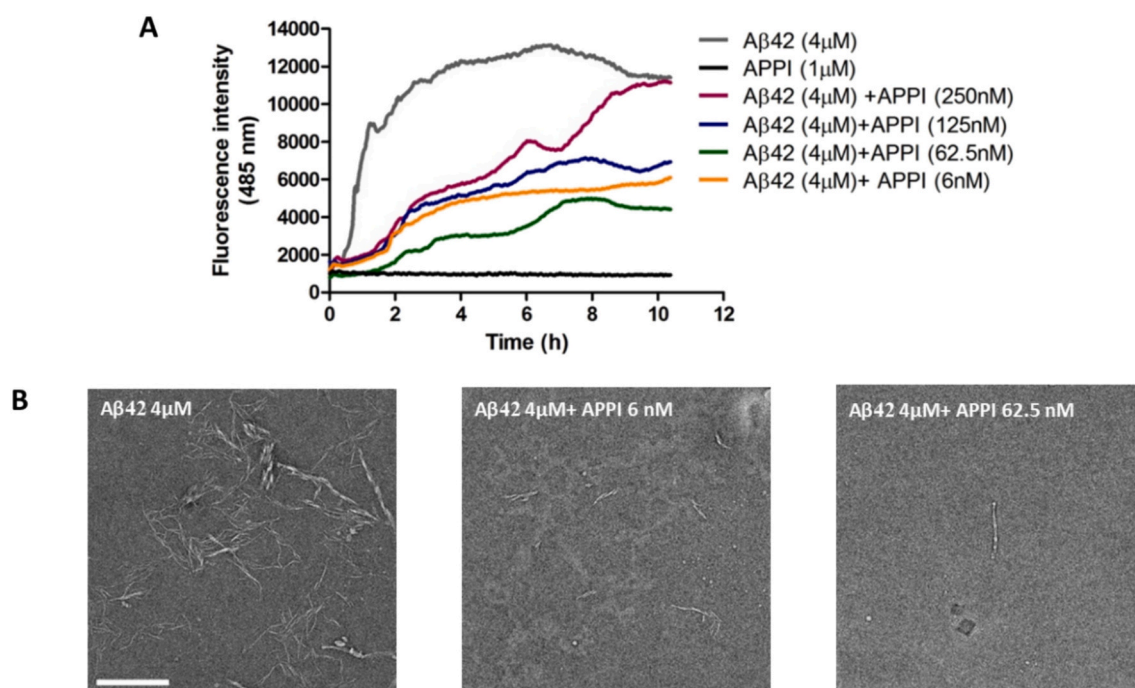


Fig. 4. APPI inhibits Aβ42 aggregate formation in an inverse concentration-dependent manner. (A) ThT assay of the Aβ42 aggregation conducted over 10.5 h with various concentrations of APPI, ranging between 6 and 125 nM. The controls indicated the formation of Aβ42 fibrils in the absence of an inhibitor (gray curve) and no detectable aggregates in the presence of high concentration (1 μM) of APPI alone (black curve). (B) TEM images of Aβ42 fibrils in samples from the ThT assay after 10.5 h of incubation. The scale bar for all the images is equivalent to 200 nm.

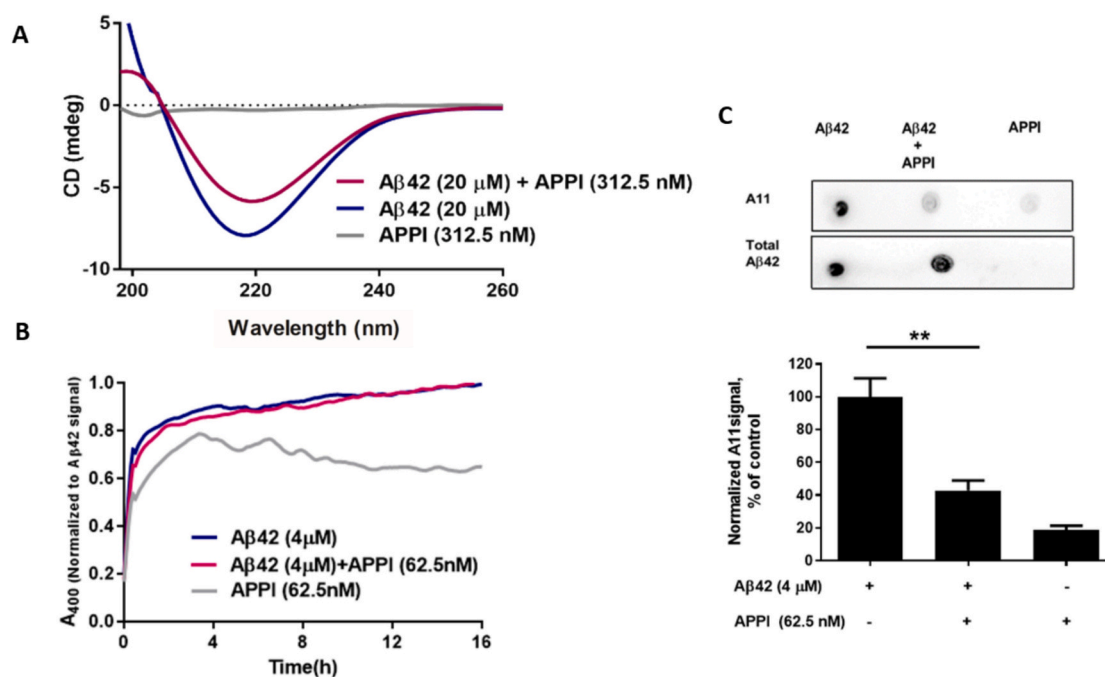


Fig. 5. APPI modulates the Aβ42 aggregation pathway to form less toxic species. (A) Far-UV CD spectra (at 198–260 nm) of treated and untreated Aβ42; blue curve – signature curve of 20 μM Aβ42; purple curve – 20 μM Aβ42 treated with 312.5 nM APPI; and gray curve – signature curve of 312.5 nM APPI. The curves represent the average values of four repetitions. (B) Detection of Aβ42 oligomerization by light scattering spectroscopy (at 400 nm). The samples were incubated and monitored for 16 h at 37 °C with constant shaking. The values were normalized to the absorbance signal of untreated Aβ42. (C) Upper panel: Samples prepared for the light scattering spectroscopy experiment were loaded on a nitrocellulose membrane, and the presence of toxic oligomeric Aβ42 was detected using the A11 antibody. Lower panel: The results were normalized to the intensity of untreated Aβ42. Data ($n = 3$) are presented as means $\pm$ SD, and statistical analysis was performed using an unpaired Student's t -test; $^{**}P \leq 0.01$.

pattern (i.e., oligomerization pattern) of untreated A β 42, which is consistent with that in previous studies [45,46]. This pattern was not affected by APPI, indicating that A β 42 oligomers were also formed in the presence of APPI. To evaluate the toxicity of this oligomeric A β 42, the samples prepared for the light-scattering study were also subjected to a dot blot assay using a conformation-specific antibody, namely, A11, that identifies on-pathway cytotoxic A β oligomers (i.e., oligomeric species that promote the elongation of A β 42 into mature amyloid fibrils) [47]. As shown in Fig. 5C, APPI significantly diminished the recognition of the oligomers by the A11 antibody, with the signal being reduced to 40 %. This result indicates that APPI does not abolish A β 42 oligomerization but instead changes the A β 42 oligomeric pathway towards the formation of off-pathway oligomeric species, previously shown to be less prone to form amyloid fibrils [48,49] and less cytotoxic [50,51] in comparison with untreated A β 42. Taken together, these findings indicate that APPI modulates the A β 42 aggregation pathway.

