

Protective effects of β -sheet breaker α/β -hybrid peptide against amyloid β -induced neuronal apoptosis in vitro

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Alzheimer's disease is most common neurodegenerative disorder and is characterized by increased production of soluble amyloid- β oligomers, the main toxic species predominantly formed from aggregation of monomeric amyloid- β (A β). Increased production of A β invokes a cascade of oxidative damages to neurons and eventually leads to neuronal death. This study was aimed to investigate the neuroprotective effects of a β -sheet breaker α/β -hybrid peptide (BSBHp) and the underlying mechanisms against A β_{40} -induced neurotoxicity in human neuroblastoma SH-SY5Y cells. Cells were pretreated with the peptide A β_{40} to induce neurotoxicity. Assays for cell viability, cell membrane damage, cellular apoptosis, generation of reactive oxygen species (ROS), intracellular free Ca²⁺, and key apoptotic protein levels were performed in vitro. Our results showed that pretreatment with BSBHp significantly attenuates A β_{40} -induced toxicity by retaining cell viability, suppressing generation of ROS, Ca²⁺ levels, and effectively protects neuronal apoptosis by suppressing proapoptotic protein Bax and up-regulating antiapoptotic protein Bcl-2. These results suggest that α/β -hybrid peptide has neuroprotective effects against A β_{40} -induced oxidative stress, which might be a potential therapeutic agent for treating or preventing neurodegenerative diseases.

KEYWORDS

Alzheimer's disease, amyloid- β peptide, apoptosis, human cerebrospinal fluid, neuroprotection, SH-SY5Y cell, β -sheet breaker α/β -hybrid peptide

1 | INTRODUCTION

Alzheimer's disease (AD), a protein-misfolding neurodegenerative disease, is characterized by progressive loss of memory and cognitive ability in elderly population.^[1] The central event in AD is the formation of soluble oligomers of A β from dysregulated proteolytic cleavage of amyloid precursor protein (APP) by two proteases β - and γ -secretases, respectively,

Abbreviations: AD, Alzheimer's disease; A β O, amyloid- β oligomers; Ant, anthranilic acid; APP, amyloid precursor protein; BSBp, β -sheet breaker peptide; BSBHp, β -sheet breaker α/β -hybrid peptide; CAA, cerebral amyloid angiopathy; h-CSF, human cerebrospinal fluid; NFT, neurofibrillary tangles; ROS, reactive oxygen species; SPs, senile plaques.

which impair hippocampal synaptic plasticity and cause cognitive dysfunction.^[2] Extensive accumulation of A β peptide in brain parenchyma and cerebral vessel walls, called cerebral amyloid angiopathy (CAA), and senile plaques (SPs), plays a key role in Alzheimer's disease.^[3] Neuropathological hallmarks in AD brain are aggregation and fibrillization of A β leading to extracellular deposition of neuritic plaques and hyperphosphorylated microtubule-associated Tau protein, called neurofibrillary tangles (NFT).^[4] In addition, synaptic dysfunction, alteration of neuronal metabolism, loss of neurotransmission, and ultimately loss of neuronal cell occur.^[5,6]

A β is a 4.3-kDa peptide composed of 39–42 amino acids. Aggregation and misfolding of A β is the reversible transformation of nonpathogenic soluble native peptide first into

soluble toxic oligomers and further to insoluble amyloidogenic fibrils composed of β -sheet structures.^[7] Several studies reported that soluble oligomers are more toxic to neurons in cultures than insoluble amyloidogenic peptide and are responsible for neuronal cell death by synapse loss, membrane disruption, altered mitochondrial metabolism, impaired Ca^{2+} homeostasis, DNA damage, and inflammatory processes.^[8–10]

$\text{A}\beta$ -induced neurotoxicity and its underlying mechanism is complex and remains obscure, but several pieces of evidence imply that enhancement of oxidative stress provoked by $\text{A}\beta$ is associated with AD.^[11] It has been reported that $\text{A}\beta$ can fragmentize and thus generate free radicals with potent lipid peroxidation. In addition, $\text{A}\beta$ -induced toxicity in cell culture supports that oxidative stress induced by ROS plays a vital role in $\text{A}\beta$ -mediated neuronal degeneration. The excessive production of ROS can cause cellular damage and subsequent cell death; thus, ROS is a sensitive marker of oxidative injury. Therefore, pharmacological intake of ROS modifying β -sheet breakers peptide can be considered as one of the therapeutic strategies to treat $\text{A}\beta$ -induced toxicity in AD and could protect neurons from $\text{A}\beta$ -induced oxidative stress.

A class of synthetic peptide called β -sheet breaker peptide (BSBp) can inhibit the formation of β -sheet-rich pathological conformers of $\text{A}\beta$ peptide and reverse the misfolded conformation of pathogenic $\text{A}\beta$ peptide. Previously, Soto et al. introduced a series of BSBps specially $\text{iA}\beta 5$ that is comprised of a recognition motif homologous to pathogenic $\text{A}\beta 42$ and also contains a proline-rich β -breaker element.^[12] Most of the previously reported BSBps contain either α -amino acids or α -amino acid derivatives. They are degradable by proteolysis. Because β -amino acids are nonproteinogenic, β -amino acid-containing peptides are better choice for drug design for their better stability against proteolytic degradation.^[13]

We have demonstrated the design and synthesis of a new class of β -sheet breaker peptide (e.g., Ac–Leu–Ant–Phe–Phe–Asp– NH_2) that contains an anthranilic acid (Ant) as a β -sheet breaker unit. Ant is a noncoded aromatic β -amino acid, which is nonproteinogenic, stable against proteolytic degradation, and also a precursor for the biosynthesis of tryptophan. Unlike proteinogenic amino acids, Ant and its homologs are conformationally rigid and strongly favor helix or turn conformation when formed hybrid peptide with the α -amino acids.^[14–16] Collectively, we named them as β -sheet breaker α/β -hybrid peptide (BSBHp). Such BSBHps have been identified as non-amyloidogenic and exhibited inhibitory and disruptive efficacy against $\text{A}\beta$ aggregation.^[17] Therefore, Ant-containing α/β -hybrid peptides can be a benign candidate for drug design against AD and various protein-misfolding diseases.

Herein, we have used human neuroblastoma SH-SY5Y cells as in vitro cellular model of AD as it differentiates into

mature neuron-like structure, resembles with morphology and biochemistry of human neurons. It reveals expression of several neurospecific markers including synaptic protein sv-2, nuclear marker NeuN, as well as the presence of synapses. Reports from other laboratories have shown that these cells also express mature tau isoforms and tau protein. So, this cellular in vitro model is useful for primary screening of drugs particularly in AD.

In this study, we have investigated the effects of the BSBHp on $\text{A}\beta_{40}$ -induced neurotoxicity on human neuroblastoma SH-SY5Y cells. We evaluated the neuroprotective effects and the underlying mechanism of BSBHp against $\text{A}\beta$ -mediated neuronal cell death, generation of ROS, intracellular Ca^{2+} release, through mitochondrial apoptotic pathway (Bax, Bcl-2, caspase 9, caspase 3, cytoplasmic and mitochondrial cytochrome-C) in vitro. We reported here that BSBHp exerted the neuroprotective effects against $\text{A}\beta$ not only by regulating the expressions of pro-apoptotic proteins, but also through restoring the intracellular levels of Ca^{2+} and ROS generation. We also demonstrated the effect of BSBHp for disruption of $\text{A}\beta$ aggregates present in h-CSF of Alzheimer's patients of late stage.

2 | MATERIALS AND METHODS

2.1 | Reagents and solvents

Rink amide MBHA resin (loading 0.7 mmol/g), Fmoc amino acids, coupling reagents (BOP and PyBOP), and human Alzheimer's β -amyloid ($\text{A}\beta_{40}$) were purchased from GL Biochem (Shanghai). DIPEA, thioflavin T, Congo red, and 5(6)-carboxyfluorescein were purchased from Sigma. All the lipids were purchased from Avanti Polar Lipid, Inc. DMF, DCM, and acetonitrile of HPLC grade were obtained from Merck (India). Human cerebrospinal fluid (h-CSF) samples (late stage) were obtained from Guwahati Neurological Research Center (GNRC) and Hospital, Guwahati, India, for performing biophysical studies according to the bioethics policy of the hospital.

