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Isobavachalcone and bavachinin from *Psoraleae Fructus* modulate Aβ42 aggregation process through different mechanisms in vitro

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ABSTRACT

Spontaneous aggregation of Aβ is a key factor in the development of Alzheimer's disease. In searching for Aβ aggregation inhibitors from traditional Chinese herbal medicines, we identified two active compounds from *Psoraleae Fructus*, namely isobavachalcone and bavachinin. We further demonstrated that the two compounds modulate Aβ42 aggregation process through different mechanisms. Isobavachalcone significantly inhibits both oligomerization and fibrillization of Aβ42, whereas bavachinin inhibits fibrillization and leads to off-pathway aggregation. Both of the compounds attenuated Aβ42-induced toxicity in a SH-SY5Y cell model. These findings may provide valuable information for new drug development and Alzheimer's therapy in the future.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by extracellular amyloid plaques and intraneuronal fibrillary tangles in the brain [1]. Abnormal accumulation and aggregation of the amyloid β (Aβ) peptides, especially the peptides with 40 (Aβ40) or 42 residues (Aβ42), are believed to be responsible for the amyloid plaques [2]. Oligomerization and fibrillization of Aβ peptide, especially Aβ42, is the key event leading to development of AD plaques as well as progressive neuronal loss. Inhibition of aggregation of Aβ peptide is a potential therapeutic approach for the treatment of AD [3–5]. Varieties of Aβ aggregation inhibitors have been discovered, including natural products from plants, for example, the plant phenolic compounds that show strong inhibitory effect on Aβ aggregation both in vitro and in vivo [6,7].

Psoraleae Fructus, the dried fruits of *Psoralea corylifolia* L., is a yang-tonifying herb commonly used in clinical practices of Traditional Chinese Medicine (TCM). Recent studies have shown that it possesses multiple benefits, including anti-bacteria [8], anti-oxidant [9], anti-tumor [10,11], immune-modulation [11], estrogenic and bone-strengthening, etc. [12–14]. Chemical composition of *Psoraleae Fructus* is also well studied. It mainly contains coumarins and prenylflavone derivatives [15–18]. Choi et al., recently reported that the compounds from *Psoraleae Fructus* strongly inhibit BACE1 (β-secretase) activity [19], a key enzyme in Aβ production in vivo. However, the effect of these compounds on Aβ aggregation remains unclear.

Recently we established a yeast two-hybrid system to screen the inhibitors of Aβ42 aggregation [20]. In a screening of traditional Chinese herbal medicines, we found the methanol extract of *Psoraleae Fructus* showed strong inhibitory effect on Aβ42 aggregation. In this study, we further confirmed the anti-aggregation effect of *Psoraleae Fructus* and demonstrated isobavachalcone and bavachinin as the major active compounds effectively inhibiting the on-pathway aggregation of Aβ42 through two different mechanisms.

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-beta peptide; ThT, thioflavin T; PFE, methanol extract of *Psoraleae Fructus*; BKC, bakuchiol; PLD, psoralidin; BCN, bavachinin; IBC, isobavachalcone; NBIF, neobavaisoflavone; BC, bavachin; IPL, isopsoralen; PL, psoralen

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2. Materials and methods

2.1. Reagents and chemicals

Aβ42 (purity >95%) was synthesized by ChinaPeptides (Shanghai, China). Thioflavin T (ThT) and hexafluoroisopropanol (HFIP) were obtained from Sigma (St. Louis, USA). EGCG, curcumin, psoralen, isopsoralen, psoralidin, bavachin, bavachinin, bakuchiol, neobavaisoflavone, and isobavachalcone were purchased from Shanghai Yuanye Bio-Technology Co. (Shanghai, China). All of chemical compounds were dissolved in DMSO for Aβ42 inhibition assay, or in methanol for CD Spectroscopy analysis. Compound structures are shown in Fig. 1.

2.2. Plant materials and extracts

Psoraleae Fructus was purchased from a local TCM drug store and identified as the dried fruits of *Psoralea corylifolia* L. The herb sample was grinded into fine powder. Five grams of the powder was extracted twice with 50 ml methanol by 30 min ultra-sonication and 5 min refluxation in a microwave extractor. The methanol extracts were filtered, vacuum-dried at 40 °C, then stored at −30 °C. For Aβ42 inhibition assay, fresh samples dissolved in DMSO were used without frozen storage.