2.6. APPI enhances the viability of SH-SY5Y cells expressing intracellular A β 42

To examine the ability of APPI to inhibit intracellular A β 42 aggregates (i.e., A β 42 expressed within the cell) and the toxicity of the aggregates, we cloned the APPI gene into a pHAGE2 plasmid with an N-terminal hemagglutinin (HA) tag. Western blot analysis of transiently transfected SH-SY5Y cells validated the expression of the full-size APPI protein (~10 kDa) by the cells (SI Appendix, Fig. S5A). To visualize A β 42 aggregate formation in the presence or absence of APPI, we co-transfected cells either with the A β 42-GFP gene and an empty plasmid or with A β 42-GFP and APPI genes. Confocal microscopy imaging performed 48 h post-transfection revealed that in the absence of APPI, high-intensity GFP signals, indicating accumulation of dense A β 42 aggregates, were observed within the cell (designated by white arrows; Fig. 6A, left panel). However, when APPI was expressed together with A β 42-GFP, the GFP signal was spread out throughout the cell (Fig. 6A, right panel). Notably, western blot analysis confirmed that the cells expressed equal amounts of intracellular A β 42-GFP in the presence or absence of APPI (SI Appendix, Fig. S5B).

For viability determination, an SH-SY5Y cell line stably expressing A β 42 fused to GFP (A β 42-GFP) was generated as described previously [45]. Western blot analysis revealed that the SH-SY5Y cells stably expressed the A β 42-GFP (SI Appendix, Fig. S5C). Thereafter, naïve SH-SY5Y cells and SH-SY5Y cells stably expressing A β 42-GFP were co-transfected with a pHAGE plasmid containing APPI or a plasmid lacking an insert (i.e., an empty plasmid that served as a negative control). Cell viability was determined by an XTT assay 48 h post-transfection. As

shown in Fig. 6B, stable expression of A β 42 significantly reduced cell viability to 71 % in comparison to control cells. However, APPI expression abolished A β 42 toxicity, as indicated by 100 % viability of the SH-SY5Y cells. Notably, the expression of APPI alone did not impair cell viability.

2.7. Extracellular APPI reduces A β 42 internalization and toxicity in SH-SY5Y cells

In addition to the neurotoxicity attributed by intracellular A β 42, extracellular A β 42 has also been shown to exert neurotoxic effects [52–54]. To determine whether APPI inhibits the toxicity of extracellular A β 42 and to make progress in elucidating the mechanism of this inhibition, we performed a confocal microscopy imaging experiment to follow A β 42 aggregation and toxicity in cells. Purified recombinant APPI was labeled with Alexa Fluor 680 (designated APPI-680), and conjugation was confirmed using 15 % SDS-PAGE, followed by InstantBlue™ staining (SI Appendix, Fig. S6). Then, A β 42 labeled at the N-terminus with HiLyte™ Fluor 488 (designated A β 42-488) was pre-incubated for 18 h in the presence or absence of APPI-680. Thereafter, the pre-incubated protein samples were added to the cells, and after 48 h the localization of the proteins was detected by confocal microscopy. Fig. 7A shows that untreated A β 42-488 accumulated within the cells as inclusion bodies (upper panels), whereas APPI-680 was localized on the cell membrane and could not penetrate into the cells (middle panels). Notably, the vast majority of A β 42-488 fragments that had been treated with APPI were also detected on the cell membrane, indicating that APPI treatment prevented A β 42 penetration and accumulation within the cells (lower panels).

We also assessed cell viability following different extracellular treatments by using the XTT assay (Fig. 7B). After 48 h of incubation, cells exposed to pre-formed A β 42 aggregates exhibited a 15 % reduction in viability. However, the viability of cells that were treated with the APPI-A β 42 mixture was similar to that of the control (untreated) cells and to that of the cells treated with APPI alone.

Previous studies have shown that accumulation of extracellular A β 42 within cells induces apoptosis [55]. To evaluate the ability of APPI to inhibit A β 42-mediated apoptosis in SH-SY5Y cells, we performed a flow cytometry experiment, in which treated and untreated cells were double-stained with annexin-V APC, which binds strongly to the phosphatidylserine that is exposed on the cell surface during apoptosis [56], and SYTOX™ green, which has a high affinity to nucleic acids and specifically detects dead cells [57]. In this analysis, annexin-V APC-positive/SYTOX-negative cells were in an early apoptotic stage; cells that were positively stained for both dyes were in the late apoptotic

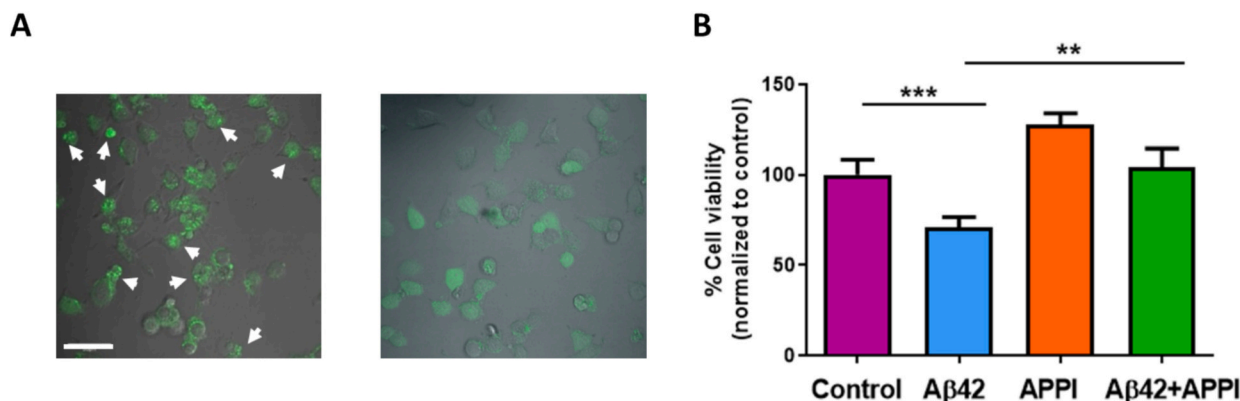


Fig. 6. APPI reduces the formation and toxicity of intracellular A β 42 aggregates in SH-SY5Y cells. (A) Live-imaging microscopy of SH-SY5Y cells expressing A β 42-GFP (left panel) and A β 42-GFP together with APPI (right panel). White arrows indicate the formation of dense, intracellular A β 42 aggregates. Scale bar is equivalent to 50 μ m. (B) Viability of SH-SY5Y cells stably expressing A β 42 was determined by an XTT assay at 48 h post-transfection. The values were normalized to those for SH-SY5Y cells treated with an empty plasmid. Data ($n = 5$) are presented as means $\pm$ SD, and statistical analysis was performed using an unpaired Student's t -test; ** $P \leq 0.01$; *** $P \leq 0.005$.

