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Engineered peptidic constructs metabolize amyloid β by self-assembly-driven reactions†

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Inspired by the unique mechanism of proteolytic maturation of host cell factor-1, we designed small peptide-based constructs that selectively recognized amyloid β (Aβ) and cleaved it in a non-catalytic manner initially around the α-secretase cleavage-site, thus termed as "artificial α-secretases". "Artificial α-secretases" also cleaved Aβ on the cleavage-sites of other Aβ processing enzymes by prolonged treatment, as evidenced by time-resolved MALDI-TOF-mass analyses.

Proteases cleave peptide linkages either by a catalytic triad-based mechanism or assisted by a metal.[1] Mimicking enzyme action has been achieved by mimicking the triad-based catalytic site by protein engineering[2] that essentially required the specific supramolecular arrangement of large protein constructs[3] making them more bulky, immunogenic, and cost intensive. To bypass these challenges, we planned to achieve the proteolytic function of a protease explicitly for a specific target. Therefore, we would be sacrificing all other functions, even the catalytic activity of the protease, to reduce the possibility of unrelated effects keeping the total mass minimum. Thus a function specific mimic of a protease or proteases would be generated.

To demonstrate the utility of the concept we selected Alzheimer's amyloid β peptide (Aβ), its precursor protein (APP) and its oligomers as the targets. Alzheimer's disease (AD), caused by the deposition of insoluble Aβ plaques outside neurons[4] and hyper-phosphorylated tau protein in the neuron along with other mechanisms, has no cure to date despite enormous efforts in diverse directions.[5,6] In spite of the prevailing controversy, it is usually accepted that inhibition of formation and metabolism of these peptide aggregates have therapeutic relevance.[7] However, the inhibition of formation and metabolism of disease- causing peptide aggregates cannot be achieved yet by externally added compounds or medicines. We engineered a small peptide to selectively recognize and cleave Aβ and its toxic oligomers by supramolecular interactions.[8] We termed them as "artificial α-secretases" as they function at the α-secretase cleavage site first, thus mimicking its Aβ-specific proteolytic function. Interestingly, with prolonged treatment at physiological pH and temperature, "artificial α-secretases" also cleave Aβ at the cleavage sites of other metabolic enzymes, finally dissolving the amyloid and rendering it non-toxic.

To the best of our knowledge, only one protein reported to date follows a solely Glu aided cleavage mechanism *in vivo*. Host cell factor-1 (HCF-1), a transcriptional co-regulator of human cell-cycle progression, undergoes proteolytic maturation in which any of its six repeated sequences is cleaved by the *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferase (OGT).[9] OGT usually glycosylates Ser or Thr side chains. Therefore, OGT driven proteolysis seemed mysterious initially. However, recently Walker *et al.* demonstrated that it occurs *via* an unusual glycosylation of a glutamate side chain by OGT followed by an internal pyroglutamate formation that undergoes spontaneous peptide bond hydrolysis.[10] We wanted to mimic the Glu-based site-selective proteolysis mechanism of HCF-1. However, nucleophilic attack of the backbone nitrogen at the glycosylated side chain carboxylate of Glu is feasible for the favorable formation of the intramolecular five-membered cyclic pyroglutamate *in vivo*, but replicating it in an intermolecular fashion is another challenge. Therefore, we replaced *O*-GlcNAc by a more reactive benzyl (*O*-Bn) group. Furthermore, we replaced Glu with a Lys attached to a benzyl adipate (BnAdp). Adipic acid is a six carbon-containing linear dicarboxylic acid. While one end was attached to the Lys side chain, in order for it to be long enough to reach the peptide backbone of the target Aβ or APP, the other end was benzylated. Finally, we attached such constructs at the N-terminus of a self-assembling partial sequence of Aβ, KLVFF ($A\beta_{16-20}$), which is also a part of APP ($APP_{687-691}$).[11] These peptide conjugates ("artificial α-secretases", Fig. 1) are intended to self-assemble with homologous parts of Aβ and APP by supramolecular interactions and cleave at or around the α-secretase cleavage site, primarily. As "artificial α-secretases" are designed to act by a self-assembly-driven proteolysis mechanism, they are also able to truncate and dissolve aggregated Aβ, mimicking the proteolytic function of NEP, ECE, IDE, P, ACE, and MMPs. Thus, "artificial α-secretases" are supposed to act as a function-specific mimic of α-secretase and these Aβ degrading enzymes at once.

