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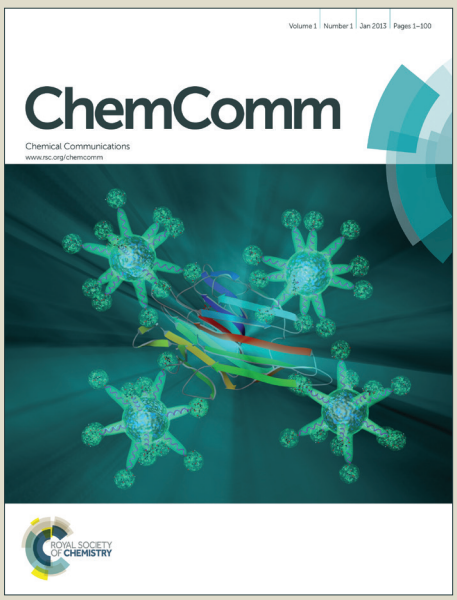

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COMMUNICATION

Inhibition of Alzheimer's Amyloid-β Peptide Aggregation and its Disruption by a Conformationally Restricted α/β Hybrid Peptide

Ashim Paul, Krishna Chaitanya Nadimpally, Tanmay Mondal, Kishore Thalluri, and Bhubaneswar Mandal*

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Insertion of an anthranilic acid in an amyloidogenic peptide sequence generates a novel conformationally restricted α/β-hybrid peptide that inhibits amyloid formation of $A\beta_{(1-40)}$ and disrupts preformed fibrillar aggregates *in vitro*. Such β-sheet breaker hybrid peptides (BSBHps) may be useful for designing novel physiologically important compounds relevant to diverse amyloidoses and for studying the process of aggregation.

Alzheimer's disease (AD) is characterized by the deposition of amyloid comprised of Amyloid-β peptide (Aβ, comprised of 39-42 residues) in interneuronal spaces.[1] Amyloid aggregation is the reversible[2] transformation of the soluble native peptide, first to soluble Aβ oligomers, and eventually to insoluble amyloidogenic cross-β-sheet structures.[3] Soluble Aβ oligomers are recognized as more toxic than the insoluble amyloids and are responsible for disruption of cell membrane causing cell death.[4] Therefore, preventing the conversion of Aβ by stabilizing the soluble monomer using conformationally restricted peptides has emerged as an important strategy. For example, Soto *et al*. introduced β-sheet breaker peptides (BSBp) comprised of a recognition motif and a β-breaker element (proline).[5] Dehydrophenylalanine[6] and α-aminoisobutyric acid (Aib)[7] containing conformationally restricted BSBps are also reported. Most of the reported BSBps contain either an α-amino acid or a modified α-amino acid which are unstable against proteolytic degradation. Since β-amino acids are non-proteinogenic and thus more stable against proteolytic degradation, they are attractive for peptide drug design.[8]

Herein, we report the design, synthesis, and anti-aggregation potential of a new class of β-sheet breaker peptides which contain an anthranilic acid unit (**Ant**, *ortho*-aminobenzoic acid, a non-coded aromatic β-amino acid) as the β-breaker element. **Ant** is found in many biologically active molecules, and also serves as a precursor for the biosynthesis of tryptophan.[9] The known propensity of **Ant** to induce either helix or turn conformation[10] and its stablity towards proteolytic degradation prompted us to use it as a novel β-breaker element in β-sheet breaker peptides. Collectively we termed them β-sheet breaker α/β hybrid peptides (BSBHps). We have demonstrated their ability to inhibit and even reverse the self aggregation of $A\beta_{1-40}$ peptide. To the best of our knowledge, such aromatic β-amino acids were not used as β-sheet breaker unit before.