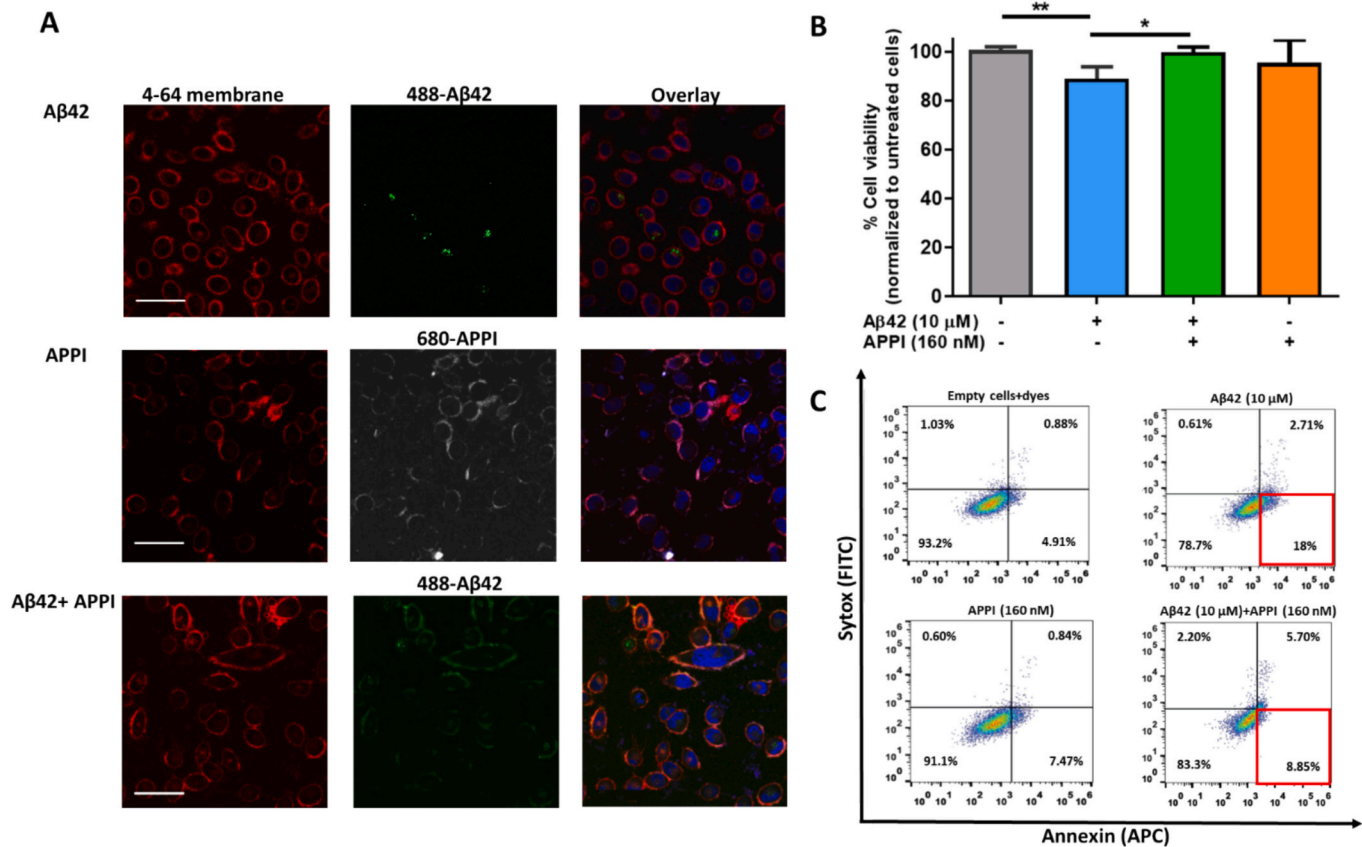


Fig. 7. Recombinant extracellular APPI reduces extracellular Aβ42 internalization and toxicity in SH-SY5Y cells. (A) Live imaging of SH-SY5Y cells treated with 0.5 μM HiLyte Fluor 488-labeled Aβ42 (upper panel), 7.8 nM Alexa Fluor 680-labeled APPI (middle panel) or a combination of the two (lower panel). Scale bar is equivalent to 50 μm. (B) Cell viability was determined by the XTT assay after 48 h of incubation with different treatments. Data ($n = 3$) are presented as means $\pm$ SD, and statistical analysis was performed using an unpaired Student's *t*-test; * $P \leq 0.01$, ** $P \leq 0.05$. (C) Flow cytometry analysis of annexin-V APC and SYTOX green staining of SH-SY5Y cells after 48 h of incubation with different treatments. The cells subjected to different treatments (indicated above each panel) were analyzed using Gallios instrument, and the results were further visualized using FlowJo software. The red squares indicate cells in the early apoptotic stage.

stage; and annexin-V APC-negative/SYTOX-positive cells were necrotic. Fig. 7C shows that approximately 90 % of the control cells were living cells (annexin-V negative and SYTOX green negative), and approximately 6 % were in the early apoptotic stage (annexin-V positive). The percentage of necrotic cells in these control cells was very low (approximately 1 %), indicating that APPI did not induce apoptosis or necrosis in SH-SY5Y cells, even after treatment for 48 h. Notably, our results revealed that 18 % of SH-SY5Y cells treated for 48 h with pre-formed Aβ42 aggregates were in the early apoptosis stage, while a lower percentage of cells (~9 %) undergoing early apoptosis was found after treatment with Aβ42 plus APPI. Taken together, these results show that treatment with recombinant extracellular APPI does not interfere with Aβ42 binding to the cell membrane, but it does prevent penetration, accumulation and subsequent toxicity of Aβ42 in SH-SY5Y cells (Fig. 7A, lower panel).

3. Discussion

The significance of this study centers on the elucidation of the dual neuroprotective role played by APPI, which is a constituent of the two alternatively spliced APP isoforms, APP751 and APP770, that are widely distributed in the brains of AD patients, but not of the APP695 isoform that is predominantly expressed in healthy human brains. This dual role is manifested as the inhibition of KLK6-induced cleavage of APP to form Aβ42 fragments and the inhibition of the aggregation of Aβ42 fragments into neurotoxic oligomers.

Biochemical confirmation and characterization of APP cleavage by

KLK6 and inhibition of KLK6 by APPI was undertaken in experiments using purified APPI, KLK6 and the extracellular domains of APP695 and APP751. In an experiment using the SH-SY5Y neuroblastoma cell line, we identified the APP695 isoform as the substrate cleaved by KLK6 (as demonstrated in Fig. 1). This finding is supported by the previous study of Little et al. [24], which showed that overexpression of both KLK6 and APP695 in HEK293 cells led to the formation Aβ peptide fragments. In an extension of that finding, we showed here, for the first time, that overexpression of KLK6 and APP695 in HEK293 cells significantly reduced cell viability, thereby suggesting direct links between the expression of KLK6, its proteolytic activity on APP695, and the toxicity of cleaved APP695 in cells.

We then examined the inhibitory effect of APPI on the cleavage of APP695 and APP751 and showed that, as a soluble protein and part of the extracellular APP sequence (in APP751), APPI efficiently inhibited the cleavage of APP695 and APP751, respectively (Fig. 2). Of note, the inhibitory effects of soluble APPI and APP751 on KLK6 activity were shown to be similar (Fig. 3). This finding suggests that APPI maintains its inhibitory function when fixed as a domain within APP751. It is reasonable to assume that KLK6 is inhibited by the APPI domain of APP751 and not by soluble APPI, because KLK6 neither cleaves APP751 nor releases APPI from its extracellular region such that there is no soluble APPI in the reaction milieu (Fig. 2B). This maintenance of APPI's inhibitory activity when it is part of the entire extracellular region of APP751 was also demonstrated by the comparable values of the inhibition constants (K_i) in the low nanomolar range for APPI as a soluble protein – determined in this study, and in previous work [34] – and for

the APPI domain within APP751 (4.38 ± 0.11 , 2.24 ± 0.11 , and 70.75 ± 0.94 nM, respectively).