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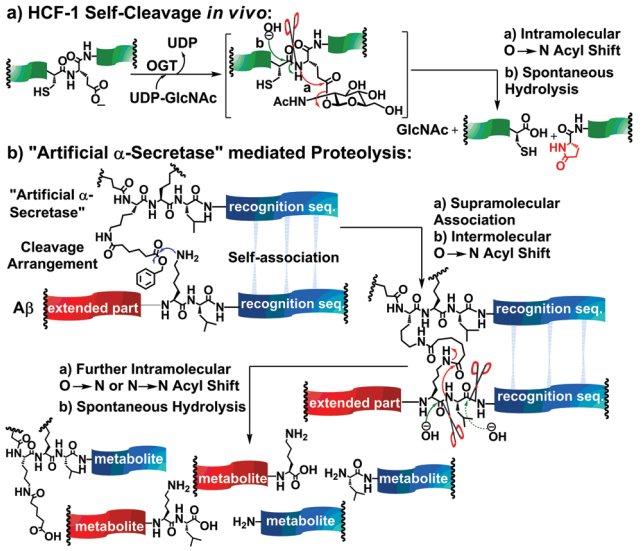

Fig. 1 (a) Mechanism of HCF-1 self-cleavage, and (b) anticipated mode of proteolysis of Aβ by "artificial α-secretases".

Four "artificial α-secretases" were designed altering the positions of Lys(BnAdp) (Table 1, AαS1–4 stands for "artificial α-secretase"1–4). "Artificial α-secretase"4 was designed to achieve a synergistic effect. To obtain an initial idea of their proteolytic activity, a partial sequence of Aβ ($Aβ_{12–20}$, which is also $APP_{683–691}$), which contains the α-secretase cleavage site, the peptide bond between Lys16 and Leu17,[12] and the self-assembling $Aβ_{16–20}$ part was synthesized and acted as a model aggregating peptide (mAβ). One of the C-terminal Gly mimicked the highly disease-prone Flemish Ala21 Gly mutation;[13] the other served as a spacer for the synthetic advantage. Proteolytic activity of the "artificial α-secretases" was finally confirmed by commercial $Aβ_{1–40}$. To examine the selective nature of "artificial α-secretases", we also synthesized a 28 residue-long, unrelated negative control peptide (NC) based on the mutant diphtheria toxin (DTP28)[14] by replacing Val8 and Lys23 by Pro. Molecular docking studies (Fig. S21 and S22, ESI†) in AutodockVina 1.1.2[15] suggested that "artificial α-secretases" fitted well in the binding pockets of both helical and fibrillar $Aβ_{1–40}$.[16] As "artificial α-secretase"4 exhibited the lowest binding affinity, it was also docked into the helical C-terminal domain of APP. The interacting amino acids were Leu17, Val18, Phe19, and Phe20, as desired.