2.2 | Peptide synthesis

The described peptides were synthesized by standard Fmoc/tBu-based solid-phase peptide synthesis method on MBHA-Rink amide resin (loading 0.7 mmol/g). For each amino acid attachment, 2 equiv of Fmoc amino acids, 2.5 equiv of coupling reagent (BOP), and 5 equiv of base (DIPEA) were used for incomplete reaction, and coupling cycles were repeated, followed by capping with acetic anhydride (2 equiv) and *N*-methyl imidazole (3 equiv). Fmoc deprotection was performed with 20% piperidine in DMF. The final peptide was cleaved from the resin using a cleavage cocktail (90% TFA, 5% DCM, and 5% H_2O) for

5 hr. The crude peptide was precipitated by cold diethyl ether followed by centrifugation to achieve crude solid peptide.

2.3 | Liquid chromatography and mass spectrometry

Crude peptides were purified by RP-HPLC (Waters 600E) using a C18- μ Bondapak column at a flow rate of 5 ml/min. Binary solvent systems were used, solvent A (0.1% TFA in H₂O) and solvent B (0.1% TFA in CH₃CN). A Waters 2,489 UV detector was used with dual detection at 214 and 254 nm. A total run time of 20 min was used and gradient used for purification was 5%–100% CH₃CN for 18 min followed by 100% CH₃CN till 20 min. Purity of the peptides was confirmed from Waters UPLC-MS system (ESI +ve mode), and Micromass Q-TOF equipped with Masslynx software was used. Mobile phase comprised solvent A (0.1% TFA in H₂O) and solvent B (0.1% TFA in CH₃CN) on C18 column with a flow rate of 0.25 ml/min. Dual wavelengths were selected at 214 nm and 254 nm. Linear gradient of 5%–100% A was used in a total run time of 8 min.

2.4 | Carboxyfluorescein dye-loaded large unilamellar vesicles (LUVs) leakage study

The LUVs were prepared using three different lipids, DMPC, cholesterol, and GM1 with 68:30:2 molar ratios in 50 mM HEPES buffer of pH 7.4.^[20,21] The formation of LUVs was confirmed by transmission electron microscope (TEM). For the leakage study, the peptide and lipid were taken in 1:20 molar ratios, and the dye (carboxyfluorescein) release was monitored using Fluoromax-4, Horiba instrument. The excitation was at 485 nm and emission was monitored at 516 nm with 3 nm of bandwidth. At the end of the experiment, 10 μ l of Triton-X-100 was added to obtain complete dye release from the vesicle and the final fluorescence was measured. The % of leakage (dye release) was calculated as

$$\% \text{ Leakage} = \frac{(\text{observed fluorescence} - \text{initial fluorescence})}{(\text{total fluorescence} - \text{initial fluorescence})} \times 100\%$$

From the instrument, the text file was taken and graph was plotted using ORIGIN PRO 8 software. For each data point, three different sets of replicate solutions were scanned separately and the average was taken with observed standard deviation.

2.5 | Transmission electron microscopy (TEM)

We first prepared a stock solution of large unilamellar vesicles (LUVs) of concentration of 2 M. The prepared stock LUV solution (2 M) was diluted to 20 μ M for TEM analysis. 10 μ l

of aliquot (20 μ M) was added over the dark side of carbon-coated copper grid and allowed to float for 1 min. Then, 2% uranyl acetate solution (10 μ l) was added onto it and allowed to float for another 1 min. Then, the excess solution was removed using a blotting paper. The sample was dried at room temperature and kept in desiccators before taking TEM analysis on JEOL (Model: JEM 2100) instrument at 200 kV.

In case of amyloidogenic study for the peptide solution, the samples were prepared in a similar fashion as described above.

2.6 | Peptide preparation and treatment

Commercially available A β ₄₀ was dissolved in small volume (20 μ l) of TFA to obtain disaggregated A β ₄₀. TFA was evaporated using nitrogen gas. To remove TFA completely, HFIP was added and evaporated using nitrogen gas. This process was repeated twice and the disaggregated A β ₄₀ was kept at –20°C before biophysical studies. For cell-based studies, commercially available A β ₄₀ peptide was dissolved in sterile deionized water at a concentration of 50 mM, incubated in a capped vial at 37°C for 2 days to induce the generation of toxic species.^[19] Monomeric A β ₄₀ peptide was also co-incubated with different concentration of BSBHp for 2 days and added to SH-SY5Y cells. All assays were performed after 24-hr co-incubation with or without A β ₄₀ and BSBHp.

2.7 | Cell culture

The human neuroblastoma SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, CRL-2266) and grown in DMEM/F12 medium (Cat. No. Gibco/BRL, Life technologies, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Cat. No. Gibco/BRL, Life Technologies, NY, USA), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C and 5% CO₂.^[18] Cells were differentiated with 10 μ M of all-trans retinoic acid (RA) (Sigma-Aldrich, St. Louis, MO, USA) for 5 days. Cells were seeded in a FBS-free media for 12 hr prior to the treatment with A β ₄₀ and BSBHp peptides. A β ₄₀ was dissolved in deionized distilled water at a concentration of 10 μ M. The stock solution was diluted to desired concentrations immediately before use. The control cells were added with the same medium without A β ₄₀. Confluent cells were used in all assays, and each experiment was performed in triplicate.

2.8 | MTT reduction assay for cell viability

Cell viability was assessed using conventional MTT reduction assay. SH-SY5Y cells were seeded into a 96-well plate at a density of 1.5 \times 10⁴ cells/well and quantified mitochondrial activity of living cells by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

assay.^[22] Cells were preincubated with different concentrations (0.5, 1, 3, 5, and 10 μM) of BSBHp for 24 hr to study the self-toxicity. After that, $\text{A}\beta_{40}$ (10 μM) was co-incubated with or without BSBHp for 24 hr at 37°C. MTT (Cat. No. M5655; Sigma, St. Louis, MO, USA) was prepared at a concentration of 5 mg/ml in PBS and a total of 100 μl MTT solution was added to each well. The cells were incubated at 37°C and 5% CO_2 for 24 hr. After incubation, the MTT solution was aspirated and 200 μl of dimethylsulfoxide (DMSO, Cat. No. GRM 5856; Himedia, India) was added to each well to sufficiently dissolve the formazan precipitate. The amount of formazan was evaluated by measuring absorbance in a microplate reader (Bio-Rad, CA, USA) at 570 nm, and reference filter was used at 690 nm. All data were normalized to control cells from three independent experiments.

2.9 | Lactate dehydrogenase (LDH) release assay for cell membrane damage

LDH is a soluble cytosolic enzyme present in most eukaryotic cells, and its release into the culture medium is a key marker for cell death due to the damage of plasma membrane. Cell death was assessed by measuring the release of LDH into the medium.^[23] The increase in the LDH activity in the culture supernatant is proportional to the number of lysed cells. SH-SY5Y cells were seeded into FBS-free medium for 12 hr and then $\text{A}\beta_{40}$ alone or with different concentrations of BSBHp (0.5, 1, 3, 5, and 10 μM) co-incubated with 10 μM of $\text{A}\beta_{40}$ for 24 hr. After 24-hr treatment, 50 μl culture supernatants were collected from each well. Extracellular LDH release was evaluated by using colorimetric assay kit (Cat. No. ab102526 Abcam, Cambridge, MA, USA) as per manufacturer's protocol. Total LDH release was determined by adding 1% (V/V) Triton-X-100 into the cell for 1 hr. This represents maximal LDH release as the positive control with 100% cytotoxicity. The absorbance was measured at test wavelength of 490 nm with 660 nm as a reference filter. LDH leakage was expressed as the percentage (%) of the total LDH activity (LDH in the supernatant + LDH in the cell lysate), according to the equation:

$$\% \text{LDH released} = (\text{LDH activity in the medium} / \text{total LDH activity}) \times 100.$$

2.10 | Identification of apoptotic cells

The morphologies of nuclear chromatin were assessed by Hoechst 33258 staining after co-incubation of SH-SY5Y cells with $\text{A}\beta_{40}$ and BSBHp or $\text{A}\beta_{40}$ alone. Hoechst 33258 is a DNA-binding dye. Apoptotic cells were identified by their characteristics such as condensed chromatin, fragmentation of nucleus, and bright staining, whereas nuclei from normal cells demonstrated a normal uniform chromatin

pattern. The protocol was followed as described previously with some modifications.^[24] Briefly, Hoechst 33258 (Cat. No. 861405 Sigma, St. Louis, MO, USA) stain was added to the culture medium at a concentration of 10 $\mu\text{g}/\text{ml}$ for 10 min at 37°C. After that, cells were washed twice with phosphate-buffered saline (PBS) containing 1% (V/W) paraformaldehyde. Cells were mounted on a clean glass slide with mounting solution (0.1 M citrate, 0.25 M NaH_2PO_4 , glycerol) and the slide kept it in a dark place in 4°C for 20 min. Nuclear morphology was observed under fluorescence microscope (Olympus $\times 51$, Japan) with excitation filter of 350 nm and emission of 460 nm. The number of cells with condensed or fragmented nucleus was counted for the indication of apoptosis.