To test our hypothesis, we designed and synthesized three BSBHps, **1** [LSL**X**LSLG-NH_2], **2** [Ac-**X**LSLG-NH_2], and **3** [Ac-L**X**FFD-NH_2], each comprised of an oligopeptide as a recognition motif and an **Ant (X, $C_7H_7NO_2$)** as the breaker element (standard amino acids are represented with one letter code, Ac = Acetyl group). A previously reported BSBp, widely known as Soto's peptide (**4**, Ac-L**P**FFD-NH_2**)**, was also synthesized and used as a control. The switch peptide **5** [LSL**(H^+)**SLSLG-NH_2], which is known as the functional mimic of the Aβ peptide, was used as a model aggregating peptide.[11] The BSBHps **1** and **2** were designed to share sequence homology with the corresponding aggregating peptide **5** whereas the -F-F- portion of BSBHp **3** shares sequence homology with $A\beta_{19-20}$ for proper recognition. The C-terminal Asp of the BSBHp **3** is kept to maintain sequence homology with the control peptide **4**, for proper comparison. Commercially available $A\beta_{1-40}$ was used as the native aggregating system.

We first examined the amyloidogenic nature of the BSBHps by various biophysical methods. The presence of fibrillar structure under transmission electron microscope (TEM) is a characteristic property of amyloid formed by a peptide.[12] None of the BSBHps were found to exhibit such fibrillar assembly (Fig. S1, ESI). Another characteristic property of amyloid is the expression of green gold birefringence under cross polarized light after staining with Congo red.[8] No such green gold birefringence was noticed for the BSBHps (Fig. S2, ESI). The conformation of the peptides was monitored by CD and FTIR spectroscopy (Fig. S3 and S4, ESI), but the characteristic β-sheet profile was not observed. Therefore, it was concluded that the **Ant** containing α/β-hybrid peptides (**1**, **2,** and **3**) do not form β-sheets and are non-amyloidogenic in nature.

Next, we investigated the aggregation inhibitory efficacy of the BSBHps. We first tested BSBHps **1** and **2** on the model amyloidogenic peptide **5** before testing with the $A\beta_{1-40}$ peptide as $A\beta_{1-40}$ is costly and its synthesis and purification are difficult. The conversion of **5** from its iso-peptide state to the native state was observed by LC-MS at pH 7.0 (Fig. 1a, it was too fast to detect at pH 7.4). The kinetics of amyloid formation of **5** was monitored by a time dependent thioflavin T (ThT) fluorescence assay. The increase in fluorescence intensity of a peptide upon binding with ThT is a characteristic property of the fibrillar nature of the peptide and the fluorescence intensity is directly proportional to the amount of fibril present.[13] The fluorescence intensity of **5** was

found to increase with time in the absence of a breaker peptide (black, Fig. 1b), but was suppressed in the presence of **1** (red, Fig. 1b) and **2** (blue, Fig. 1b) indicating the inhibitory nature of the BSBHps. Peptide **5** alone showed clear fibrillar structure when viewed under TEM (Fig. 1c, i) and green gold birefringence under cross polarized light when stained with Congo red (Fig. 1d, i). But, when **5** was co-incubated with **1** or **2** (2 fold molar excess) for 5 days under physiological conditions *in vitro* (PBS of pH 7.4 at 37 °C), no characteristic fibrillar structure was observed in TEM (Fig. 1c, ii and iii) and Congo red birefringence studies (Fig. 1d, ii and iii). The conformational change of **5** was also monitored by parallel CD and FTIR absorption (Fig. S5 and S6, ESI) in the absence and presence of **1** or **2**. The results were in agreement with those of the fluorescence experiments. Thus, the inhibitory effect of the BSBHps (**1** and **2**) upon amyloid formation of the model amyloidogenic peptide **5** was demonstrated.