To check the proteolytic efficiency, first "artificial α-secretases" 1–3 were co-incubated with mAβ in 1 : 1 molar ratio at 37 °C and pH 7.4 (PBS) on a water bath. Aliquots were tested by a MALDI-TOF mass spectrometer in a time-dependent manner. "Artificial α-secretases" were stable for even as long as 15 days; however, debenzylated products started appearing after only one day. After three days, peaks corresponding to the mass of various intermediates and fragments could be observed. Plausible routes of proteolysis of mAβ by "artificial α-secretases" were drawn (Fig. S23–S50, ESI†) based on the mass values. Each of the "artificial α-secretases" followed a similar mechanism. Routes followed by "artificial α-secretase"2 are depicted in Fig. 2 as a representative example. At first, the amine group of the side chain of Lys of mAβ became attached to the nucleophilic carbonyl of the pendant adipate of "artificial α-secretase"2 by the expulsion of the good leaving group, *O*-Bn, generating the intermediate C″ (C′, C″ and C‴ for "artificial α-secretase"1, 2, and 3, respectively) *via* intermolecular *O* to *N* acyl migration (Fig. 2a and b inset). The intermediate C″ then followed either route $1^{mAβ–AαS2}$ to cleave itself between Leu17 and Val18 of mAβ to generate segment D″ and intermediate E″ that was again sliced to form I″ or followed route $2^{mAβ–AαS2}$ to cut between Lys16 and Leu17 of mAβ to generate J″ and another intermediate K″. K″ might further convert to O″ *via* L″ and N″. The time-dependent persistence map of the intermediates and fragments (Fig. 2c–e) revealed that while "artificial α-secretase"1 and 2 started fragmenting mAβ as early as after three days, this took "artificial α-secretase"3 ∼13 days. Also, while "artificial α-secretase"2 generated most of the fragments by seven days, "artificial α-secretase"1 took >13 days. Thus, the order of reactivity is "artificial α-secretase"2 > 1 > 3, and this depends on the position of the pendant group.

However, mAβ remained after as long as 30 days in all three cases, but it disappeared completely with two-fold molar excess of "artificial α-secretase"4 by ten days. "Artificial α-secretase"4 retained the site-selectivity and followed similar mechanistic pathways (routes $1–5^{mAβ–AαS4}$, Fig. S51–S56, ESI†), but produced more intermediates and fragments having a range of lifetimes at a faster rate than the other analogues. "Artificial α-secretases" also generated self-degradation products, *e.g.*, Q″ and R″ by "artificial α-secretase"2, following route $1^{SD–AαS2}$ and route $2^{SD–AαS2}$, respectively, confirmed by similar experiments in the absence of mAβ (Fig. 2a and Fig. S57–S63, ESI†). The self-degradation products of mAβ (control, Fig. S64–S67, ESI†) are different from the "artificial α-secretase"-mediated proteolysis products. This result, along with the observation of the corresponding mass of C″ and consistency of appearance of the fragments, collectively supports the proteolytic role of "artificial α-secretases" excluding the probability of fragmentation by the spectrometer.

DFT calculation revealed the most stable conformation of C″ (Fig. 2f) and C‴′ (Fig. S68, ESI†). The bond distances between

Table 1 Peptides and their roles in the current study

Name	Peptide sequence	Role of the peptide
AαS1	Ac-K(BnAdp)LVFF-NH_2	Site-selective cleavage
AαS2	Ac-K(BnAdp)GLVFF-NH_2	Site-selective cleavage
AαS3	BnAdp-AALVFF-NH_2	Site-selective cleavage
AαS4	BnAdp-K(BnAdp)K(BnAdp)LVFF-NH_2	Site-selective cleavage
mAβ	Ac-VHHQKLVFFGG-NH_2	Prototype of Aβ & APP
$Aβ_{1–40}$	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-NH_2	Target aggregating peptide
Mutant DTP28	GSSDSIGPLGYGKTVDHTKVNSPLSLFG-NH_2	Negative control