2.11 | Measurement of generation of intracellular reactive oxygen species (ROS)

The intracellular ROS generation was measured by dichlorodihydrofluorescein diacetate (DCFH-DA) as described previously^[25] with some modifications. In brief, following the drug treatment, the medium was aspirated and cells were incubated with DCFH-DA (Cat. No. D6883; Sigma, St. Louis, MO, USA) at a concentration of 10 $\mu\text{M}/\text{L}$ for 40 min at 37°C. The nonfluorescent molecule DCFH-DA is cleaved intracellularly by nonspecific esterases and turns to highly fluorescent 2',7'-dichlorofluorescein (DCF) upon oxidation by ROS. Cells were then washed twice with PBS and cellular DCF was determined by a fluorescent microplate reader (FLX 800, Biotek, USA), and the fluorescence intensity was measured by using an excitation wavelength at 490 nm and emission wavelength at 520 nm.

2.12 | Measurement of intracellular calcium (Ca^{2+})

The intracellular free Ca^{2+} levels in suspension of SH-SY5Y cells were measured by Ca^{2+} -sensitive dye Fura-2AM by previously defined method.^[26] Shortly, after treatment with respective peptides, 5 μM of Fura-2AM (Cat. No. 14591 Cayman, USA) was added to the culture medium at 37°C for 45 min. After that, each plate (1×10^6 cells) was washed twice with Krebs-Ringer-HEPES (KRH) buffer (135 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 0.4 mM KH_2PO_4 , 1 mM CaCl_2 , 5.5 mM glucose, 20 mM HEPES, pH 7.4). Fluorescence intensity was measured by using fluorescent microplate reader (FLX 800, Biotek, USA) with excitation and emission wavelengths of 488 and 525 nm respectively. The results are the mean \pm SEM of quadruplicate measurements from three separate experiments and are expressed as the percentage increase in relative fluorescence units (RFU) relative to the reading at baseline.

2.13 | Measurement of cytochrome-C

Cytochrome-C (cyt-C) expression was measured in both the cytosolic and mitochondrial fractions. Cytosolic and mitochondrial fractions were prepared using mitochondrial/cytosol fractionation kit as per manufacturer's instruction (Cat. No. K256-25 Biovision, USA). Briefly, treated cells were centrifuged at 700 *g* for 5 min at 4°C. Cells were washed in PBS and centrifuged, and the supernatant was discarded. Then, 0.5 ml of cytosolic extraction buffer was added that contains DTT and protease inhibitor. After incubation for 10 min, cells were homogenized and centrifuged at 700 *g* for 10 min. Supernatant was collected as cytosolic fraction of protein. The pellet was resuspended with 120 μ l of mitochondrial fractionation buffer with DTT and protease inhibitor. The mixture was vortexed for 30 s and marked as mitochondrial protein fraction. The protein expression from each fraction was analyzed by Western blot technique described below.

2.14 | Western blot analysis

Western blot analysis was performed for Bax, Bcl-2, caspase-9, caspase-3, cytochrome-C (cyto and mito) protein expression. After treatment with BSBHp and A β 40 peptides with indicated dosage for 24 hr, about 5×10^6 cells were washed twice with cold PBS and homogenized in ice-cold lysis buffer (50 mM Tris, pH-8.0 150 mM NaCl, 1% NP-40, 5 mM EDTA, pH 8.0, 1% deoxycholic acid) containing protease cocktail inhibitor (Cat. No. P8340; Sigma, St. Louis, MO, USA). Respective protein samples were electrophoresed on 15% (W/V) SDS-polyacrylamide gel (Mini-PROTEAN[®] tetra cell with mini-trans Blot[®], Bio-Rad, USA) and subsequently transferred from gel onto a nitrocellulose membrane (Millipore, USA). The membrane was incubated with fresh blocking buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 containing 5% nonfat-dried milk) for 1 hr at room temperature and then probed with respective monoclonal primary mouse anti-Bcl-2 (Cat. No. SC-7382; Santa Cruz Biotechnology, Inc., USA; 1:800 dilution in 3% BSA), mouse anti-Bax (Cat. No. SC-7480; Santa Cruz Biotechnology, Inc., USA; 1:800 dilution in 3% BSA), polyclonal rabbit anticaspase-3 (Cat. No. ab13847; Abcam, Cambridge, MA, USA; 1:700 dilution in 3% BSA), monoclonal rabbit anticaspase-9 (Cat. No. ab32539; Abcam, Cambridge, MA, USA; 1:1,000 dilution in 3% BSA), polyclonal rabbit anticytochrome-C (Cat. No. ab90529; Abcam, Cambridge, MA, USA; 1:1,000 dilution in 3% BSA) at 4°C overnight. Nitrocellulose membranes were washed three times in TBST and incubated in the respective HRP-conjugated goat anti-mouse (Cat. No. Sc-2005; Santa Cruz Biotechnology, Inc., USA; 1:5,000 dilution in 3% BSA), goat anti-rabbit (Cat. No. ab97051; Abcam, Cambridge, MA, USA; 1:5,000 dilution in 3% BSA)

secondary antibody at room temperature for 2 hr and washed it three times in the TBST buffer. After stripping the membrane with stripping solution, anti- β -actin rabbit polyclonal antibody (Cat. No. ab8227; Abcam, Cambridge, MA, USA; 1:5,000 dilutions in 3% BSA) was used as a control to reduce internal blot variability. Immunoreactive bands were visualized by using the ECL substrate solution (Cat. No. 32106; Thermo Scientific, Rockford, USA). The electrophoresis image analysis system (Smartview 2001, S/N: SV-0002202, Japan) was used for analysis of the optical density (OD) value of each band and normalized against corresponding β -actin band.

2.15 | Thioflavin T fluorescence assay of h-CSF sample from AD patients

A stock solution of thioflavin T (ThT) of 50 μ M concentration in PBS (50 mM, pH 7.4) was prepared and stored at 4°C with proper protection to prevent degradation from light. For disaggregation of A β ₄₀ present in human cerebrospinal fluid (CSF), CSF sample alone and with 10-fold molar excess of breaker peptides in PBS (pH 7.4) was incubated at 37°C in a water bath. For fluorescence assay, 40 μ l of aliquot from the stock was mixed with 200 μ l of ThT solution (50 μ M) and total volume was made up to 400 μ l using PBS. Fluorescence emission was measured at 485 nm and excitation at 440 nm, using a slit of 5 nm on a Fluoromax-4, Horiba instrument.

2.16 | Congo red-stained birefringence

A 20 μ l aliquot from the stock was placed over a glass slide followed by 20 μ l of the saturated Congo red solution. The excess solution was removed using a blotting paper, dried at room temperature, and analyzed under a Leica ICC50 HD polarizable microscope.

2.17 | Statistical analysis

All data were represented as mean \pm standard error of mean (SEM) for three independent experiments. Data were statistically analyzed with ANOVA followed by Student's *t* test using GRAPHPAD PRISM 6.0 Software (GraphPad Software, Inc., San Diego, CA, USA). Mean values were considered to be statistically significant at $p < .05$.

3 | RESULTS

We have synthesized a β -sheet breaker α/β -hybrid peptide (BSBHp, Ac-Leu-Ant-Phe-Phe-Asp-NH₂) and a known control breaker peptide (CBp, Ac-Leu-Pro-Phe-Phe-Asp-NH₂)^[12] via solid-phase peptide synthesis (SPPS) following Fmoc/tBu orthogonal protection strategy. The synthesized peptides were characterized by LC-MS (Figure 1). The