Since previous studies have shown that AD patients, compared to healthy individuals, exhibit elevated levels not only of KLK6 but also of APP751 and APP770 [8,9], it is plausible that expression levels of APP751 and APP770 (both containing the APPI domain) increase as a response to elevated KLK6 levels in an effort to balance its proteolytic and pro-amyloidogenic activities. More specifically, in comparison with APP695, which lacks the APPI domain, the APPI-containing APP isoforms (i.e., APP751 and APP770) may interfere, via their APPI domain, with the proteolysis of APPs (i.e., APP751, APP770 and also APP695) in the brain, reducing the formation of toxic A β cleavage products.

In previous studies, the APPI domain has been shown to reduce the formation of A β peptide fragments via a different mechanism to that suggested here. For example, several studies have demonstrated that APP dimerization promotes non-amyloidogenic cleavage by α -secretase to generate soluble APP fragments but not neurotoxic A β peptide fragments [58,59]. Specifically, Byun et al. [60] have shown in in-vitro experiments that homodimerization of purified APP751 (containing APPI) predominates over that of purified APP695 (lacking APPI). Since heterodimerization between APP695 and APP751 is energetically less favorable, it was concluded that the APPI domain has a direct impact on dimeric APP751 stabilization. In that regard, molecular dynamics simulations confirmed that the APP751 homodimer is much more stable than the APP695 homodimer or the APP751-APP695 heterodimer due to the direct involvement of the APPI domain in APP dimerization [60]. By facilitating APP751 dimerization, APPI may reduce sequential cleavage of APP by β - and γ -secretases (i.e., amyloidogenic processing) and thus reduce the formation of A β peptide fragments [58,59].

In keeping with the above premise, we previously reported that a short cyclic peptide derived from the β -hairpin domain within APPI (i.e., residues 18–35) reduced A β 42-mediated cytotoxicity by accelerating A β 42 aggregation to form fibrils in preference to the neurotoxic low-molecular-weight A β 42 oligomers [37]. We referred to this inhibitory activity as promoting an ‘alternative/off-pathway mechanism’ for A β 42 aggregation [61,62]. This mechanism may be attributed to the aggregation of A β 42 without the formation of intermediate neurotoxic oligomeric A β 42 species [63]. In contrast, full-length APPI reduced A β 42-mediated cytotoxicity by inhibiting A β 42 aggregation and formation of neurotoxic low-molecular-weight A β 42 oligomers. We hypothesized that the β -sheet structure in the full-length APPI protein [64] interacted with the elongated A β 42 aggregates and that the bulky 3D structure of the full-length APPI protein (in contrast to the cyclic peptide) probably prevented A β 42 self-assembly, primarily due to a steric hindrance between A β 42 units. The presence of APPI would thus reduce the formation of neurotoxic A β 42 oligomers that occurs via the on-pathway mechanism of A β 42 oligomerization [62]. Indeed, while synthetic A β 42 underwent rapid (within minutes) nucleation and elongation into fibrillar aggregates [65], treatment with purified APPI efficiently halted A β 42 aggregation (Fig. 4A). Importantly, an inverse dose-dependent effect for inhibition of aggregation was observed for the inhibition of A β 42 aggregation by APPI. Specifically, as the concentration of APPI decreased, the inhibition of A β 42 aggregation increased, reaching maximal inhibition at a molar ratio of 1:64 (APPI:A β 42). This observation can be partially explained by the results of our light scattering assay (Fig. 5B), showing that APPI monomers – when reaching a specific concentration threshold – can self-associate, resulting in reduction in the ability of APPI to interact with A β 42 oligomers and inhibit their aggregation into amyloid fibrils. Indeed, TEM images showed that APPI disrupts the self-assembly of A β 42 fibrils, reducing their length (otherwise >200 nm; Fig. 4B, left panel) and quantity and disrupting their well-organized unbranched configuration, as was also previously shown by others [44,45] (Fig. 4B, middle and right panels). Support for this finding was obtained, at the molecular level, using CD spectroscopy, which showed a reduction in the β -sheet content of A β 42 aggregates due to interference by APPI with the self-assembly of β -sheet-containing A β 42

oligomers (Fig. 5A). Complementary information was provided by our a light scattering experiment, which revealed that APPI – despite its inhibition of the formation of A β 42 fibrils (Fig. 4) – facilitated the formation of A β 42 oligomers (Fig. 5B) that were not neurotoxic, as shown by immunoblotting with A11 antibody (Fig. 5C) [26,47]. We thus concluded that APPI interacts with the neurotoxic A β 42 oligomers that have a high β -sheet content, in analogy with the activity of other beta-sheet blockers [66–68]. This line of thinking leads us to hypothesize that treatment with APPI inhibits the A β 42 ‘on-pathway’ mechanism, as previously described for other beta-sheet blockers; for example, an A β 42 variant carrying two mutations (A β _{DM}) recently developed in our group, inhibited A β 42 aggregation and significantly reduced A β 42-mediated cell cytotoxicity [45]. However, despite its promise as therapeutic lead compound, the A β _{DM} variant exhibited a propensity to self-aggregate, which diminished its inhibitory effect on A β 42. APPI, on the other hand, retained its monomeric structure and solubility over time—a property that may offer superiority over other A β 42 inhibitors as a potential therapeutic [69,70].

While the above-mentioned biophysical assays (i.e., ThT, TEM, CD and light scattering) are widely used to monitor A β 42 fibril formation in vitro, they have several limitations that may hinder a full and comprehensive understanding of the misfolding pathway and the aggregation dynamics. For example, ThT fluorescence primarily detects β -sheet-rich fibrillar structures and may therefore fail to reveal early oligomeric or non-fibrillar aggregates that are also biologically relevant. Likewise, sample preparation for TEM involves drying and staining, which may introduce artifacts or distort the native fibril morphology of A β 42. Additionally (as is the case for ThT), TEM has limited sensitivity for detecting small oligomeric intermediates, which make a critical contribution to understanding early aggregation events and the toxicity of A β 42. In this regard, future studies would benefit from integrating complementary computational approaches, which fall beyond the scope and objectives of this work.

Following our observation that the structure and oligomeric state of synthetic A β 42 change upon interaction with purified APPI in solution, we aimed to test whether such changes also take place in cells. Indeed, we found that in SH-SY5Y cells A β 42 accumulation and aggregation was inhibited, and cell viability was increased upon co-expression of A β 42 with APPI (as shown in Fig. 6), suggesting that APPI may be therapeutically effective not only as a soluble protein but also as an intracellularly expressed protein.

We stress here that although the interaction of APPI with A β 42 results in a structural change in A β 42 and a reduction in neuronal cell apoptosis (in comparison to cells treated with A β 42 alone), the mechanisms for inhibition of toxicity differ for intracellular and extracellular A β 42: in the former APPI inhibits aggregation of A β 42 and formation of oligomeric toxic species, but in the latter APPI inhibits internalization of A β 42 into the cell. Indeed, microscopy images in the current study suggest that the reduction in cell death and prevention of A β 42 internalization is a result of inhibition by APPI (at an APPI:A β 42 molar ratio of 1:64) of A β 42 binding to the cell membrane. This is an important finding, since multiple studies have suggested that intracellular accumulation of A β 42 is a result of the uptake of extracellular aggregates into neuronal cells, which, in turn, is a key factor in inducing neuronal cell death [20,71]. For example, Chafekar et al. [72] showed that inhibition of oligomeric A β 42 uptake increased SK-N-SH neuroblastoma cell viability. Moreover, Han et al. [73] demonstrated that exposure of primary cultured mouse cerebral cortical neurons to extracellular A β 42 induced apoptosis, by disrupting the mitochondrial membrane potential, and increased levels of intracellular reactive oxygen species (ROS). Our XTT and apoptosis results correlate strongly with these observations, suggesting that APPI prevents A β 42 internalization and thus protects the cells from A β -mediated cell death (Fig. 7B and C) [55]. These experiments also established APPI as an inhibitor of A β 42 aggregation without causing any toxic effects on the tested cells (Fig. 7B and C).