2.3. HPLC analysis and quantification of the extracts

HPLC analysis was performed on an Agilent 1260 Infinity LC system with a DAD detector and an Alltima C18 column (Alltech, 4.6 × 250 mm, 5 μm). Separation was conducted at 25 °C with a binary mobile phase consisting of 0.1% aqueous acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1.0 ml/min [18]. A gradient elution program was applied as follows: 40–50% B in 15 min, 50–60% B in 20 min, 60–80% B in 20 min, and 80% B for 5 min. Elution peaks were detected at wavelength 245 nm. Psoralen, isopsoralen, psoralidin, bavachin, bavachinin, bakuchiol, neobavaisoflavone, and isobavachalcone were used as reference standards for quantification.

2.4. Yeast two-hybrid inhibition assay

A yeast two-hybrid system for screening Aβ42 inhibitors has been described previously [20]. Briefly, the transformed yeast strain AH109 (Clontech) carries two Aβ42-fusion plasmids (Aβ42 fused to GAL4-DB of pGBKT7 and GAL4-AD of pGADT7) which was maintained on the high-stringency SD^{4-} media (synthetic media depleted of leucine, tryptophan, adenine and histidine). The fresh yeast clone was inoculated into SD^{4-} liquid media, grown and harvested in early log-phase (OD_{600} = 0.5–1.0), and diluted to OD_{600} = 0.1. The yeast cells were aliquoted and grown at 30 °C for 48 h in the presence or absence of the test compounds. The reduction in growth rate of each compound was calculated according to DMSO (vehicle) control and used as an index for Aβ42 inhibition.

2.5. Inhibition of Aβ42 aggregation

Synthetic Aβ42 peptide was prepared as described previously with minor modifications [21]. Aβ42 was first dissolved in 100% HFIP and dried under nitrogen gas. The dried peptide was reconstituted in DMSO (or 10 mM NaOH for CD spectroscopy, 5 mg/ml Aβ42), then sonicated for 10 min, and diluted in PBS (pH 7.4) to 50 μM. The peptide was centrifuged at18 000×*g* for 60 min at 25 °C. The supernatant was aliquoted and incubated at 37 °C in the presence or absence of compounds for 0–7 days without agitation.

2.6. ThT fluorescence assay

Aβ42 samples (10 μl) were mixed with 40 μl ThT solutions (25 μM in 0.2 M Glycine–NaOH buffer, pH 8.5), placed in the dark for 10 min, and then measured at 440/490 nm (excitation/emission) in a fluorescence spectrophotometer (F96Pro, Shanghai Lengguang Technology Co. Ltd., China). To pellet the aggregates, Aβ42 samples were centrifuged at 18 000×*g* for 30 min at 25 °C and the pelleted fraction (5% of starting volume) was analyzed by ThT fluorescence assay.

2.7. SDS–PAGE

Aβ42 samples were mixed with loading buffer, and separated in a 15% Tris–glycine PAGE gel at 60 V. Gels were then stained by Coomassie Blue [22].

2.8. Dot blot assay

Two conformation-specific antibodies were applied for dot blot assay to detect formation of Aβ42 oligomers (A11, Invitrogen) and fibrils (OC, Millipore) as described previously [21]. Briefly, Aβ42