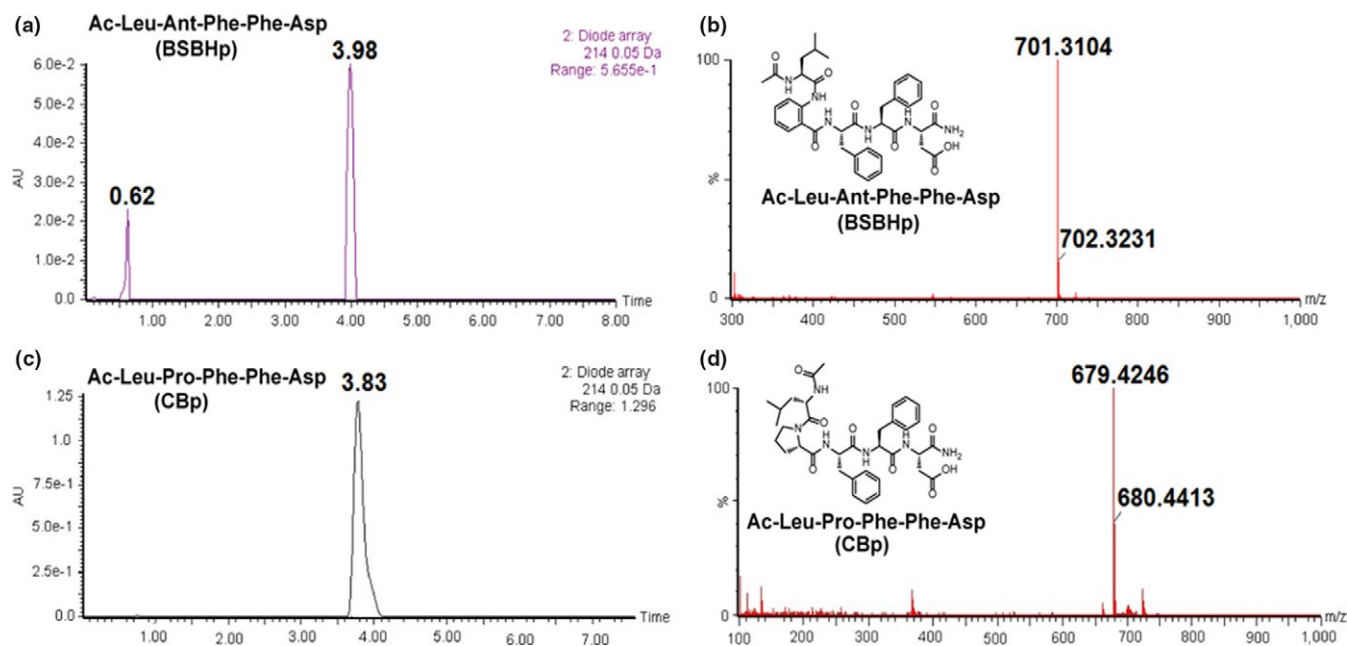


FIGURE 1 LC-MS picture of the purified BSBHp: Calculated mass for $C_{37}H_{45}N_6O_8$ is $701.32 [M + H]^+$, while observed $701.31 [M + H]^+$ (a, b). LC-MS picture of the purified CBp: Calculated mass for $C_{35}H_{47}N_6O_8$ is $679.34 [M + H]^+$, while observed $679.44 [M + H]^+$ (c, d) [Colour figure can be viewed at wileyonlinelibrary.com]

BSBHp was found non-amyloidogenic and efficiently disrupts the amyloid aggregates of $A\beta_{40}$ in vitro.^[17] As the soluble oligomers are more toxic than the mature amyloid,^[27] it was important to check whether the BSBHp disrupts the amyloid aggregates of $A\beta$ into toxic soluble oligomers or to nontoxic species. To address this problem, we have carried out a dye-loaded large unilamellar vesicle (LUV) leakage assay in vitro before conducting cell-based neurotoxicity studies. LUV leakage study is a proof of presence of toxic oligomeric species^[27] in a solution. Amount of dye leakage is proportional to the amount of the toxic soluble oligomeric species as toxicity is originated by pore formation in cell membrane or vesicle. LUVs were prepared as described in Materials and Methods section and characterized by TEM (Figure 2a). For the LUV leakage study, we prepared five different sets of solutions with two replicas of those solutions. We added the breaker peptides (BSBHp and CBp) to the preaggregated (3 days old) $A\beta$ solution and incubated them for a total of 10 days (3 + 7 days), before LUV leakage study. Five sets of solutions were used for the vesicle leakage studies, namely untreated (a) and untreated LUVs, (b) $A\beta_{40}$ (incubated for 12 hr), (c) $A\beta_{40}$ (incubated for 10 days), (d) $A\beta_{40}$ and BSBHp (1:10, added after 3 days), and (e) $A\beta_{40}$ and CBp (1:10, added after 3 days).

The pore formation on LUVs followed by dye release was monitored by time-dependent fluorescence assay (Figure 2a (i) and (ii), b, c). We observed that the 12-hour-old $A\beta_{40}$ (magenta, Figure 2b, c) caused more dye leakage ($\sim 53\%$ in 72 hr), which confirmed the presence of toxic oligomeric species (in solution a). Again, 10-day-old $A\beta_{40}$ sample (cyan, Figure 2b, c) caused less LUV leakage

in comparison with 12-hour-old $A\beta$ fibril. The results confirmed that soluble oligomers caused more toxic effect on LUVs by pore formation and dye leakage in comparison with the mature fibrils. We observed lesser extent of dye leakage when BSBHp (blue, Figure 2b, c) was present with $A\beta_{40}$ and that leakage was almost comparable with that from the untreated LUVs. Therefore, it was concluded that presence of BSBHps disaggregates the amyloid fibrils of $A\beta$ into nontoxic species.

To determine the toxic effect of soluble oligomeric misfolded $A\beta$ peptide ($A\beta_{40}$) for 24 hr in SH-SY5Y cells, MTT reduction assays were used to determine the cell viability. As shown in Figure 3a, BSBHp at each of these concentrations (0.5–10 μM) alone did not produce any cytotoxicity in SH-SY5Y cells. Cells were treated with 10 μM $A\beta_{40}$ with or without different concentrations of BSBHp (0.5–10 μM). After 24-hr exposure to $A\beta_{40}$ (10 μM) alone, cell viability was significantly ($p < .01$) decreased by $36.5 \pm 3.1\%$ as compared to control (no treatment) cells. However, this toxic effect was significantly ($p < .01$) ameliorated when co-incubated with BSBHp at a concentration of 3 μM ($79 \pm 4.5\%$), 5 μM ($84 \pm 3.05\%$), and 10 μM ($87 \pm 2.2\%$) on cell viability assessed by MTT reduction assay (Figure 3b).

Aggregated $A\beta_{40}$ induces neuronal cell death by disrupting the cell membrane. To assess the membrane damage, LDH release assay was performed. Lactate dehydrogenase is a cytosolic enzyme; after treating with 10 μM $A\beta_{40}$ alone in SH-SY5Y cells for 24 hr, $45 \pm 2.8\%$ increase in LDH release was found into the medium, which is an indicator of cytotoxic damage of cells. However, when cells were incubated with

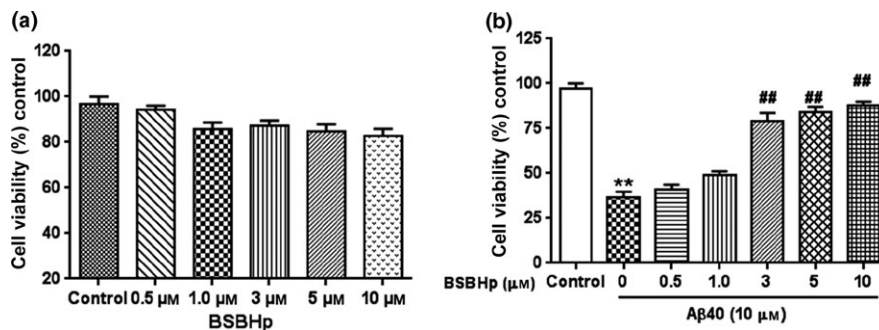
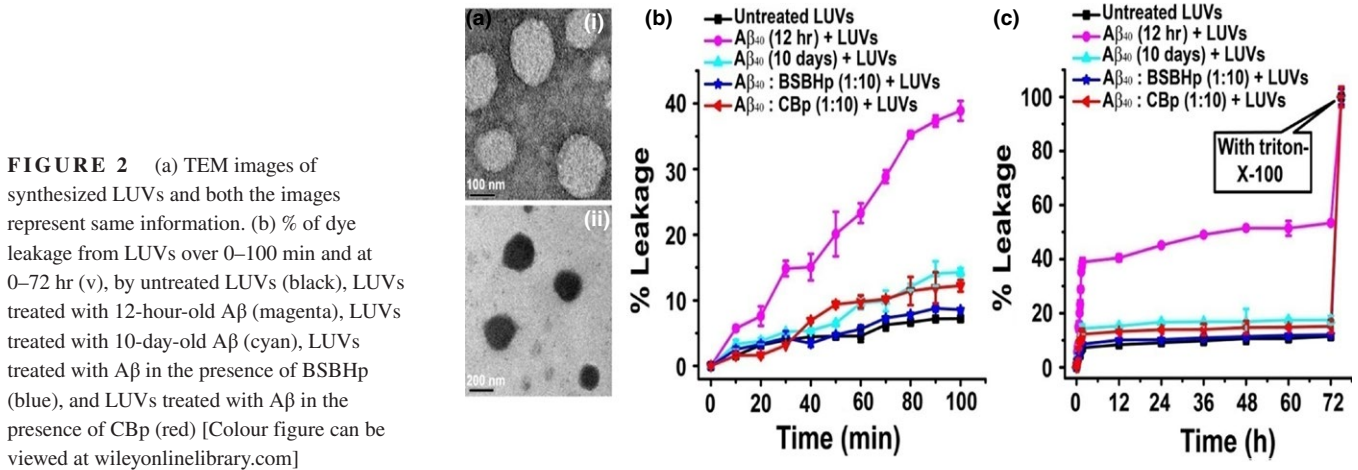