Overall, our findings contribute to a better understanding of the regulatory and inhibitory mechanisms that affect A β pathology and underscore the interplay between APP isoforms and proteolytic processing in Alzheimer's disease.

4. Conclusion

In summary, our study shows that the APPI domain, either as part of APP or in isolation, reduced KLK6-dependent cleavage of APP both in solution and in cells. Additionally, upon APPI overexpression in neuroblastoma cells or upon addition of purified APPI to the extracellular milieu there was a reduction in the formation of A β 42 aggregates and hence an enhancement of neuroprotection mediated by intracellular and extracellular A β 42, respectively. Our research suggests that APPI has the potential to act as a dual inhibitor, targeting two key processes related to the toxicity of A β 42 and of other A β peptide fragments, i.e., the formation and accumulation of potentially neurotoxic A β species and the aggregation of A β peptide fragments into neurotoxic oligomers.

5. Materials and methods

5.1. Reagents and peptides

Synthetic amyloid beta peptides, including A β 1–42 (A β 42) and A β 42 labeled at the N-terminus with HiLyte™ Fluor 488 (A β 42-HiLyte 488), were purchased from Anaspec (Fremont, CA, USA). The alkaline phosphatase substrate, 4-nitrophenyl phosphate (pnPP), was obtained from Abcam (Cambridge, UK). The KLK6 substrate, BOC-Phe-Ser-Arg-AMC (BOC-FSR-AMC), was purchased from Bachem (Bubendorf, Switzerland). Anti A β 42 (ab11132) and 6 $\times$ His tag (ab49936) antibodies were purchased from Abcam. Anti-HA antibody was obtained from Biolegend (San Diego, CA, USA). A11 antibody was obtained from ThermoFisher Scientific (Waltham, MA, USA). Crystal violet powder was purchased from Sigma-Aldrich (MO, USA).

5.2. Cell culture

The HEK293 human embryonic kidney and SH-SY5Y human neuroblastoma cell lines were maintained at 37 °C in a 5 % CO $_2$ in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Kibbutz Beit-Haemek, Israel) supplemented with 10 % tetracycline-free fetal bovine serum (FBS; Biological Industries), 2 mM L-glutamine (Biological Industries), and penicillin (100 units/mL)/streptomycin (0.1 mg/mL) (Gibco, Waltham, MA, USA).

5.3. Gene cloning in mammalian expression plasmids

For expression in *Pichia pastoris*, APPI-6 $\times$ His was cloned as described previously [33]. In brief, the extracellular regions of APP695 and APP751 genes fused to an N-terminal 6 $\times$ His tag were cloned into the pPIC9K plasmid with *Sna*BI and *Avr*II restriction sites using the forward primer 5'-AAAATACGTACATCACCATCATCACCACTGGAGGTACCCACT GAT-3' and the reverse primer, 5'-TTTCTCTAGGTTACGCTATGACAACACCGC-3'.

For expression in SH-SY5Y neuroblastoma cells, the following three gene constructs were generated:

(i) A β 42-GFP and APPI were cloned into a pHAGE vector for expression in SH-SY5Y cells. The pHAGE plasmid was used as a mammalian vector for expressing APPI linked to blue fluorescent protein (BFP) via an internal ribosome entry site in SH-SY5Y cells under the control of the human cytomegalovirus promoter. A β 42-GFP was cloned as described previously [45]. The APPI gene was cloned into the pHAGE2 plasmid with the addition of an N-terminal fragment of human influenza HA tag to detect APPI expression and the Kozak sequence, which served as a translation initiation site. The forward primer was 5'-ATCATGCGGCCGCGCCACCATGTACCCATACGATGTTT CAGATTACGCTGAAGTTTGTCTGAACAAGCTG-3', and the reverse primer, 5'-CGCGG

ATCCAATAATAGCAGAACCACAAACAGC-3'. The amplified gene construct was inserted into the plasmid, using *Not*I and *Bam*HI restriction enzymes (New England Biolabs, MA, USA), to generate the final gene construct.

(ii) The KLK6 construct was designed to include the KLK6 proteolytic domain and a 6 $\times$ His tag. The gene was cloned into the pHAGE plasmid with the addition of the interleukin-2 signal peptide, by using the Gibson assembly method (New England Biolabs) with the forward primer, 5'-TCACGAATTCGATATCGGCCATGGATAT GAAGAAGCTGATGGTGGTG-3' and the reverse primer, 5'-GGGGGGGGGAGGGATC CTCACACCACTTTGTACAAGAAAGCTG-3'.

(iii) mCherry-APP695-GFP in the pCDNA3.1 plasmid and APP695 fused to SEAP at the N-terminal (SEAP-APP695) in the pEAK12 plasmid were a generous gift from the lab of Ayelet Fishman (Technion-Israel Institute for Technology, Haifa, Israel). SEAP-APP695 was cloned into the pLenti vector with the *Asi*SI and *Not*I restriction enzymes (New England Biolabs) using the forward primer 5'-ATCGCTGCGATCGCATGCTGCTGCTGCTGC-3' and the reverse primer 5'-ATTCT-TATGCGGCCGCGTTC TGCATCTGCTCAAAGAA-3'.

5.4. Evaluation of the APP isoform-dependent cleavage by KLK6 in SH-SY5Y cells

SH-SY5Y cells were plated at a density of 3 $\times$ 10 ^{5} cells per well in a six-well plate. The following day, the cells transiently transfected with the following plasmids at a final quantity of 2 μ g in each well: SEAP-APP695, KLK6-6 $\times$ His, a combination of APP695 and KLK6-6 $\times$ His, and an empty plasmid as control. The cells were incubated in phenol red-free DMEM supplemented with 5 % FBS, 2 mM L-glutamine, and penicillin (100 units/mL)/streptomycin (0.1 mg/mL) for 48 h. For samples to be examined for KLK6 inhibition by APPI, soluble APPI was added at final concentrations of 1, 5, and 10 μ g. Thereafter, the media were collected and centrifuged at 300 $\times$ g to remove cell debris. To denature endogenous alkaline phosphatase, the media were heat inactivated at 65 °C for 30 min. For the detection of SEAP activity, the chromogenic pnPP substrate was prepared by dissolving pnPP at 1 mg/mL final concentration in pnPP buffer (0.1 M glycine, 1 mM MgCl $_2$, 1 mM ZnCl $_2$, pH 10.4). Aliquots of 100 μ L were then loaded into the wells of a 96-flat bottom transparent well plate, to which treated medium, 100 μ L/well, was added. Cleavage of the substrate was monitored by tracking the increase in absorbance at 405 nm in a Synergy2 microplate spectrophotometer (BioTek, Winooski, VT, USA).