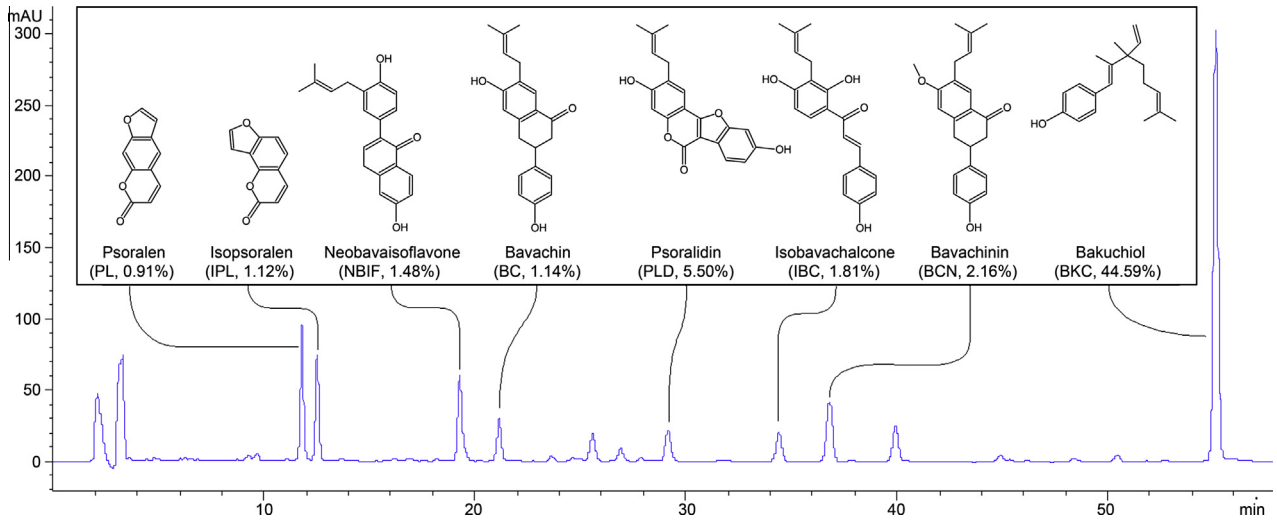

Fig. 1. Representative HPLC chromatograph of the methanol extract of *Psoraleae Fructus* and quantification of the major constituents. The chemical structures and contents of the eight standards are shown at the top.

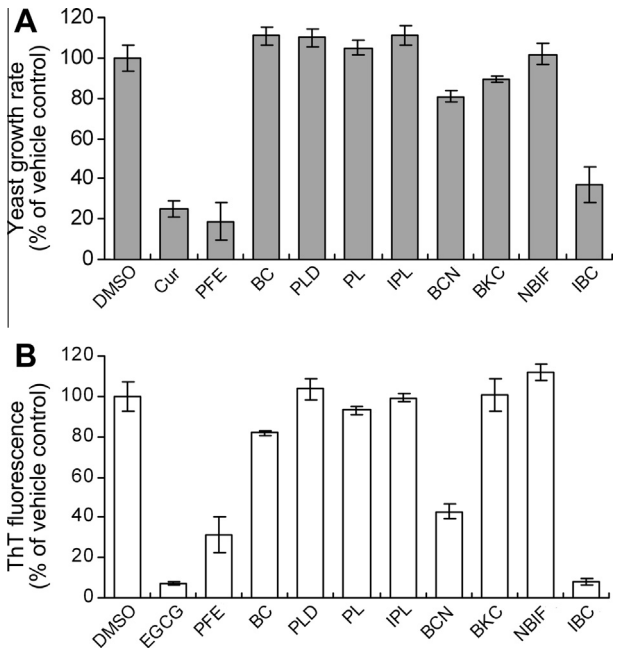

Fig. 2. Effect of PFE and its major compounds on Aβ42 aggregation. (A) Yeast two-hybrid analysis. Yeasts were grown in SD^{4-} medium for 48 h with the test substances adding at non-toxic concentrations (PFE: 10 μg/ml; NBIF and IBC: 3 μM; other compounds: 30 μM). Curcumin was used as a positive control (Cur, 150 μM). (B) ThT fluorescence assay. Aβ42 (50 μM) were incubated with PFE (100 μg/ml) or test compounds (50 μM) for 1 day. EGCG (50 μM) was used as a positive control. DMSO was served as a vehicle control for both assay ($n = 3$).

samples (5 μl) were spotted onto nitrocellulose membranes and air dried. The membranes were blocked 2 h at room temperature with 10% non-fat milk in Tris–buffered saline containing 0.01% Tween 20 (TBST). The membranes were then washed and probed with either oligomer-specific A11 or fibril-specific OC overnight at 4 °C, which was diluted 1:1000 in TBST containing 5% milk. The membranes were washed again and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies diluted 1:5000 in TBS-T containing 5% milk for 2 h at room temperature. Then the blots were washed three times with TBS-T for 5 min and developed with the ECL chemiluminescence kit (SuperSignal West Pico, Thermo Fisher Scientific Inc.).

2.9. Circular dichroism spectroscopy (CD)

The CD spectra of Aβ42 samples were measured by a Jasco J-810 CD spectrometer at 25 °C. Each spectrum is the average of at least 5 readings.