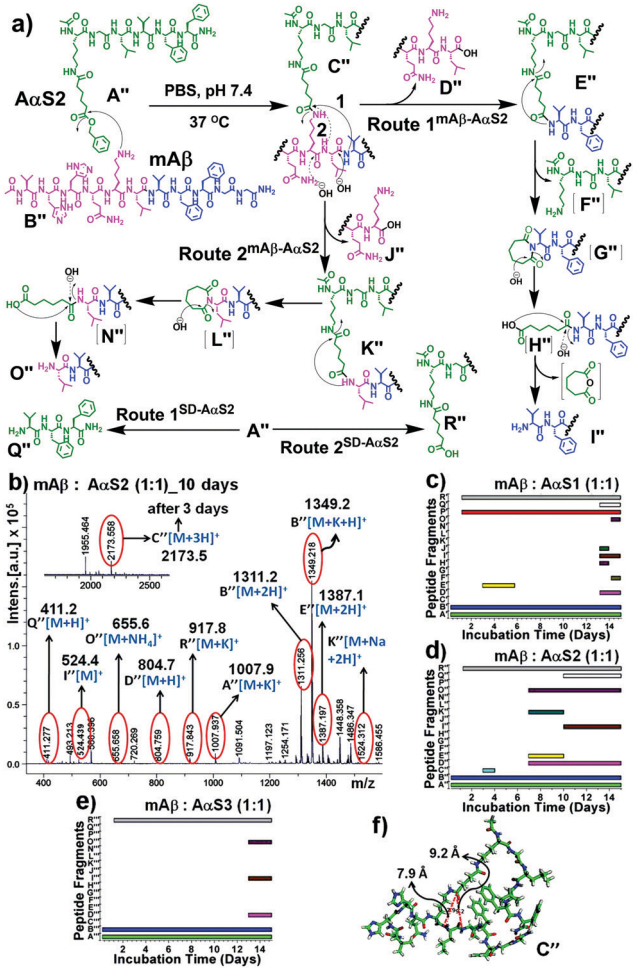

Fig. 2 (a) Plausible routes of proteolytic cleavage of mAβ into various fragments. (b) MALDI-TOF mass spectra of mAβ in the presence of "artificial α-secretase"2 (1 : 1) after ten days [inset: after three days] of incubation in PBS pH 7.4 at 37 °C. (c–e) Time-dependent persistence map of various fragments of mAβ in the presence of "artificial α-secretases". (f) The most stable conformation of the intermediate C″ (image generated by PyMOL) obtained from DFT calculation [B3LYP, basis set: 6-31G]. AαS stands for "artificial α-secretase".

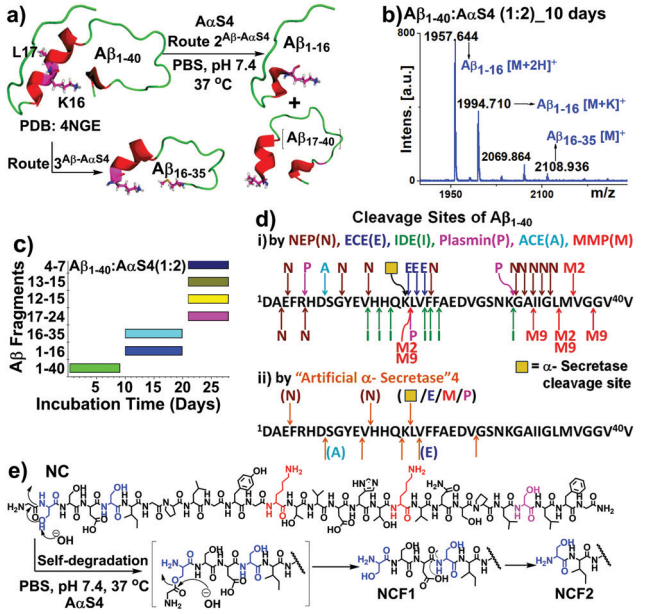

Fig. 3 (a) Some of the probable routes of proteolytic cleavage and (b) MALDI-TOF mass spectra of $A\beta_{1-40}$ in the presence of "artificial α-secretases"4 (1 : 2) at ten days in PBS pH 7.4 at 37 °C. (c) Time-dependent persistence map of various fragments. (d) Multiple proteolytic enzyme activities of "artificial α-secretases"4. (e) Plausible routes of self-degradation of NC into various fragments (NCFs).