5.5. Live cell imaging of mCherry-APP695-GFP cleavage by overexpressed KLK6

SH-SY5Y cells were seeded at 10,000 cells/well on 1-cm micro-slides (ibidi, Martinsried, Germany) and incubated at 37 °C. On the following day, cells were transfected with either 0.5 μ g pPhage IL2-KLK6 and 0.5 μ g mCherry-APP695-GFP pCDNA3.1 or 0.5 μ g mCherry-APP695-GFP pCDNA3.1 and 0.5 μ g empty pPhage plasmid. The transfection procedure was conducted using a jetOPTIMUS transfection kit (Polyplus, USA) for SH-SY5Y cells, according to the manufacturer's instructions. Two days post-transfection, the cells were washed twice with PBS (Sartorius, Göttingen, Germany) and fixed with 4 % PFA for 30 min. Images were taken by an LSM880 confocal microscope (Zeiss, Germany) equipped with x63/1.4 oil objective. Cells were imaged in Z-stack mode (20 slices with 0.3- μ m steps) and three fluorescent channels (DAPI, EGFP and mCherry). At least five individual fields of view were imaged in each group. To quantify the fluorescent images, the images were analyzed in CellProfiler version 4.2.5 by using a custom-made script. Briefly, nuclei were segmented by the DAPI channel. Cell territories were defined by thresholding of the EGFP channels and related to predefined nuclei. A standard set of fluorescence intensity features was measured for each cell, for both GFP and mCherry signals, and the results were exported as a CSV file.

5.6. Viability of SEAP-APP695- and SEAP-APP751-expressing cells following proteolysis of APP by KLK6

HEK293 cells were seeded at 250,000 cells/well in a 6-well plate and incubated at 37 °C. On the following day, the cells were transfected using polyethylenimine (PEI; Polysciences Inc., PA, USA) with 2 µg total DNA containing one of the following combinations: 1 µg pHAGE IL2-KLK6 plus 1 µg pLenti SEAP-APP695; or 1 µg pHAGE IL2-KLK6 plus 1 µg pLenti SEAP-APP751, 1 µg pLenti SEAP-APP695, 1 µg pLenti SEAP-APP751, or 1 µg pHAGE IL2-KLK6. In the latter three cases, 1 µg of empty pHAGE plasmid, lacking an insert, was added to maintain a constant amount of DNA. Cells transfected with 2 µg empty pHAGE plasmid served as a control. Two days post-transfection, the cells were gently washed twice with PBS (Sartorius) and fixed with 100 % methanol for 10 min. Thereafter, the methanol was removed, and the plates were allowed to dry completely. Then, 800 µL of a crystal violet solution (0.5 % w/v crystal violet in 20 % v/v methanol/water solution) was added to each well for 30 min. Following the staining, three washing cycles with doubly distilled water removed the excess crystal violet solution. Finally, the fixed crystal violet stain was dissolved out with 2 % aqueous SDS solution, 500 µL/well, and the wells were read at a wavelength of 590 nm using a Synergy2 microplate spectrophotometer (BioTek). Three repetitions for each treatment were performed. The values were normalized to the control group.

5.7. APPI, APP695, and APP751 expression and purification

P. pastoris strain GS115 was transformed with pPICK9K plasmids containing either APPI or APP isoform genes as previously described [33]. The yeast cells were first inoculated into 50 mL of BMGY medium overnight (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) and then into 1 L of BMMY medium (BMGY, but with 0.5 % methanol instead of glycerol) for 3 days, with 2 % methanol being added every 24 h to maintain induction. For APP695 and APP751, the protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (PMSF; Roche, Basel, Switzerland) and cOmplete™ ULTRA Tablets, EDTA-free, Protease Inhibitor Cocktail (Roche; one tablet) were also added to the growth medium. Following four days of induction, the cultures were centrifuged at 3800 ×g for 10 min, and the supernatants containing the secreted recombinant inhibitors were prepared for purification by nickel-immobilized metal affinity chromatography (IMAC). The supernatants were adjusted to 10 mM imidazole and 0.5 M NaCl at pH 8.0 and left to stand for 1 h at 4 °C. Thereafter, filtration was performed to remove additional precipitates using a 0.22-µm Steritop bottle-top filter (Millipore, MA, USA). The filtered supernatants were loaded onto a 5-mL HisTrap column (GE Healthcare, Piscataway, NJ, USA) at a flow rate of 0.7 mL/min for 24 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) in an ÄKTA™ pure instrument (GE Healthcare).

As the final step of APPI purification, the buffer of the eluted protein was replaced with Tris buffer (100 mM Tris, 1 mM CaCl₂, pH 8.0) using a 3.5-kDa cutoff dialysis kit (Gene Bio Application, Israel). For APPI, gel filtration chromatography was performed on a Superdex 75 16/600 column (GE Healthcare) equilibrated with Tris buffer at a flow rate of 1 mL/min in an ÄKTA start instrument (GE Healthcare). For APP695 and APP751, the elution buffer was replaced with heparin chromatography buffer (Buffer A; 50 mM Tris, 20 mM EDTA, pH 7.4) using the HiTrap desalting column (Cytiva, MA, USA). Then, the proteins were loaded separately on a HiTrap heparin HP column (Cytiva, MA, USA) at a flow rate of 0.5 mL/min in an ÄKTA start instrument. Thereafter, the proteins were eluted with a gradient starting with 100 % buffer A and ending with 100 % buffer B (equivalent to buffer A but supplemented with 1 M NaCl) in 20 column volumes at a flow rate of 1 mL/min. The purity and

molecular weight (M_w) of the proteins were analyzed by 15 % and 10 % SDS-PAGE gels (for APPI and APP isoforms, respectively), followed by InstantBlue staining (Expedeon, Cambridge, UK). The calculated molar mass of the recombinant proteins was 9.2, 71.6, and 80 kDa for APPI, APP695, and APP751, respectively. Protein concentrations were determined using extinction coefficients of 13325, 61600, and 71945 M⁻¹ cm⁻¹ for APPI, APP695, and APP751, respectively. The yields were approximately 20, 8, and 2 mg purified APPI, APP695, and APP751 proteins per liter of yeast culture, respectively. Finally, SDS-PAGE analysis of selected fractions confirmed the correct size and high purity of these proteins (SI Appendix, Fig. S1C and S3C).

5.8. KLK6 expression and purification

Recombinant KLK6 was expressed and purified from the Sf21 insect cell line at the Protein Expression and Purification Core Facility, EMBL Heidelberg, Germany, as described previously [74].

5.9. Gelatinase zymography assay for KLK6 activity

Recombinant KLK6 (5 µg) was mixed with a non-reducing sample buffer and loaded on 15 % SDS-PAGE mixed with 1 % gelatin (Sigma-Aldrich). After electrophoresis, the gels were first rinsed for 1 h in 2.5 % Triton X-100 (Sigma-Aldrich) at room temperature with gentle agitation. Then, the solution was removed, and the gels were incubated for 24 or 48 h at 37 °C in a development buffer [50 mM Tris-HCl (pH 7.5), 10 mM CaCl₂, 0.02 % NaN₃]. Finally, the gels were washed three times with doubly distilled water and stained with SimplyBlue™ SafeStain (ThermoFisher Scientific, Waltham, MA, USA). The areas degraded by KLK6 were visible as clear bands against a dark blue background.

5.10. Monitoring APP695 or APP751 protein cleavage by KLK6

APP695 (3 µM) was incubated in the absence or presence of KLK6 (0.3 µM) in PBS buffer. To examine the inhibition effect of APPI on KLK6 cleavage, samples of APP695-KLK6 mixture were incubated with 30 µM or 60 µM of APPI. Thereafter, 40 µL of the reaction mixture was loaded on 12.5 % SDS-PAGE, followed by InstantBlue staining. All samples were incubated for 20 h at 37 °C.