2.10. Atomic force microscopy (AFM)

Aβ42 samples were diluted 1:4 in PBS (pH 7.4) and spotted on freshly cleaved mica. The samples were adsorbed 30 min at room temperature and then washed three times with water and dried overnight. Images were taken at DFM mode using a Seiko SPA 400 AFM system with Tap300Al cantilevers (BudgetSensors).

2.11. Aβ42 induced cell toxicity

MTT assay was performed to determine the cell toxic effects of Aβ42 samples in SH-SY5Y human neuroblastoma cells. The cells were seeded at a concentration of 4000 cells/well (200 μl/well) in 96-well plates in full medium (DMEM/F12) and incubated 48 h at 37 °C. The medium was replaced with 100 μl of serum-free medium containing 10% Aβ42 aggregation samples. The cells were treated for 48 h at 37 °C before MTT was added to each well (0.5 mg/ml, final concentration) and incubated for 4 h at 37 °C. The culture solution was removed and 150 μl of DMSO was added. The plates were shaken for 10 min and measured at 492 nm using a microplate reader.

3. Results

3.1. The major constituents in the methanol extract of Psoraleae Fructus

HPLC analysis of the methanol extract of *Psoraleae Fructus* (PFE) showed that peaks of the major constituents were well resolved. Eight compounds were quantified using reference standards (Fig. 1). Bakuchiol is the most abundant (BKC, 44.59%), followed by psoralidin (PLD, 5.5%), bavachinin (BCN, 2.16%), isobavachalcone (IBC, 1.61%), neobavaisoflavone (NBIF, 1.48%), bavachin (BC, 1.14%), isopsoralen (IPL, 1.12%) and psoralen (PL, 0.91%). This is consistent with previous reports [16,17].

3.2. PFE and the compounds IBC, BCN inhibit Aβ42 dimerization in yeast two-hybrid model

An established yeast two-hybrid system was first applied to investigate the inhibitory effect of PFE and the eight compounds on Aβ42 self interaction. The yeast model is based on the heterodimerization of Aβ42-fusion protein, which reconstituted GAL4 transcriptor to drive its downstream reporter genes compensating for the multiple auxotrophic phenotype of the yeast strain [20]. Therefore, the yeast growth in high-stringency SD^{4-} media is completely dependent on Aβ42 self interaction thus was used as a read out for evaluation of Aβ42 inhibitors. Toxicity dependent false-positive effects were identified by a parallel test replacing SD^{4-} with complete SD media. As shown in Fig. 2A, PFE (10 μg/ml) and curcumin (150 μM), a known inhibitor, significantly reduced the yeast growth in SD^{4-} medium, indicating that they strongly inhibit Aβ42 dimerization. Among the eight test compounds, IBC (3 μM) and BCN (30 μM) were found to be active at non-toxic concentrations.

3.3. PFE and the compounds IBC, BCN inhibit formation of Aβ42 fibrillar aggregates in vitro

To further confirm whether PFE and the compounds can inhibit Aβ42 aggregation, a standard ThT fluorescence assay was performed. ThT is known to specifically bind to Aβ42 fibrillar conformers and generate a unique fluorescence spectrum peaking near 490 nm. As shown in Fig. 2B, both the positive control EGCG and PFE significantly reduced ThT fluorescence, indicating they inhibited formation of Aβ42 fibrillar aggregates. IBC also exhibited strong inhibitory effect on ThT fluorescence, which is comparable with EGCG. Interestingly, while BCN only showed mild reduction on yeast growth, it decreased ThT fluorescence more efficiently. We further determined the IC50 of IBC and BCN in the ThT assay and they are about 25 and 45 μM, respectively.

3.4. IBC inhibits formation of high molecular weight aggregates of Aβ42 whereas BCN promotes conversion of low molecular weight conformers

To explore how IBC and BCN modulate Aβ42 aggregation, SDS–PAGE analysis was carried out. Two major bands corresponding to monomers and low molecular weight conformers (trimers/tetramers) were observed in Aβ42 samples before incubation

(Fig. 3A). After 6 h incubation, high molecular weight aggregates markedly accumulated in the samples. Addition of IBC greatly prevented the formation of high molecular weight aggregates, further confirmed its anti-aggregation effect (Fig. 3A and B). Interestingly, BCN treatment led to no significant effect on high molecular weight aggregates but noticeably reduced low molecular weight conformers. This result suggests that IBC and BCN modulate Aβ42 aggregation in two different manners. PFE treatment produced an intermediate effect compared with the two compounds.