FIGURE 3 The characterization of BSBHp on the viability of SH-SY5Y cells. (a) To study the self-toxicity of synthesized BSBHp on cell viability of SH-SY5Y cells. Cells were incubated with different concentrations (0.5–10 μ M) of BSBHp for 24 hr, which indicates that BSBHp has no toxic effect and up to 85% cells were alive. (b) A β_{40} monomer (10 μ M) was co-incubated with 0.5, 1, 3, 5, and 10 μ M or without BSBHp for 48 hr at 37°C. After 24 hr of treatment, cell viability was measured by MTT assay. Values are represented as mean \pm SEM of three separate experiments. * p < .01, compared to control group and ## p < .01 compared to A β_{40} -treated group, one-way ANOVA

respective concentrations of BSBHp, only 3 μ M ($25 \pm 1.7\%$), 5 μ M ($21 \pm 2\%$), and 10 μ M ($19 \pm 2.9\%$) significantly (p < .01) reduce LDH release as compared to A β_{40} -treated ($45 \pm 2.8\%$) cells (Figure 4a). This result suggests that BSBHp has neuroprotective effect on A β_{40} -induced cytotoxicity in SH-SY5Y cells. Therefore, 3 μ M dose of BSBHp was used for all experiments. Cellular morphology was also observed in phase-contrast microscope after 24-hr co-incubation with A β_{40} (10 μ M) plus BSBHp (3 μ M) and A β_{40} (10 μ M) alone (Figure 4b).

Aggregated A β_{40} induces apoptosis and apoptotic cells are characterized by condensed or fragmented nuclei. To study the cytoprotective effect of BSBHp on apoptosis induced by A β_{40} , cells were stained with DNA-binding dye Hoechst 33258. 10 μ M of A β_{40} was incubated with or without 3 μ M of BSBHp for 24 hr. Fluorescence microscopic images showed that A β_{40} -treated (52 ± 2.3) cells exhibit significant (p < .01) nuclear fragmentation and increased number of apoptotic cells as compared to untreated control cells (12 ± 1.4). However, cells treated combined with A β_{40} and BSBHp had intact nuclei and exhibit significant (p < .05) reduction in the number of apoptotic cells (29 ± 3.0) (Figure 5a, b).

To determine the effect of BSBHp on oxidative stress induced by aggregated A β_{40} in SH-SY5Y cells, intracellular ROS generation was measured by fluorescent dye DCFH-DA. It was found that cells treated with 10 μ M of A β_{40} for 24 hr significantly (p < .01) exhibit increase (208 ± 4.3) in the intensity of DCF fluorescence as compared to untreated cells. Incubation of cells with A β_{40} and BSBHp significantly (p < .05) inhibited (156 ± 4.2) A β_{40} -induced ROS production in SH-SY5Y cells (Figure 6).

Impeding of Ca $^{2+}$ homeostasis leads to impairment of neuronal activity, neurogenesis, and neurotransmitter release that causes the progression of pathogenesis of AD and also induces mitochondrial apoptotic cascade.^[28–30] We measured intracellular free Ca $^{2+}$ by fluorescent Ca $^{2+}$ indicator, Fura-2AM after incubation of cells with A β_{40} (10 μ M) alone or co-incubated with BSBHp (3 μ M). Our result showed that A β_{40} significantly (p < .01) elevates intracellular Ca $^{2+}$ level ($158 \pm 2.7\%$) in SH-SY5Y cells as compared to untreated cells ($64 \pm 4.4\%$). Moreover, after incubation with A β_{40} and BSBHp, elevated Ca $^{2+}$ levels significantly (p < .01) reduced (96.5 ± 4.6) as compared to A β_{40} -treated cells as shown in

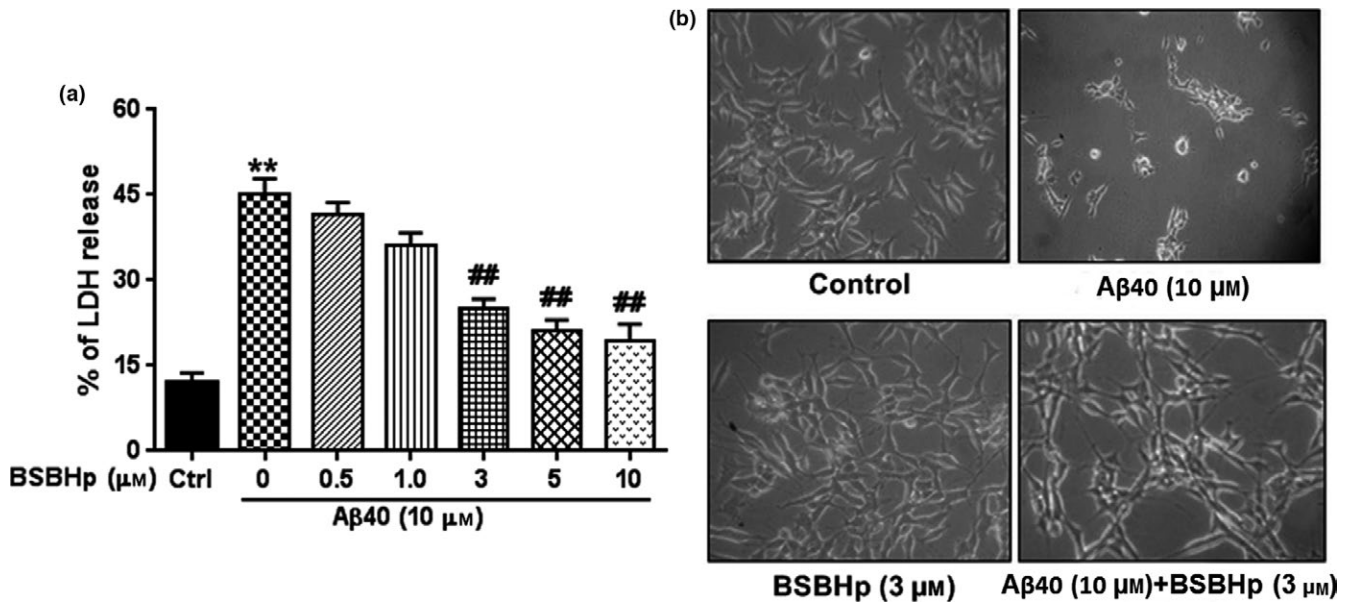


FIGURE 4 Effect of BSBHp on A β_{40} -induced cytotoxicity in respect to LDH release on SH-SY5Y cells. A β_{40} (10 μM) was co-incubated with (0.5–10 μM) or without BSBHp for 48 hr at 37°C and then added to culture medium. (a) After 24 hr of treatment, cytotoxicity was measured by % of LDH release from the damaged cell membrane. (b) Phase-contrast micrograph image (40X) shows cellular morphology of SH-SY5Y cells after treatment with 3 μM of BSBHp alone or with A β_{40} for 24 hr at 37°C. All values are represented as mean \pm SEM of three separate experiments. ** p < .01 compared to control group and ## p < .01 compared to A β_{40} -treated group, one-way ANOVA