To examine the inhibition of the APPI domain within APP751, samples of APP695 or APP751 (4.7 µM) were incubated in the absence or presence of KLK6 (0.47 µM). APP695-KLK6 and APP751-KLK6 sample mixtures to which soluble APPI (4.7 µM) had been added served as a control for inhibition. All samples were incubated for 16 h at 37 °C. Then, aliquots of 20 µL and 5 µL of the samples were loaded on two different 15 % SDS-PAGE gels followed by InstantBlue and silver staining, respectively.

5.11. Monitoring Boc-FSR-AMC cleavage by KLK6

Aliquots of 50 µL of KLK6 were pre-incubated for 1 h at 37 °C in KLK6 cleavage buffer (50 mM Tris-HCl, 100 mM NaCl, and 0.2 % BSA, pH 7.3) with one of the following (50 µL): APP695, APP751, or a combination of soluble APPI with APP695. Subsequently, each sample (100 µL) was added to a solution of Boc-FSR-AMC. The sample containing a mixture of KLK6 and Boc-FSR-AMC served as the positive control for substrate cleavage. The final reaction concentrations were 1 nM KLK6, 300 nM for each APPI and the relevant APP isoform, and 50 µM Boc-FSR-AMC. Fluorescence was measured at 355 and 460 nm for excitation and emission, respectively. All samples were monitored at 37 °C for 5 h at 5-min intervals using a Synergy2 microplate spectrophotometer (BioTek). The experiment was conducted in triplicate.

5.12. KLK6 inhibition by APPI and APP751

The inhibition constant K_i of APPI and APP751 in complex with

KLK6 was determined according to previous work [34]. Briefly, tight binding experiments were conducted at a fixed concentration of the substrate Boc-FSR-AMC (1 mM). Samples of KLK6 (50 μ L) and APPI or APP751 (50 μ L) were mixed and equilibrated at 37 °C for 1 h in a 96-well microplate (Greiner, Kremsmünster, Austria) before the addition of the substrate (50 μ L). The final protein concentrations in the reaction buffer (50 mM Tris-HCl, 100 mM NaCl, and 0.2 % BSA, pH 7.3) were 1 nM for KLK6, and 0–50 nM and 0–100 nM for APPI and APP751, respectively. The fluorescence signal of the reactions was monitored in Synergy 2 microplate reader at 355 and 460 nm for excitation and emission, respectively.

Reactions were followed for 5 h at 5-min intervals of reads at 37 °C using a Synergy 2 microplate spectrophotometer (BioTek). The intrinsic fluorescence of the substrate was subtracted from all samples. The inhibition constants were calculated using the equation:

$$\frac{V_0 - V_i}{V_i} = \frac{[I]_0}{K_I(1 + [S]_0/K_m)}$$

where V_i and V_0 are the steady-state rates in the presence and absence of the inhibitor, respectively; K_m is the Michaelis constant for substrate cleavage; and $[S]_0$ and $[I]_0$ are the initial concentrations of the substrate and inhibitor, respectively.

5.13. Detection of A β 42 aggregation by the ThT assay

A series of APPI concentrations (ranging between 6 nM to 250 nM) was added into 350 μ L of a stock solution of phosphate buffer [20 mM sodium phosphate (pH 7.4), 150 mM NaCl] plus ThT (Sigma-Aldrich, MO, USA) and A β 42 at final concentrations of 10 μ M and 4 μ M, respectively. Aliquots of 120 μ L of the reaction mixture were added to a black 96-well plate (Greiner, Kremsmünster, Austria). The samples were monitored at 37 °C with constant orbital shaking at 250 rpm for 10.5 h in a Tecan Infinite M1000 plate reader (Männedorf, Switzerland; Cytometry and Proteomics Unit, NIBN, BGU). The fluorescence signals were detected using excitation and emission wavelengths at 440 and 485 nm, respectively. All measurements were performed in triplicate.

5.14. Visualization of A β 42 aggregation by using TEM microscopy

Samples for TEM imaging were prepared as described previously [45]. After 10.5 h of incubation, 2.5- μ L aliquots of the samples taken from the above ThT assay were deposited on carbon-coated copper 300-mesh grids. After 1 min, excess liquid was carefully blotted off with filter paper, and the grid was held at ambient temperature for another minute. Uranyl acetate (2 % in aqueous solution, 5 μ L) was added to each sample for 1 min, and then any excess solution was carefully removed with filter paper. Imaging was performed using a Tecnai G2 12 BioTWIN (FEI, ThermoFisher Scientific, Paisley, UK) TEM with an acceleration voltage of 120 kV (Ilse Katz Institute for Nanoscale Science & Technology). Different magnifications were used for visualization, depending on the size of the aggregates, and multiple fields in each grid were analyzed.

5.15. CD spectroscopy

A β 42 samples (20 μ M in 300 μ L) diluted in phosphate buffer were incubated in a Thermo Shaker Incubator in the presence or absence of 312.5 nM APPI for 16 h at 500 rpm at 37 °C. For smoothing the data, the spectrum of each sample was recorded four times, and the spectral scans were averaged by a Jasco J715 spectropolarimeter (Shimadzu Co., Kyoto, Japan). The spectra of the proteins were recorded in a range between 260 and 190 nm using a quartz cuvette with a path length of 1 mm, a scanning speed of 50 nm/min and a data interval of 1 nm. For baseline correction, a protein-free phosphate buffer was used.

5.16. Detection of A β 42 oligomerization by light scattering

To each well (in triplicate) of a black 96-well plate with a transparent bottom were added 100 μ L of phosphate buffer containing 62.5 nM APPI with or without 4 μ M A β 42. The reactions were monitored using a Tecan Infinite M1000 plate reader with orbital constant shaking of 250 rpm at 37 °C. The changes in the absorbance at 400 nm were monitored over 16 h.

5.17. Characterization of A β 42 oligomers by using immunoblotting with a structural antibody

The three repetitions of each light scattering sample (see above) were merged, and 5 μ L of each sample (i.e., A β 42, APPI and A β 42-APPI mixture) were loaded onto a nitrocellulose membrane. The membrane was blocked with 5 % skim milk (Sigma-Aldrich) in Tris-buffered saline plus Tween (TBST; Biolab, Jerusalem, Israel). The membrane was washed with TBST three times for 5 min each. Then, the membrane was incubated with rabbit anti-oligomer antibody A11 diluted 1:1000 in PBS (ThermoFisher Scientific, Waltham, MA, USA). Thereafter, a secondary HRP-linked anti-rabbit antibody diluted 1:3000 in 5 % skim milk (Jackson, PA, USA) was added, and the chemiluminescence signal was detected using the EZ-ECL kit (Sartorius). To confirm that A β 42 was loaded equally in each sample, the membrane was blocked again with 5 % skim milk and incubated with mouse anti-A β 42 antibody diluted 1:1000 in PBS (Abcam, Cambridge, UK) followed by a secondary HRP-linked anti-mouse antibody diluted 1:3000. Here again, the membrane was developed using the EZ-ECL kit (Sartorius). The chemiluminescence signals were detected in both immunoblotting procedures with the Fusion FX imaging system (Vilber Lourmat, Germany). The intensity of the images was quantified using ImageJ software, and the intensity values of the oligomers detected by A11 were normalized to the untreated A β 42 samples. The experiment was performed in triplicate.