3.5. IBC dose dependently inhibits formation of both OC and A11 positive conformers, whereas BCN generates similar effect on OC positive conformers but complicated effects on A11 positive conformers

Aβ aggregation is a complicated process involving independent oligomerization and fibrillization pathways [21,24], although the two pathways are closely linked by the same dynamic buffer pool of Aβ monomers. To elucidate which pathway of Aβ42 aggregation was modulated by IBC and BCN, we performed dot blot assay using oligomer-specific A11 antibody and fibril-specific OC antibody. OC antibody recognizes fibrillar intermediates and mature amyloid fibrils that are less toxic, ThT-positive and β-sheet-rich structures [21,23]. When Aβ42 was incubated alone, OC positive conformers accumulated significantly as time progressed (Fig. 4). However, this accumulation was almost completely stopped when either BCN or IBC was added at a molar ratio of 5:1 (BCN or IBC to Aβ42) and the inhibition by either compound was dose-dependent (Fig. 4). This demonstrated both compounds inhibited Aβ42 fibrillization process. In contrast to OC, A11 antibody recognizes soluble oligomers that are more toxic and ThT-negative [21,24,25]. In agreement with previous findings [26], A11-positive conformers appeared to be transiently present in Aβ42 samples during incubation period, and they were recognized by A11 at day 3 but not day 0 or day 7 of incubation. Addition of IBC significantly inhibited A11 blotting signal indicating that IBC also inhibited Aβ42 oligomerization (Fig. 4). Surprisingly, BCN significantly enhanced A11 blotting signal at all time points at a molar ratio of 1:1 (Fig. 4 1×), indicating that BCN may rapidly promote and further stabilize the formation of A11-positive oligomers. Even more strikingly, this enhancing effect diminishes as concentration of BCN increases (Fig. 4) or decreases (data not shown).

3.6. IBC inhibits both oligomerization and fibrillization of Aβ42 whereas BCN promotes oligomerization and off-pathway aggregation

In order to further understand the mechanisms of IBC and BCN inhibiting Aβ42 fibrillization, we performed pellet-ThT fluorescence assay and CD spectrum analysis. After 7 days of incubation, Aβ42 alone formed significant amount of aggregates that can be pelleted by centrifugation and recognized by ThT indicated by the increase of fluorescence at 490 nm (Fig. 5A). CD analysis further revealed that Aβ42 at day 7 had a predominant β-structure, as indicated by a minimum at 217 nm in CD spectra (Fig. 5C). However, addition of either IBC or BCN to Aβ42 greatly reduced both ThT-positive pellets (Fig. 5B) and β-sheet content (Fig. 5C), suggested that the "on-pathway" aggregation of Aβ42 were significantly inhibited by both compounds. Interestingly, majority of Aβ42 peptides were converted to random coil conformers by IBC, as indicated by a characteristic minimum near 200 nm in CD spectra (Fig. 5C).

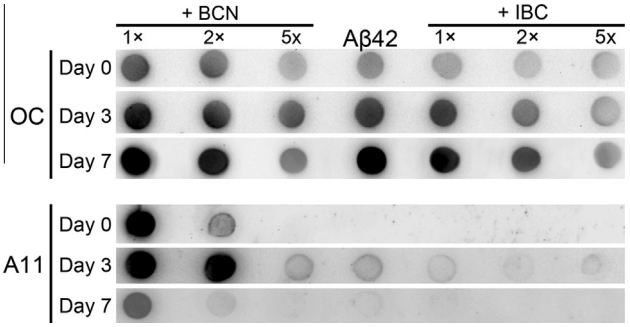

Fig. 4. Dot blotting analysis using OC and A11 antibodies. Aβ42 samples (50 μM) were incubated for 0–7 days with IBC or BCN (1×, 2×, 5×: a molar ratio of 1:1, 2:1, 5:1 to Aβ42). Each image is a representative of at least three independent experiments.