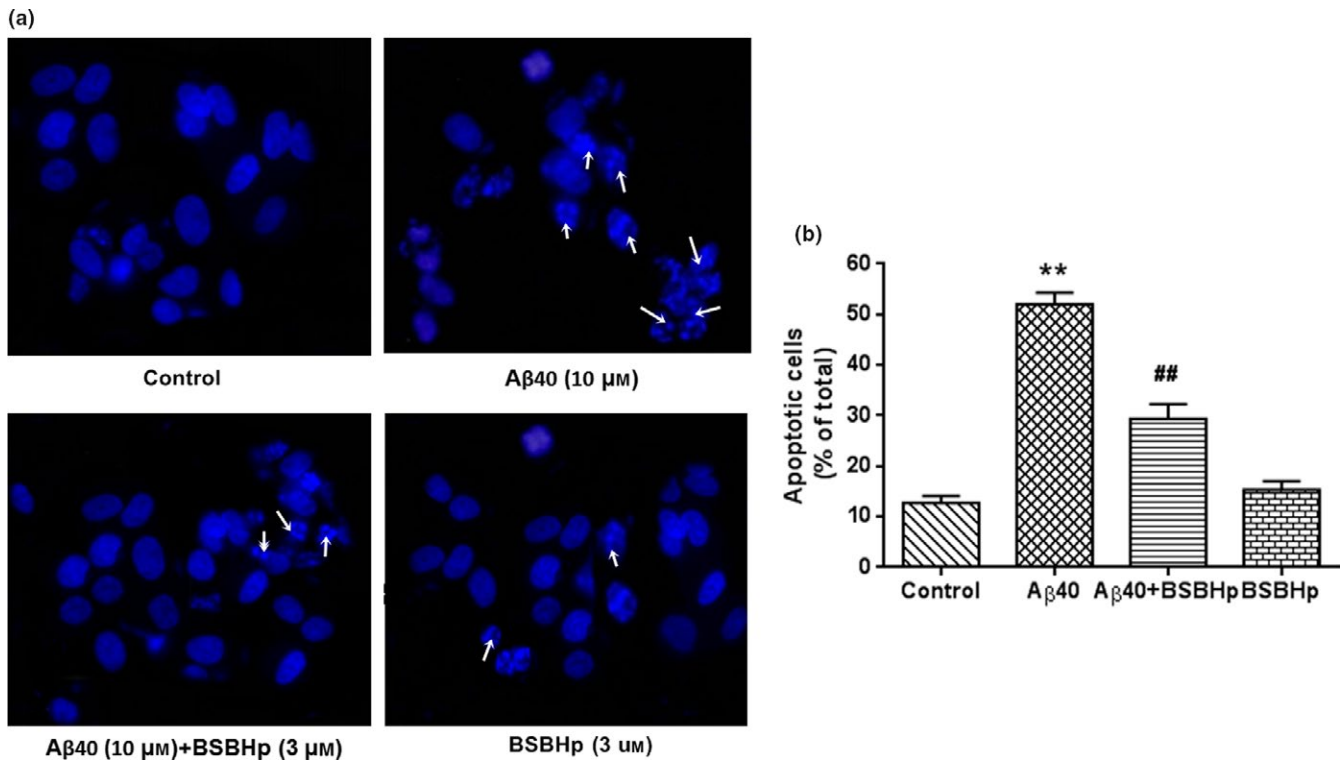


FIGURE 5 BSBHp prevents apoptosis of SH-SY5Y cells from toxic oligomers of aggregated A β_{40} . (a) Shown cells were exposed to A β_{40} (10 μM) alone or BSBHp (3 μM) co-incubated with A β_{40} (10 μM) for 24 hr. Cells were stained with Hoechst 33258 and viewed under fluorescence microscope. Arrow indicates condensed and fragmented nuclei. (b) Apoptotic cells with altered nuclear structure were quantified after A β_{40} treatment with the presence or absence of BSBHp. Results are shown from three separate experiments. Data are mean \pm SEM of three independent experiments. ** p < .01 compared to control group and ## p < .05 compared to A β_{40} -treated group [Colour figure can be viewed at wileyonlinelibrary.com]

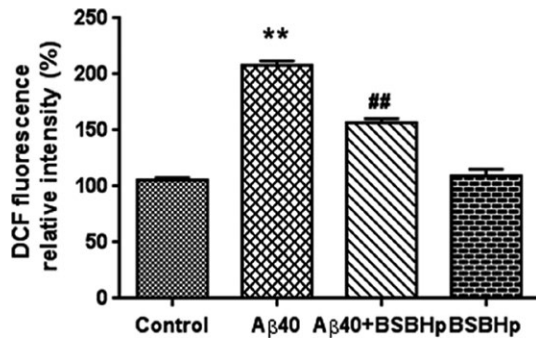


FIGURE 6 Effect of BSBHp on A β_{40} (10 μ M)-induced reactive oxygen species (ROS) generation in SH-SY5Y cells after 24-hr incubation. Cells were treated with A β_{40} (10 μ M) or BSBHp (3 μ M) alone and/or co-incubated with both the peptides. The amount of intracellular ROS formation was determined by oxidation of DCFH-DA to DCF by fluorescent microplate reader. Values are mean \pm SEM of three separate experiments. ** $p < .01$ compared to control group and ## $p < .05$ compared to A β_{40} -treated group

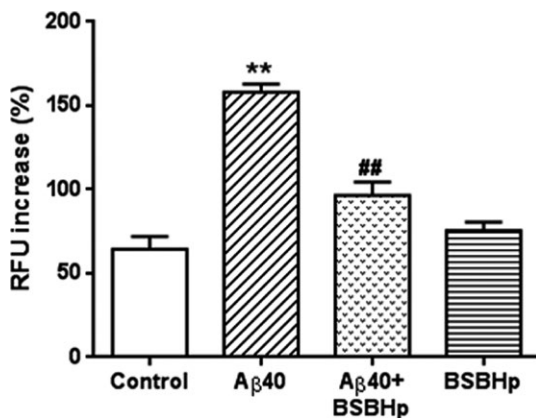


FIGURE 7 Effect of BSBHp (3 μ M) on A β_{40} (10 μ M)-induced elevation of intracellular calcium (Ca²⁺) in SH-SY5Y cells. After respective treatment, cells were incubated with Fura-2AM and relative fluorescent intensity was measured by fluorescence microplate reader. Results are shown as mean \pm SEM of three separate experiments. ** $p < .01$ compared to control group and ## $p < .01$ compared to A β_{40} -treated group

Figure 7. This result suggests that BSBHp has a protective role in Ca²⁺ homeostasis by blocking the aggregation of A β_{40} .

Following the treatment with A β_{40} and BSBHp on SH-SY5Y cells, Western blot analysis was performed to assess the mechanistic role of proteins associated with mitochondrial apoptotic cascades. Mitochondria-mediated apoptosis was regulated by Bcl-2 family of proteins including pro-apoptotic regulator Bax and antiapoptotic factor Bcl-2. Under stressed condition, Bax regulates the release of cytochrome-C from mitochondria followed by the activation of caspase-9 and caspase-3 for the induction of apoptosis.^[31] Our results showed that A β_{40} -treated cells up-regulated Bax expression, showed a slight change in Bcl-2 protein level, and thereby a significant increase ($p < .05$) in the ratio of Bax/Bcl-2 expression

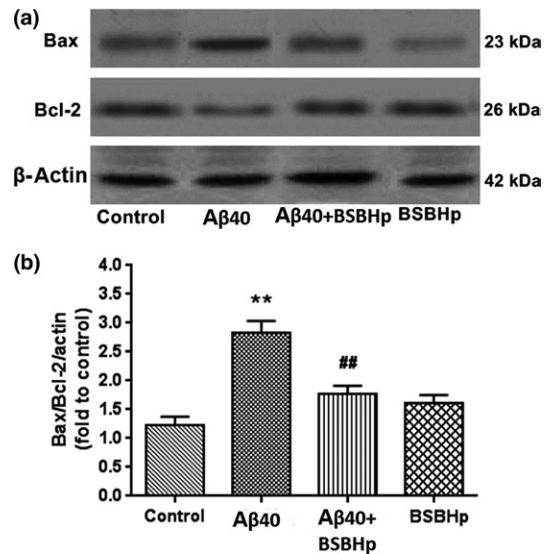


FIGURE 8 Effect of BSBHp on A β_{40} (10 μ M)-induced Bcl-2 and Bax expression in SH-SY5Y cells. A β_{40} (10 μ M) was co-incubated with or without BSBHp (3 μ M) for 24 hr. (a) Shown representative Western blot analysis of Bax and Bcl-2 expression. (b) and (c) shown Bax and Bcl-2 expression (changes fold to control), respectively. The protein bands were quantified using Smartview image analysis software, and values are expressed as mean \pm SEM ($N = 3$ animals per group) of three independent experiments. ** $p < .05$ compared to control group and ## $p < .05$ compared to A β_{40} -treated group

approximately 2.8-fold as compared with the untreated control cells. However, the expression of Bax protein was down-regulated by reducing the Bax/Bcl-2 ratio after co-incubation of A β_{40} with BSBHp-treated SH-SY5Y cells (Figure 8).

As caspases are the important proteases that are activated by diverse stimuli by the process of apoptosis, and Western blot analysis was performed to investigate the expression profile of caspase-9 and caspase-3, following the respective treatments. Our results indicate that treatment with A β_{40} significantly increases ($p < .05$) the expression of caspase-9 and caspase-3 protein level as compared to control cells. Consequently, BSBHp significantly decreases ($p < .05$) the caspase-9 and caspase-3 levels after 24-hr co-incubation with A β_{40} (Figure 9).