5.18. Detection of APPI and A β 42-GFP expression in SH-SY5Y cells

Naïve SH-SY5Y cells or SH-SY5Y cells stably expressing A β 42-GFP were seeded on 10-cm plates. For transient expression of APPI, the cells were transiently transfected on the following day with 5 μ g pHAGE-APPI by using the jetOPTIMUS® transfection reagent (Polyplus, Illkirch, France) according to the manufacturer's manual. The cells were harvested and centrifuged (at 1,200 rpm for 5 min) 48 h post-transfection (or incubation) of cells transiently transfected with APPI (or cells stably expressing A β 42-GFP). Then, the cell pellets were resuspended in 10 $\times$ RIPA buffer (25 mM HEPES-NaOH, 150 mM KCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % NP-40, pH 7.4) and a fresh solution of protease inhibitor (PI) cocktail diluted 1:100 (APE $\times$ BIO, Boston, MA, USA). Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA), and 50 μ g of total protein from the cell lysates were loaded on 15 % SDS-PAGE and then transferred to nitrocellulose membrane for western blot analysis. For determination of APPI expression, the membrane was probed with rabbit-anti-HA antibody diluted 1:4000 in PBS. For A β 42-GFP detection, the membrane was probed with mouse-anti-A β 42 antibody diluted 1:1000 in PBS (Abcam, Cambridge, UK). In both cases, HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) were used.

5.19. APPI labeling with Alexa Fluor 680

APPI buffer (Tris buffer) was replaced with PBS (Sartorius) using a 3.5 kDa cut-off Vivaspinn (GE Healthcare Life Sciences, UK) to eliminate Tris molecules that can interfere with NHS chemical reaction. Purified APPI (300 μ g) was incubated for 1 h in the dark at room temperature with 300 μ g Alexa Fluor 680 amine-reactive dye dissolved in DMSO to a concentration of 10 μ g/ μ L, where Alexa Fluor 680 contains a hydroxysuccinimide ester that forms a covalent bond with primary amines

(ThermoFisher Scientific, Waltham, MA). The unconjugated dye residuals were removed using a 3.5-kDa cutoff dialysis kit (Gene Bio Application, Israel). To confirm protein labeling, 10 μ L of labeled APPI was loaded on 15 % SDS-PAGE, followed by InstantBlue staining.

5.20. Generation of pre-incubated protein samples

APPI, A β 42 or a mixture of APPI and A β 42, unlabeled or labeled with Alexa Fluor 680 or HiLyte Fluor 488 (APPI-Alexa Fluor™ 680 and A β 42-HiLyte™ Fluor 488), were prepared as follows: samples in several concentrations, which were experiment dependent but maintained a 1:64 (APPI:A β 42) molar ratio, were mixed with phosphate buffer [20 mM sodium phosphate (pH 7.4), 150 mM NaCl] and incubated in a Thermo Shaker Incubator for 18 h (500 rpm, orbital shaking) at 37 °C.

5.21. Detection of intracellular and extracellular effects of APPI on A β 42 aggregation by using live cell confocal microscopy

In all experiments, SH-SY5Y cells were seeded at 10,000 cells/well in 1-cm micro-slides (ibidi) and grown at 37 °C with 5 % CO₂. For intracellular expression of APPI and A β 42, the cells were transfected one day after seeding with either 1 μ g of a mixture of A β 42-GFP and empty plasmids or a mixture of A β 42-GFP and APPI pPHAGE plasmids by using the jetOPTIMUS transfection reagent, according to the manufacturer's manual. After 48 h the cells were analyzed.

For extracellular treatment with both APPI and A β 42, the cells were incubated one day after seeding with 1 μ M extracellular pre-formed A β 42-HiLyte Fluor 488 in the presence or absence of 15.8 nM of APPI-Alexa Fluor™ 680. To examine whether APPI was spontaneously internalized into the cells, pre-incubated APPI-Alexa Fluor 680 (15.8 nM) was then added to the cells. After 18 h of incubation, the media containing the proteins were carefully removed. The cells were washed twice gently with PBS (Sartorius), followed by resuspension in 200 μ L of fresh DMEM. Nuclear staining was performed using Hoechst dye diluted 1/1000 (ThermoFisher Scientific, Waltham, MA, USA) in each well, followed by membrane staining with FM™ 4-64 Dye (10 μ g/mL final concentration; ThermoFisher Scientific, Waltham, MA, USA).

In both experiments, live-cell imaging experiments were performed using an 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].

5.22. XTT viability assay for intracellular and extracellular treatment of A β 42 and APPI

In both experiments, naïve SH-SY5Y or SH-SY5Y cells that stably expressed A β 42 were seeded at a density of 10,000 cells per well in a 96-well plate with 100 μ L of DMEM. For intracellular expression of A β 42 and APPI, the cells were transiently transfected one day after seeding with 0.5 μ g DNA (empty pHAGE2 or pHAGE2 containing APPI gene). After 6 h, the cell medium was replaced with fresh DMEM to remove the transfection reagent. For extracellular addition, pre-incubated protein samples of 10 μ M A β 42, 160 nM APPI, or a mixture of 10 μ M A β 42 and 160 nM APPI were added to the cells one day after seeding.

In both experiments, 48 h post-transfection (or addition of protein samples), 20 μ L of XTT reagent (Sartorius) was added to each well. After 20 min of incubation, the plate was read at two wavelengths (i.e., 465 nm and 660 nm) using a Tecan Infinite M1000 plate reader. The latter wavelength served as the reference absorbance (i.e., to detect non-specific signals). Cell viability was determined as the difference between the values of the two readings at the two wavelengths. The intensity values are normalized to SH-SY5Y transfected with an empty plasmid.

5.23. Flow cytometry analysis of apoptotic cells

SH-SY5Y cells (5×10^5 cells per well) were seeded in a 24-well plate and incubated for 18 h with pre-formed A β 42 aggregates, 10 μ M, in the presence or absence of 320 nM APPI. Untreated cells served as a negative control. After 48 h of incubation, the cells were harvested and treated with an Annexin-V APC/SYTOX Apoptosis Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The cells were analyzed using a Beckman Gallios FACS. The following cell populations were analyzed: intact cells (annexin-V APC negative and SYTOX negative), early apoptotic cells (annexin-V APC positive and SYTOX negative), late apoptotic cells (annexin-V APC and SYTOX positive), and necrotic cells (annexin-V APC negative and SYTOX positive).

5.24. Statistical analysis

The Wilcoxon paired test was performed to quantify the fluorescent signals obtained from cells expressing mCherry-APP695-GFP in the presence or absence of KLK6. The data for A β 42 oligomers detected by the A11 antibody and XTT viability (both extracellularly and intracellularly) assays were analyzed for column statistics with GraphPad Prism, version 8.00, for Windows (La Jolla, CA, USA). In general, all data are presented as means $\pm$ SD. Statistical significance was determined by unpaired Student's *t*-test.

Abbreviations

A β	amyloid β
A β 42	amyloid β 1–42
AD	Alzheimer's disease
APP	amyloid precursor protein
APLP	amyloid precursor-like protein.
APPI	amyloid precursor protein inhibitor
CD	circular dichroism
KLK6	kallikrein-related peptidase
DMEM	Dulbecco's modified Eagle's medium
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
PBS	phosphate-buffered saline
TEM	transmission electron microscope
ThT	thioflavin T

CRediT authorship contribution statement

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

Author contributions

S.L.H. and N.P. designed the research; S.L.H., O.O., and M.R. performed the research; S.L.H. and N.P. analyzed the data; S.L.H. and N.P. wrote the paper. All authors edited the manuscript and approved the final version.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2025.146066>.

Data availability

Data will be made available on request.

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