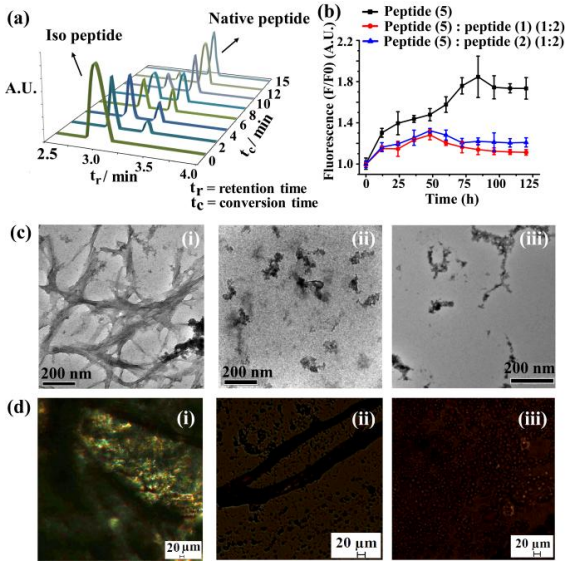

Fig. 1 (a) The kinetics of O to N acyl migration of **5**, monitored by time dependent LC-MS at pH 7.0. (b) Time dependent ThT fluorescence assay of **5** in absence (black) and presence of 2 fold excess BSBHp **1** (red) or BSBHp **2** (blue). (c) TEM, and (d) Congo red stained birefringence images of **5** in absence (i) and presence of 2 fold molar excess of **1** (ii) or **2** (iii) Peptides were incubated in PBS, pH 7.4 at 37 °C.

The inhibitory efficacy of the BSBHp **3** on the aggregation of $A\beta_{1\text{-}40}$ was investigated next. BSBHp **3** and the control peptide **4** were each co-incubated with $A\beta_{1\text{-}40}$ in PBS of pH 7.4 at 37 °C up to 7 days in parallel, and the kinetics of the amyloid accumulation was monitored using various biophysical tools. To investigate dose dependence, 2, 5, and 10 fold molar excess of the breaker peptides were used for each experiment. ThT fluorescence of $A\beta_{1\text{-}40}$ was significantly decreased in a dose dependent manner by the presence of **3** vs. the control without any breaker peptide (Fig. 2a). Two fold molar excess (red, Fig. 2a) of **3** was not sufficient to inhibit the amyloid formation of $A\beta_{1\text{-}40}$, whereas inhibition was clearly evident with 5 fold excess (blue, Fig. 2a), and even more pronounced with 10 fold excess (magenta, Fig. 2a). The amount of amyloid fibril was sharply reduced by 71-72% (blue, Fig. 2b) when **3** was co-incubated with $A\beta_{1\text{-}40}$ in 10 fold molar excess after seven days, whereas an equivalent amount of **4**, suppressed fibrillization by 47-48 % (red, Fig. 2b). These results suggest that **3** is a stronger inhibitor of amyloid formation than the existing BSBp **4** (Fig. S7-S9, ESI).

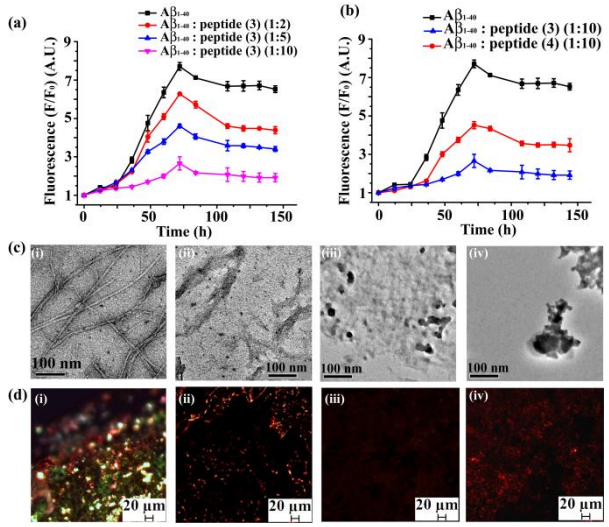

Fig.2 (a) Dose dependent ThT fluorescence assay of $A\beta_{1\text{-}40}$ in absence (black) and presence of 2 fold (red), 5 fold (blue), and 10 fold (magenta) molar excess of **3**. (b) Time dependent ThT fluorescence assay of $A\beta_{1\text{-}40}$ in absence (black) and presence of 10 fold molar excess of **3** (blue) or **4** (red). (c) TEM and (d) Congo red stained birefringence images of $A\beta_{1\text{-}40}$ in absence (i) and presence of 2 fold (ii), 5 fold (iii), and 10 fold (iv) molar excess of **3**. All the peptide solutions were incubated in PBS pH 7.4 at 37 °C.