To examine the release of cytochrome-C from both the cytosolic and mitochondrial fractions, Western blot analysis was performed after respective treatment in SH-SY5Y cells. Results showed that A β_{40} treatment significantly ($p < .05$) increases the release of cytochrome-C from mitochondria to cytosol, as compared to control. However, co-incubation with BSBHp significantly reverses ($p < .05$) the A β_{40} -induced release of cytochrome-C from mitochondria to cytosol (Figure 10). The above results demonstrated that BSBHp has antiapoptotic effect against aggregated A β_{40} -induced neuronal apoptosis mediated through mitochondrial apoptotic pathway by blocking A β_{40} aggregation.

We also performed the effect of BSBHp on the reduction in amyloid formation in human cerebrospinal fluid (h-CSF).

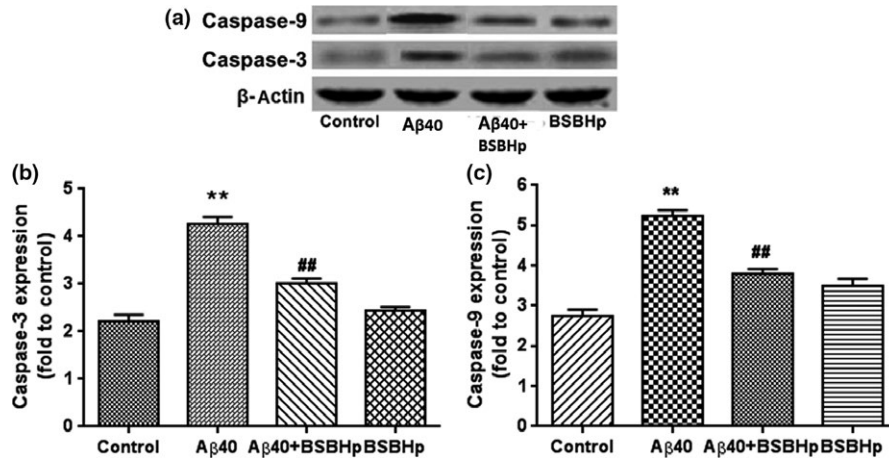


FIGURE 9 Effect of BSBHp on Aβ₄₀ (10 μM)-induced caspase-9 and caspase-3 expression in SH-SY5Y cells. Aβ₄₀ (10 μM) was co-incubated with or without BSBHp (3 μM) for 24 hr. (a) Shown representative Western blot analysis of caspase 9 and caspase 3 expression. (b) and (c) shown caspase 9 and caspase 3 expression (changes fold to control), respectively. The protein bands were quantified using Smartview image analysis, and values are expressed as mean ± SEM from three independent experiments. ***p* < .05 compared to control group and ##*p* < .05 compared to Aβ₄₀-treated group

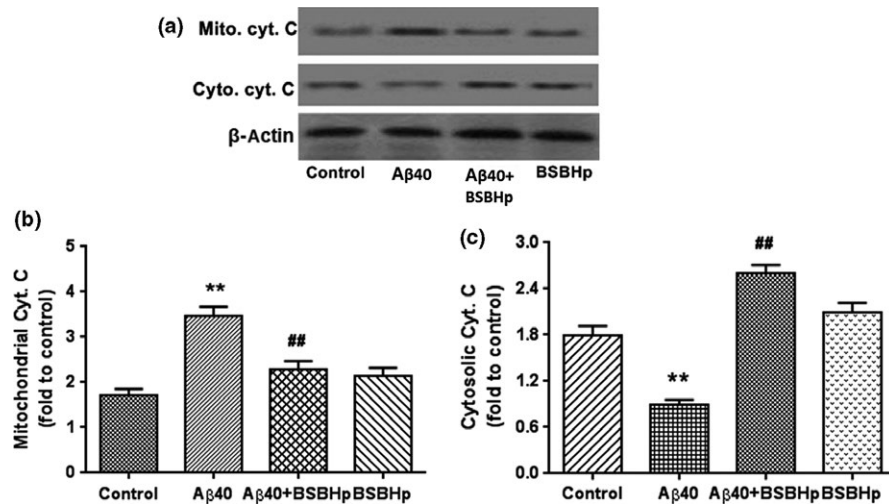


FIGURE 10 Effect of BSBHp on Aβ₄₀ (10 μM)-induced cytochrome-C release in SH-SY5Y cells. Aβ₄₀ (10 μM) was co-incubated with or without BSBHp (3 μM) for 24 hr. (a) Shown representative Western blot analysis of cytosolic and mitochondrial fractions of cytochrome-C expression. (b) and (c) shown cytosolic and mitochondrial cytochrome-C protein (changes fold to control), respectively. The protein bands were quantified using Smartview image analysis, and values are expressed as mean ± SEM from three independent experiments. ***p* < .05, compared to control group and ##*p* < .05 compared to Aβ₄₀-treated group

As Aβ aggregates as plaques in the extracellular spaces of the neuronal cells that are in direct contact with h-CSF, presence of Aβ aggregates in h-CSF can be identified by histopathological investigations.^[32] For the present study, h-CSF samples were obtained from the AD patients and the presence of amyloid was confirmed by thioflavin T (ThT) fluorescence study (Figure 11a, b). A 50 μM solution of Aβ₄₀ was prepared in PBS of pH 7.4 and incubated that at 37°C for 10 days. After 10 days, ThT fluorescence was measured with different concentrations (20, 50, and 100 μl) of Aβ₄₀ (Figure 11a) and fixed concentration of ThT. From the ThT fluorescence, we noticed that with increasing amount of Aβ₄₀, fluorescence intensity increased, which indicated that amyloid was present. Similarly, we performed ThT fluorescence study of the

h-CSF sample from the patients with AD and observed that the fluorescence intensity increased with increasing concentration of h-CSF (Figure 11b). Therefore, the presence of amyloid aggregates in h-CSF was confirmed, and from the normalized spectra, the concentration of Aβ in a CSF sample was found to be ~23 μM; 10-fold molar excess of BSBHp was added with the h-CSF sample, and the time-dependent kinetics of disaggregation of amyloid aggregates was monitored (Figure 11c). In the ThT assay, we observed that fluorescence intensity of CSF was suppressed excessively in the presence of BSBHp (blue, Figure 11c), while h-CSF alone (black, Figure 11c) showed increased fluorescence. Again, h-CSF alone showed clear fiber in TEM (Figure 11d(i)) and green gold birefringence under cross-polarized light when stained