the carbonyl oxygen of the *in situ* generated amide and the nitrogens of the backbone amides of Leu and Val are 7.9 Å and 9.2 Å, respectively (8.0 Å and 10.5 Å for C‴), which may be small enough to facilitate the nucleophilic attack by the mentioned nitrogens on the carbonyl groups that in turn become even closer and start forming a tetrahedral intermediate; this probably explains the site-selectivity of the proteolysis. It also indicates that the driving force of such a nucleophilic attack could be the release of the strain of the cyclic structure generated by the inter-strand aromatic interactions. Furthermore, the intensity of the peak corresponding to "artificial α-secretase"2 diminished but did not disappear until ten days in a time-dependent HPLC experiment (Fig. S69–S76, rt 19 min, ESI†). The same is true of mAβ (rt 13.4 min), which did not shift but changed its shape. ESI-MS analysis of the eluent revealed the presence of D″, I″, J″, O″ and mAβ (B″). All of these results collectively indicate that the "artificial α-secretases" cleaved mAβ in a site-selective manner (both amide bonds Lys16–Leu17 and Leu17–Val18) first and then at the other positions over time.

Interestingly, "artificial α-secretase"4 also cleaved the full-length $A\beta_{1-40}$ site-selectively. Various plausible routes of proteolytic cleavage (routes $1–10^{A\beta-A\alpha S4}$, Fig. 3a, b and Fig. S77–S83, ESI†) were delineated from MALDI-TOF mass analyses, as before. A time-dependent persistence map of the Aβ fragments (Fig. 3c) demonstrates the time-resolved manner of the programmed proteolysis. "Artificial α-secretase"4 cleaved $A\beta_{1-40}$ between Lys16 and Leu17, which is the cleavage site of not only α-secretase but also of ECE, MMP2, MMP9 and P; and between Leu17 and Val18, the same as ECE by the first ten days. After prolonged incubation (>21 days), "artificial α-secretase"4 cleaved $A\beta_{1-40}$ between Val12 and His13 (the cleavage site of NEP and IDE), Asp7 and Ser8 (ACE cleavage site), and Glu3 and Phe4 (NEP cleavage site). Thus, "artificial α-secretase"4 itself can cleave $A\beta_{1-40}$, mimicking the proteolytic action of multiple proteases. "Artificial α-secretase"4 also cleaved $A\beta_{1-40}$ between Glu11 and Val12 and between Val24 and Gly25, unlike any known proteolytic enzymes. None of the fragments mentioned above were observed by the time-dependent MALDI-TOF mass analysis of $A\beta_{1-40}$ in the absence of "artificial α-secretase"4 (Fig. S84–S87, ESI†); rather aggregation was noted, unlike the "artificial α-secretase"4 treated samples, indicating its amyloid-solubilizing nature. None of the expected fragments possibly generated by "artificial α-secretase"4 were present in the co-incubated (1 : 1) sample of the mutant DTP28 (NC, Fig. S88–S97, ESI†) even after ten days. Instead, the self-degradation products of "artificial α-secretase"4 and NC appeared. NC self-degraded at its Ser2 and Ser5 positions, subsequently (Fig. 3e), which was confirmed by a parallel experiment in the absence of "artificial α-secretase"4. Such cleavage may proceed *via* route 11^{NC}, which is unrelated to "artificial α-secretase"4. This result indicates the substrate-selective nature of "artificial α-secretase"4. All of the mentioned

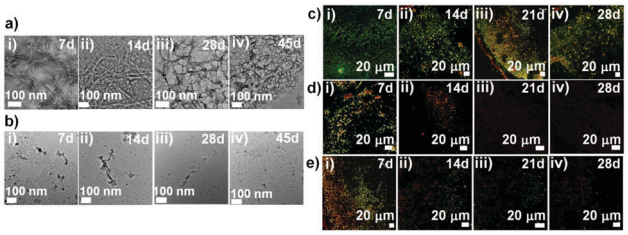

Fig. 4 Time-dependent (a and b) TEM images, and (c–e) Congo red stained birefringence images of $A\beta_{1-40}$ (a and c) in the absence and (b, d and e) in the presence of 5-fold "artificial α-secretase"4, while (b and d) show inhibition of amyloidosis, and (e) disruption of pre-existing amyloid (PBS pH 7.4 at 37 °C).

routes of cascades of chemical reactions are logically-drawn, probable routes. Many other modes of bond formation and cleavage may be operating in the sample. All of the intermediates and fragments may not be identified, but most of them could be assigned, except those in parentheses in the figures.