$A\beta_{1\text{-}40}$ alone exhibited clear fibrillar structure when viewed under TEM (Fig. 2c, i). When **3** was co-incubated in 2 fold molar excess (2c, ii) with $A\beta_{1\text{-}40}$, some fibrillar assembly was still observed. However, when the amount of **3** was increased to 5 fold (Fig. 2c, iii) and 10 fold molar excess (Fig. 2c, iv), no fibrils were observed in agreement with the results of the ThT assay. $A\beta_{1\text{-}40}$ peptide exhibited green gold birefringence under cross polarized light upon staining with Congo red (Fig. 2d, i) when incubated alone. But when **3** was co-incubated in the same molar concentrations as previously mentioned (ii, iii, and iv, Fig. 2d), similar results were obtained as described for TEM and the ThT assay. Similar results were also obtained when 2, 5, and 10 fold excess of the control **4** was co-incubated with $A\beta_{1\text{-}40}$ (Fig. S10 for TEM and Fig. S11 for birefringence, ESI). The results of the CD and FTIR absorption (Fig. S12-S14 and S15-S18, ESI) were also in agreement with the mentioned observations.

We also investigated the capability of **3** to disrupt preformed $A\beta_{1\text{-}40}$ amyloid *in vitro*. Prior ThT assays (black, Fig. 2a) determined that the growth phase for fibrillization of $A\beta_{1\text{-}40}$ was from 24 h to 96 h. Therefore, **3** was added in 2, 5, and 10 fold molar excess into the preformed fibrillar assembly of $A\beta_{1\text{-}40}$ at 60 h (PBS pH 7.4 at 37 °C) in parallel experiments. A set of replicas with control **4** was also prepared and fibrillogenesis was monitored. By ThT assay, fluorescence increased when $A\beta_{1\text{-}40}$ was incubated alone (black, Fig. 3a), but when 10 fold molar excess **3** was added to its preformed fibrillar assembly, fluorescence was markedly suppressed over time (blue, Fig. 3a). The amount of fibril formed at 60 h by $A\beta_{1\text{-}40}$ was found to be reduced by **3** (51-52% reduction, blue, Fig. 3b) more significantly than by **4** (32-33% reduction, red, Fig. 3b) after 240 h. Similar results were obtained for 2 and 5 fold molar excess of breaker

peptides **3** and **4** (Fig. S19 and S20, ESI).

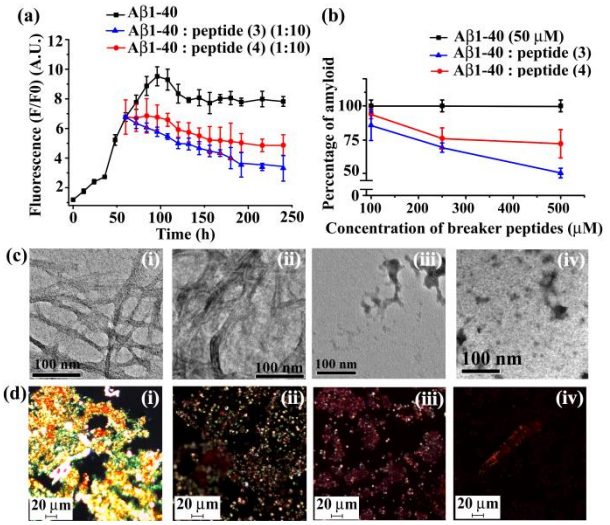

Fig. 3 (a) Time dependent ThT assay for preformed $A\beta_{1\text{-}40}$ fibril disruption in absence (black) and presence of **3** (blue) or **4** (red). (b) Concentration dependent fibril disruption in absence (black) and presence of **3** (blue) or **4** (red). (c) TEM and (d) Congo red stained birefringence images of $A\beta_{1\text{-}40}$ in absence (i) and presence of 2 fold (ii), 5 fold (iii), and 10 fold (iv) molar excess of **3**. All the images were taken after 10 (3+7) days of incubation in PBS pH 7.4 at 37 °C.