We next performed AFM analysis to monitor morphological changes of Aβ42 during the incubation. As shown in Fig. 6A, initial Aβ42 peptides are morphologically heterogeneous and mainly consisted of monomers and small size oligomers. After 7 days of incubation, the peptides assembled into noticeably larger aggregates as well as a few protofibrils and mature fibrils (Fig. 6A). However, these fibrillar aggregates were not observed in Aβ42 samples treated with either IBC or BCN, indicating Aβ42 fibrillization was significantly inhibited (Fig. 6B). Moreover, IBC dose dependently blocked formation of larger size aggregates and resulted in small size conformers comparable with the initial Aβ42 at a molar ratio of 5:1 (Fig. 6B, upper panel). This further confirmed IBC as an effective inhibitor of Aβ42 fibrillization and oligomerization. To our surprise, BCN promoted and stabilized formation of small size conformers at equimolar concentration, but strongly enhanced Aβ42 aggregation at higher concentration and produced much larger aggregates than Aβ42 alone control (Fig. 6B, lower panel).

3.7. Both IBC and BCN attenuate Aβ42-induced cell toxicity in SH-SY5Y cells

To investigate whether IBC and BCN could attenuate Aβ42 toxicity, we utilized MTT assay to assess cell viability of SH-SY5Y

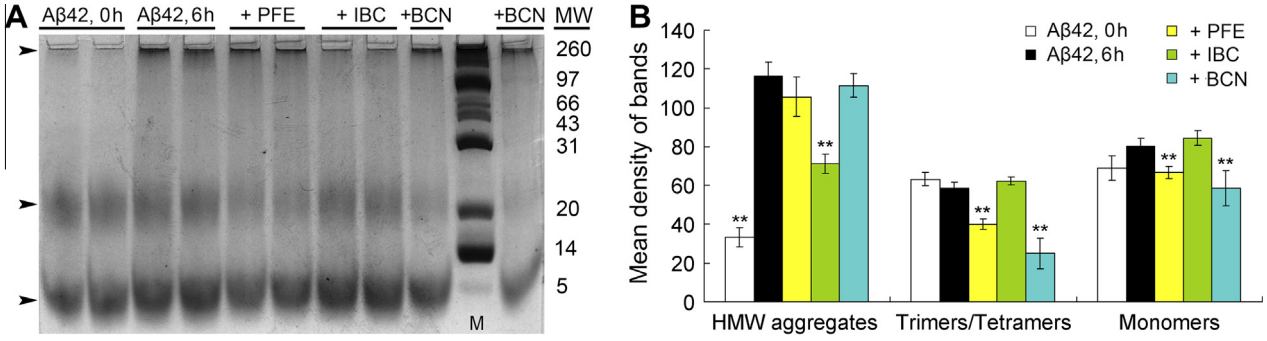

Fig. 3. SDS–PAGE analysis of Aβ42 aggregation. (A) Aβ42 samples (50 μM) were incubated for 6 h with PFE (200 μg/ml), IBC (5×, a molar ratio of 5:1 to Aβ42) or BCN (5×). Arrows indicate monomers, trimers/tetramers, and high molecular weight (HMW) aggregates. (B) Mean density of the gel bands, $n = 3$. $^{**}P < 0.01$ (versus Aβ42 alone, 6 h).

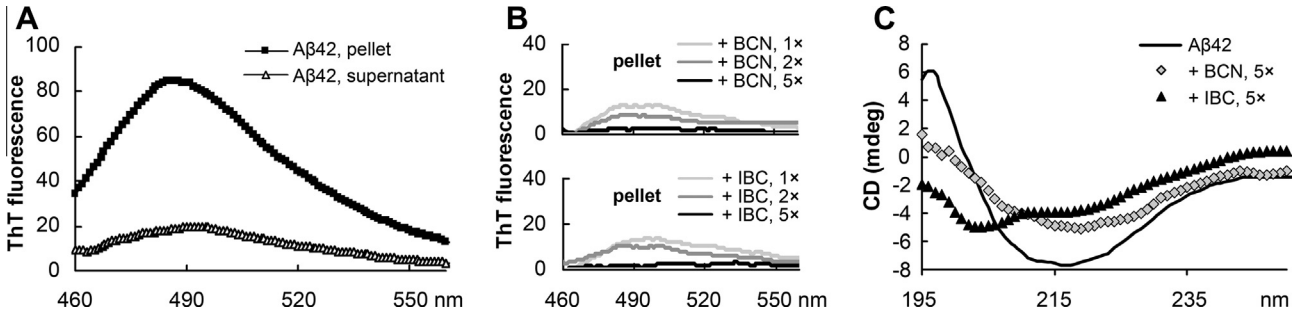

Fig. 5. IBC and BCN inhibited Aβ42 fibrillization. Aβ42 samples (50 μM) were incubated for 7 days with IBC or BCN. (A) ThT fluorescence spectra of the pellet and supernatant fraction of Aβ42 control samples. (B) ThT fluorescence spectra of the pellet fractions of BCN and IBC treated samples. (C) CD spectra of Aβ42 alone, IBC treated and BCN treated samples. Each spectrum is an average of three independent experiments.