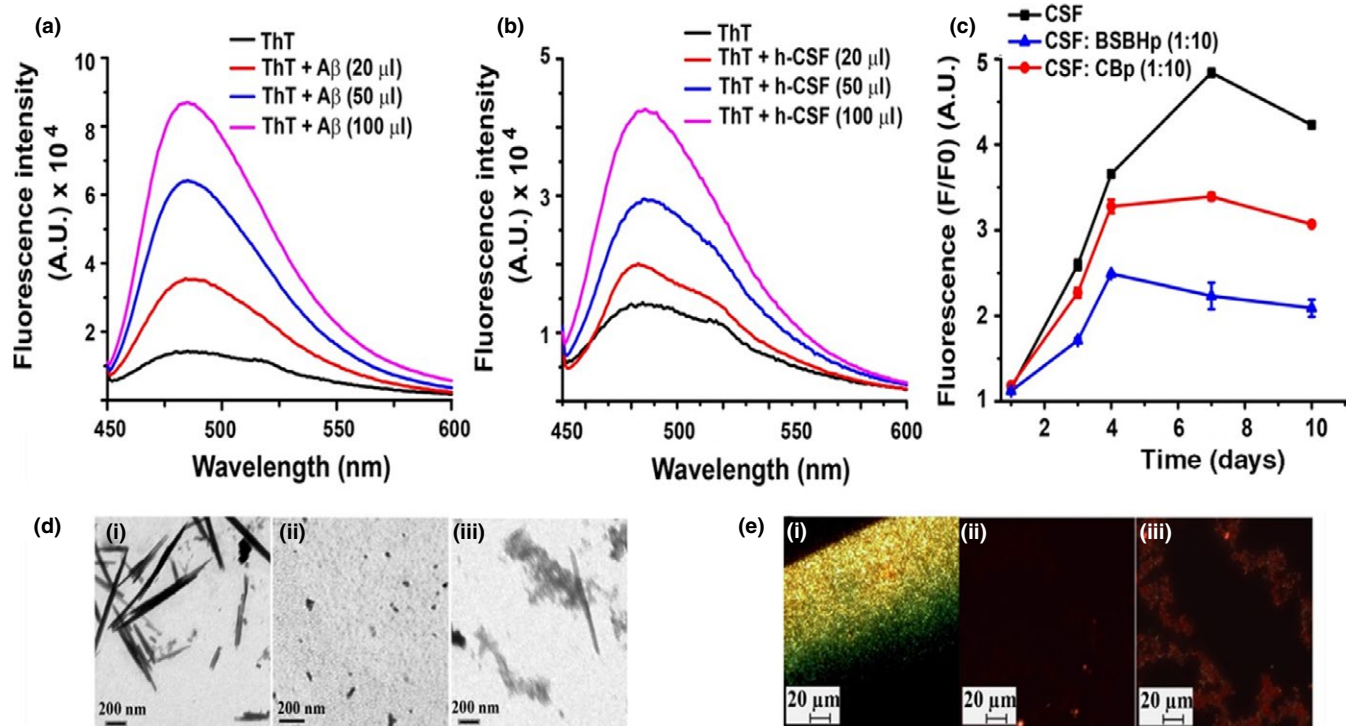


FIGURE 11 Fluorescence spectra of ThT (25 μM) in the absence (black) and presence of 20 μl (red), 50 μl (blue), and 100 μl (magenta) of $\text{A}\beta$ (a) and human cerebrospinal fluid (h-CSF) (b). (c) Time-dependent ThT assay for the disruption of the amyloid present in h-CSF in the absence (black) and presence BSBHp (blue) and CBp (red), respectively. (d) TEM images of h-CSF in the absence (i) and presence of BSBHp (ii) and CBp (iii). (e) Congo red-stained birefringence images of h-CSF in the absence (i) and presence of BSBHp (ii) and CBp (iii). All the images were taken after 10 days of incubation at 37°C. Scale bars indicated as 200 nm and 20 μm . Experiments were carried out in PBS (50 mM) at pH 7.4 and 37°C. Total cuvette volume was 400 μl (200 μl ThT (50 μM) + sample (20, 50, 100 μl) + remaining volume of PBS) [Colour figure can be viewed at wileyonlinelibrary.com]

with Congo red [(Figure 11e (i)). No such fiber [(Figure 11d (ii)) or birefringence [(Figure 11e (ii))] was observed in the presence of BSBHp, indicating the absence of amyloid.

4 | DISCUSSION

There is abundant evidence suggesting that accumulation of amyloid- β in the brain is responsible for the formation of senile plaque resulting in the neuronal degeneration, apoptosis, cognitive and memory impairments.^[33] Amyloid- β precursor protein ($\text{A}\beta\text{PP}$)-derived monomeric amyloid beta is self-assembled into highly toxic soluble $\text{A}\beta$ oligomers ($\text{A}\beta\text{O}$) and high molecular weight $\text{A}\beta$ oligomers and protofibrils are the predominant $\text{A}\beta$ species in the native soluble protein fraction of the AD brain.^[34] Thus, we consider that the anthranilic acid-containing β -sheet breaker α/β -hybrid peptide (BSBHp), which can inhibit self-aggregation, even reverse the aggregation of the pathogenic $\text{A}\beta_{40}$ can be a potential therapeutic strategy for AD.

BSBHp is a novel class of conformationally restricted β -sheet breaker peptide, which has a recognition motif of the target pathogenic peptide $\text{A}\beta_{40}$. The β -breaker element contains anthranilic acid (Ant, an aromatic β -amino acid) unit and is relatively more stable against proteolytic degradation.

Our study demonstrated that BSBHp potentially disrupts and redissolves the $\text{A}\beta_{40}$ fibrils in vitro that generate nontoxic species. Thus, inhibitory effects of BSBHp on underlying mechanisms of $\text{A}\beta_{40}$ -induced apoptotic neurodegeneration were studied on in vitro human neuroblastoma SH-SY5Y cells. To the best of our knowledge, this type of β -amino acid-containing breaker peptide was not used before for the inhibition of $\text{A}\beta_{40}$ -induced neuronal apoptosis.

It is well documented that toxic oligomers or protofibrils of $\text{A}\beta$ produce maximum toxicity than mature fibrils due to their ability of perforation in cell membrane and the induction of cytotoxicity of neuronal cell.^[8] We first examined it in large unilamellar vesicles (LUVs) that are considered to mimic cell membrane. Extent of dye-release from dye-entrapped LUVs clearly indicated immature fibrils to be more efficient for pore formation in the LUVs than mature fibrils. Also, it was found that application of BSBHp demolishes such pore formation. Our findings revealed that SH-SY5Y cells treated with 10 μM of $\text{A}\beta_{40}$ exhibit a decrease in MTT reduction and a maximum release of LDH into the medium. The novelty is that co-incubation with BSBHp protects SH-SY5Y cells from $\text{A}\beta_{40}$ -induced toxicity studied by MTT and LDH release assay. Results from LDH assay confirmed that BSBHp significantly prevents the $\text{A}\beta_{40}$ -induced membrane damage, which is corroborated with the findings of in vitro dye leakage assay

using carboxyfluorescein-entrapped LUVs. The neuroprotective effect of BSBHp was also confirmed by Hoechst staining assay, analysis of nuclear morphology, cellular apoptosis, and nuclear fragmentation. So, these findings support that BSBHp has potential role to inhibit self-aggregation and formation of toxic oligomeric species of A β_{40} . Furthermore, we investigated the protective role of BSBHp on A β_{40} -induced mitochondrial apoptotic pathway and ROS production into the neuronal cells.

Several lines of evidence showed that extensive oxidative stress is generated in AD brain during disease progression, which may cause neuronal apoptosis leading to cell death.^[35,36] It was reported that soluble oligomers of A β_{40} can penetrate phospholipids bilayer of cell membrane, and induction of neurodegenerative events like lipid peroxidation, synaptic alteration, dysregulation of Ca²⁺ homeostasis, and also activation of mitochondrial apoptotic pathway ultimately leads to cell death.^[37,38] This study demonstrated that BSBHp significantly reduces A β_{40} -induced ROS generation by both the reversion of aggregation and formation of toxic oligomeric A β_{40} . It has been pointed out that accumulation of oligomeric A β_{40} altered the Ca²⁺ homeostasis that disrupts synaptic transmission, membrane integrity and initiates apoptotic neurodegeneration.^[39,40] We found that BSBHp effectively disrupted protofibrils and maintained A β_{40} -induced Ca²⁺ homeostasis in SH-SY5Y cells.

Neuronal apoptosis caused by aggregated A β_{40} is considered to be a major pathological hallmark in AD. A β -induced neuronal apoptosis was mediated through the generation of intracellular ROS and mitochondrial impairment on neuronal cells.^[41] It is also reported that ROS are involved in the regulation of mitochondrial cascade and activation of apoptotic proteins.^[42] Bcl-2 family member proteins such as Bax and Bcl-2 are involved in the A β -induced mitochondrial pathway.^[43] Under pathological condition, elevation of Bax translocated into the mitochondrial membrane, can homodimerize there by activating several caspases, and alter the mitochondrial activity by promoting apoptotic factors into the cytoplasm.^[44,45] Thus, it is found that BSBHp alleviates Bax/Bcl-2 ratio and also A β_{40} -induced Bax expression. Another important factor is that release of cytochrome-C from mitochondria to cytosol triggers the activation of caspase-9 followed by caspase-3 and switching on the downstream signaling pathway.^[46] Our results clearly demonstrated that BSBHp effectively suppresses the A β_{40} -induced caspase-9 and caspase-3 protein expression, thereby maintaining neuronal cell death by inhibiting A β_{40} aggregation and disruption of fibrillar form of A β_{40} . We also demonstrated the effect of BSBHp on aggregated A β in the h-CSF sample of the patients with AD in vitro. The BSBHp expressively disaggregated the fibrillar assembly present in the h-CSF sample.

In conclusion, our study demonstrated that BSBHp could effectively ameliorate A β_{40} -induced oxidative stress and

apoptosis in SH-SY5Y cells by inhibiting A β_{40} aggregation. As far as we know, this is the first report to demonstrate that BSBHp has the neuroprotective effects against A β -induced neurotoxicity in SH-SY5Y cells. The protection effects of BSBHp against neuronal atrophy may help to provide the pharmacological basis of its clinical use in the treatment for neurodegeneration in AD.

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AUTHOR'S CONTRIBUTION

B.M. and A.P. designed the β -sheet breaker α/β -hybrid peptide (BSBHp) and its biophysical application in cell-free systems. A.C.M., S. Kumar, and A.K.G. designed the cell-based framework of research. AP., S. Kumar, and S. Kalita performed all the experiments. B.M., A.C.M, A.P., and S. Kumar analyzed the results and wrote the manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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