Finally, the kinetics of amyloid accumulation of $A\beta_{1-40}$, and the disruption of their preformed fibrils was monitored in the absence and presence of "artificial α-secretase"4 (Fig. 4 and Fig. S98–S106, ESI†). The comparative thioflavin T aided fluorescence, CD, TEM, AFM, Congo-red birefringence, and DLS analyses suggested that "artificial α-secretase"4 not only inhibited fibrillation of $A\beta_{1-40}$ but also dissolved preformed fibrils in a dose-dependent manner. Such destruction of the fibril and amyloid is probably due to the pre-programmed site-selective proteolysis by "artificial α-secretase"4. To the best of our knowledge this is the first report of amyloid-digestion by an externally added chemical. Smaller, soluble Aβ oligomers produced by fibril disruption were unable to rupture carboxyfluorescein dye-entrapped large unilamellar vesicles (Fig. S106, ESI†), which is usually related to the non-toxic nature of the disrupted smaller fibrils or oligomers.[6,17]

While APP processing by β- and γ-secretases generate disease-causing Aβ,[18] the natural mechanism of AD prevention lies on the site-specific APP cleavage by α-secretases to release the non-amyloidogenic neuroprotective APP fragment, known as soluble APP-α (sAPPα), preventing generation of neurotoxic and pathogenic Aβ.[19] On the other hand, enzymes such as NEP, ECE, IDE, P, ACE, and MMPs are known to degrade Aβ aggregates to facilitate their excretion.[20] When these enzymes fail, plaques form. Promoting α-secretase-mediated APP processing and Aβ metabolism is, therefore, a promising therapeutic option for AD management.[7,21] ADAM10 (a disintegrin and metalloproteinase), a transmembrane protein which is approximately 750 amino acids long, is known as one of the main α-secretases.[22] Its over-expression increased sAPPα, reduced plaque load, and improved learning and memory in the AD mouse model.[23] However, a wide variety of substrates of ADAM10 are linked to many physiological, as well as pathological conditions, including not only the immune and nervous systems but carcinogenesis has also been reported.[24] Therefore, addressing AD through the up-regulation of α-secretases may improve AD but this also increases the risk of cancer and other diseases.[25] Similarly, up-regulation of other Aβ-degrading enzymes also may cause side effects. Therefore, developing the proposed function-specific mimic of these enzymes may ease degradation and excretion of plaques.

In summary, a general method of *de novo* design of function-specific protease-mimics is disclosed. The engineered peptides, termed as "artificial α-secretases", indeed first sliced Aβ peptide at the α-secretase cleavage site followed by slicing at the cleavage sites of the other Aβ-degrading enzymes by a self-association driven repetition of an imidation–hydrolysis–transamidation cycle. One interesting feature of such "artificial α-secretases" is that the cleavage sites can be manipulated by altering the position and length of the pendant groups. Most importantly, the structurally unrelated peptide was unaffected, indicating excellent substrate-selectivity. Thus, we could achieve the proteolytic action of Aβ formation preventing enzyme (α-secretase) as well as Aβ degrading enzymes, all by "artificial α-secretases", presumably without affecting other substrates of these enzymes in a non-catalytic manner. While the amyloid-degrading enzymes cannot attack solvent inaccessible insoluble amyloid, the self-association initiated mechanism favours "artificial α-secretases" to recognize and disrupt amyloid-generating non-toxic Aβ fragments. Therefore, design and development of such constructs may be a promising approach for drug design against not only AD but also other amyloidoses. However, further optimization of the structure to improve the reactivity and pharmacokinetic profile is required to establish their physiological relevance. Such a strategy may also be extended to mimic other enzyme functions.

Conflicts of interest

There are no conflicts to declare.

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