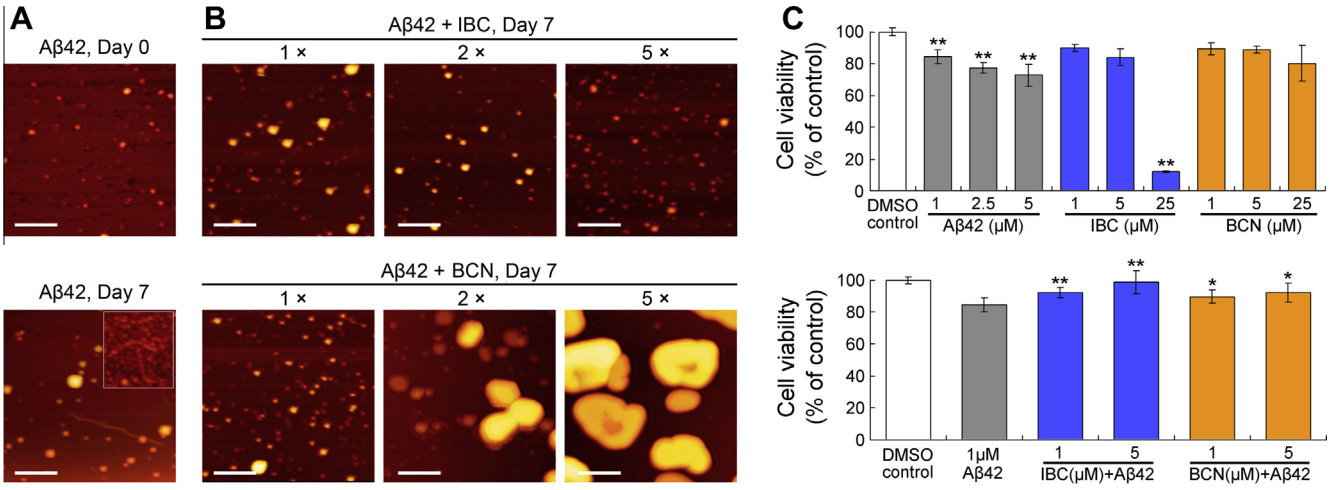

Fig. 6. IBC and BCN modified morphology of Aβ42 and attenuated its cell toxicity. Aβ42 samples (50 μM) were incubated for 0–7 days with IBC or BCN. The compounds were added at a molar ratio of 1:1 (1×), 2:1 (2×) or 5:1 (5×). (A) AFM images of Aβ42 alone samples at day 0 and day 7. (B) AFM images of IBC- and BCN-treated samples at day 7. Scale bar = 500 nm. (C) Cell viability analysis by MTT assay of SH-SY5Y cells treated with Aβ42 (day 3) or compound alone (upper panel, $^{**}P < 0.01$ versus DMSO control), and compound-treated Aβ42 samples (lower panel, $^{*}P < 0.05$, $^{**}P < 0.01$ versus 1 μM Aβ42 alone).

human neuroblastoma cells treated with Aβ42 samples. We first confirmed that aggregated Aβ42 significantly reduced SH-SY5Y cell viability in a dose-dependent manner as shown in Fig. 6C, and both IBC and BCN showed limited cell toxicity to SH-SY5Y cells at a concentration of 1 or 5 μM. However, IBC greatly reduced cell viability at 25 μM indicative of strong toxic effect (Fig. 6C, upper panell). Thus the influences of IBC and BCN on Aβ42 toxicity were only analyzed at 1 and 5 μM corresponding to molar ratios of 1:1 and 5:1 to Aβ42 (1 μM). We found that both of the compounds significantly suppressed Aβ42-induced cell toxicity (Fig. 6C, lower panel).