After 240 h of incubation in PBS pH 7.4 at 37 °C, the $A\beta_{1\text{-}40}$ alone exhibited fibrillar structure when viewed under TEM (i, Fig. 3c) indicating amyloid formation by $A\beta_{1\text{-}40}$. When **3** was mixed with $A\beta_{1\text{-}40}$ after 60 h (3rd day) in 2 fold molar excess, some fibril formation was observed (ii, Fig. 3c), indicating that 2 fold molar excess of **3** was not sufficient to significantly disrupt $A\beta_{1\text{-}40}$ fibrillization. However, no fibril was observed when the amount of **3** was increased to 5 fold (iii, Fig. 3c) and 10 fold (iv, Fig. 3c). This significant disruption of $A\beta_{1\text{-}40}$ fibril by **3** observed by TEM supported the results of the ThT assay. Similarly, $A\beta_{1\text{-}40}$ exhibited a characteristic green gold birefringence (i, Fig. 3d) when viewed under a microscope with cross polarized light. Some amount of green gold colour was observed when 2 fold molar excess (ii, Fig. 3d) of **3** was present. But with 5 fold (iii, Fig. 3d) and 10 fold (iv, Fig. 3d) molar excess of **3**, no such green gold birefringence was observed after 240 h, again indicating complete disruption of the preformed amyloid. Similar results were obtained in the case of **4** (Fig. S21, ESI). Thus, BSBHp **3** was shown to be effective at disrupting and even re-dissolving existing $A\beta_{1\text{-}40}$ amyloid fibrils in a dose dependent manner and is relatively better than the control **4** for that. The disappearance of the signature of β-sheet conformation of $A\beta_{1\text{-}40}$ by co-incubation of **3** and the presence of that in the absence of **3,** as observed by CD and FTIR absorption (Fig. S22-S25 and S26-S29, ESI), further support these findings.

Soluble oligomers or protofibrils of Aβ are known to be more toxic than the mature amyloid fibrils due to their ability of pore formation in cell membrane.[4] We carried out dye leakage assay using carboxyfluorescein entrapped large unilamellar vesicles (LUVs, Fig. S30, ESI)[14] and found that BSBHp **3** re-dissolved pre-formed $A\beta_{1\text{-}40}$ fibrils but no evidence for the presence of toxic pore-forming species was observed in the 10-day old peptide solution (Fig. S31, also text in page 5-6 and 20-22, ESI).

We have developed a novel class of conformationally restricted β-sheet breaker hybrid peptides (BSBHps) comprised of a recognition motif of the target amyloidogenic peptide and anthranilic acid (**Ant**) as the breaker element. We have demonstrated efficiency of one such peptide (BSBHp **3**) for inhibition of amyloid formation by the native $A\beta_{1\text{-}40}$ peptide at physiological pH and temperature. We have also demonstrated that **3** disrupts and re-dissolves preformed $A\beta_{1\text{-}40}$ amyloid fibrils and generates non-toxic species (possibly monomers). Since β-amino acids are relatively more stable against proteolytic degradation, this novel class of BSBHps described herein shows much promise for the design of drugs to treat protein conformational diseases, including Alzheimer's disease, Parkinson's disease, and type II diabetes. Such peptides may also prove useful for studying aggregation/disaggregation processes and may generate novel foldamers important for various applications in biology as well as in materials chemistry.

Notes and references

*Laboratory of Peptide and Amyloid Research, Department of Chemistry, Indian Institute of Technology Guwahati, Assam- 781039, India. Email: bmandal@iitg.ernet.in

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