4. Discussion

Small molecular inhibitors can modulate the process of Aβ42 aggregation [24] or remodel the conformers of Aβ42 aggregates [21] through different mechanisms. Necula et al., categorized 35 inhibitors into three classes [24]: class I compounds inhibit oligomerization without inhibiting fibrillization, class II compounds inhibit both, and class III compounds only inhibit fibrillization. In this study, we identified two active compounds from *Psoraleae Fructus*, IBC and BCN, inhibiting the "on-pathway" aggregation and the cell toxicity of Aβ42 peptides. IBC acts as a class II inhibitor, whereas BCN might belong to class III, according to Necula's classification [24].

Similar to the remodeling effects of resveratrol [26], EGCG [27], myricetin and nordihydroguaiaretic acid (NDGA) [21], BCN modulates Aβ42 into large "off-pathway" aggregates at higher concentration (molar ratio to Aβ42 at 2:1 or 5:1). This could be related to the hydrophobic property of BCN that enables tight binding to the hydrophobic core of Aβ42. Binding of BCN might also promote the hydrophobic interaction of Aβ42 monomers and oligomers leading to formation of large insoluble aggregates, as seen in AFM studies (Fig. 6B). However, these large aggregates are conformationally different from the "on-pathway" aggregates of Aβ42 peptides formed in the absence of BCN, since they were not recognized by ThT, OC or A11 antibody.

Surprisingly, BCN seems to promote the formation of A11-positive oligomers at an equimolar concentration (molar ratio of 1:1 to Aβ42, Fig. 4). Similar A11-enhancing effect has been reported previously in resveratrol [28] and NDGA [6] studies. In our study, we also found that resveratrol enhanced A11 blotting signal while EGCG inhibited it (data not shown). Since A11-positve oligomers are believed to be highly toxic, the above findings might be conflict with their protection effects as seen in Aβ42-induced cell toxicity analyses [26,28,6]. A reasonable explanation could be that these compounds may simply mediate binding of A11 antibody to Aβ42 conformers rather than modulate Aβ42 into A11-positive oligomers.

In contrast to BCN, IBC inhibited both oligomerization and fibrillization of Aβ42. This may result from its favorable structural properties as suggested by previous studies. The efficient polyphenol inhibitors are normally composed of at least two phenolic rings with two to six atom linkers, and a minimum of three hydroxyl groups on the aromatic rings [7]. Although many plant phenolic

Table 1
In silico prediction of human intestinal absorption and blood–brain barrier penetration after oral administration of IBC, BCN and selected Aβ42 aggregation inhibitors.

Compounds	Absorption level	BBB level
Baicalein	Good	Low
Myricetin	Very low	–
Piceid	Moderate	–
Rosmarinic acid	Low	–
Resveratrol	Good	Medium
EGCG	Very low	–
Tannic acid	Very low	–
IBC*	Good	High
BCN*	Good	High

"–": Undefined by Discovery Studio.
* Compounds in this study.

compounds were demonstrated to be strong Aβ aggregation inhibitors, only a few were reported to inhibit both oligomerization and fibrillization, such as piceid and tannic acid [21]. Moreover, the application of these inhibitors for AD therapy was limited by the low bioavailability due to poor absorption and low blood–brain barrier (BBB) penetration [29,30]. Compared with known phenolic inhibitors, the prenylated structures of IBC and BCN are more hydrophobic, implicating better bioavailability. We therefore predicted the oral and CNS bioavailability of IBC and BCN in comparison with the most effective phenolic inhibitors reported elsewhere [7,31], using ADMET descriptors in Discovery Studio software (Accelrys). Indeed, we found that both IBC and BCN showed good oral absorption and high BBB penetration in this model (Table 1). Although IBC can inhibit both oligomerization and fibrillization of Aβ42 in vitro, its cell toxic property is not favorable for CNS drug development. We are currently trying to analyze the structure–activity relationship of IBC by investigating its structural analogs.

In conclusion, the present study demonstrated that IBC and BCN from *Psoraleae Fructus* modulate Aβ42 aggregation process through two different mechanisms. IBC significantly inhibits both oligomerization and fibrillarization of Aβ42, while BCN converts Aβ42 into large unstructured aggregates. Both of the compounds can significantly suppress Aβ42-induced toxicity in SH-SY5Y human neuroblastoma cells.

Acknowledgments

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