Salvianic borneol ester reduces β-amyloid oligomers and prevents cytotoxicity

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Abstract

Context: The destabilization of β-amyloid (Aβ) peptide aggregates and the protection of functional cells are the attractive therapeutic strategies for Alzheimer’s disease (AD). Some active ingredients of Salvia miltiorrhiza f. alba C.Y.Wu & H.W.Li (Lamiaceae) (SM) have attracted increasing attention for the treatment of neurodegenerative diseases.

Objective: Salvianic borneol ester (SBE) is a new compound based on SM formulas. The present study was designed to examine the anti-amyloid effects and neuroprotection of SBE in vitro.

Materials and methods: The destabilizing effects of SBE and its related compounds (salvianic acid A and borneol) on preformed Aβ oligomers were measured by using fluorescence spectroscopy with thioflavin T (ThT) and the destabilizing effects of SBE were further confirmed visually by transmission electron microscopy (TEM). The neuroprotective effects of SBE against hydrogen peroxide (H2O2)-induced toxicity in human neuroblastoma cells (SH-SYSY) and motor neuron hybridoma cells (VSC 4.1) were shown by MTT assay and morphological observation.

Results: SBE showed the most significant destabilizing effect, though the mixture of salvianic acid A and borneol also destabilized Aβ1–40 oligomers. The destabilizing activity of salvianic acid A or borneol alone was not significant. SBE destabilized Aβ1–40 oligomers in dose- and time-dependent manners and the destabilizing effect could also be seen in the photographs of TEM. Furthermore, SBE could protect SH-SYSY cells and VSC 4.1 cells against H2O2-induced toxicity in a dose-dependent manner.

Discussion and conclusion: SBE had the bifunctional activities of anti-amyloid and neuroprotection. It may have therapeutic potential for AD and be an alternative lead compound for developing new drugs against AD.

Keywords: Alzheimer’s disease, β-amyloid peptide, neuroprotection, salvianic borneol ester, thioflavin T, transmission electron microscopy

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by loss of memory and cognition especially in the elderly. Since existing treatments for AD only offer limited symptom alleviation (Lleó et al., 2006; Jakob-Roetne and Jacobsen, 2009), new alternative agents are urgently needed. One of the major pathological features of AD is the appearance of senile plaques composed mainly of β-amyloid (Aβ) peptides (Jakob-Roetne and Jacobsen, 2009). Monomeric Aβ can aggregate into oligomeric and fibrillar forms and finally become senile plaques in the brain. The Aβ aggregates possess neurotoxicity in vivo and in vitro (Yankner et al., 1990; Hardy and Higgins, 1992). Recent evidence has shown that oligomeric form
of Aβ rather than the fibril is primarily responsible for the neuronal injury and death (Cleary et al., 2005). Therefore, some therapeutic efforts are targeted at finding anti-amyloid agents to disrupt Aβ aggregation, especially to reduce oligomers in AD (Hirohata and Ono, 2008; Shah et al., 2008).

Common Aβ oligomer neurotoxic mechanisms are related with oxidative stress (Behl, 1997). Aβ oligomers generated reactive oxygen species (ROS), which can cause damage to cellular lipids, proteins, or DNA and eventually lead to neuronal death (Zhu et al., 2007). As the major component of ROS, hydrogen peroxide (H2O2) is generally used as an inducer of oxidative stress in vitro models by many laboratories (Tabner et al., 2002; Zhang et al., 2007; Bi et al., 2008). Some compounds have been proven to possess neuroprotective potential as antioxidants, such as curcumin, resveratrol, lipoic acid, (−)-epigallocatechin-3-gallate (EGCG), and melatonin (Mecocci et al., 2008; Zhao, 2009).

Salvia miltiorrhiza f. alba C.Y.Wu & H.W.Li (Lamiaceae) (SM) is a traditional medicinal plant. SM formulas composed mainly of SM, borneol, and Panax notoginseng (Burkill) F.H.Chen ex C. Y.Wu & K.M.Feng (Araliaceae) are commonly used in the clinical treatment of cardiovascular diseases and cerebrovascular diseases (Zhou et al., 2005). Recently, SM and its active ingredients have attracted increasing attention for the treatment of neurodegenerative diseases. For AD, some components of SM such as hydrotanshinone and cryptotanshinone have been reported to have potential therapeutic effects as inhibitors of acetylcholinesterase in vitro (Ren et al., 2004; Lin et al., 2008). Besides, it had been reported that cryptotanshinone attenuated amyloid plaque deposition in the brain of transgenic mouse and decreased Aβ generation in rat cortical neuronal cells overexpressing human APP (Yu et al., 2007; Mei et al., 2009).

Salvianic borneol ester (SBE, benzenepropanoic acid, α,3,4-trihydroxy-(1R, 2S, 4R)-1,7,7-trimethylbicyclo[2.2.1]hept-2-yl ester, C19H26O5, MW=334.41) designed based on SM formulas is a new compound synthesized by salvianic acid A and borneol (Figure 1) (Zheng et al., 2007; Han et al., 2009). Salvianic acid A and borneol are main components of SM and borneol, respectively. To date, the role of SBE in treatment of AD has not been reported. In this study, the anti-amyloidogenic and antioxidative properties of SBE were reported. We examined the effects of SBE and its related compounds on the destabilization of preformed Aβ oligomers by using fluorescence spectroscopy with thioflavin T (ThT) and the destabilizing effect of SBE was further confirmed by transmission electron microscopy (TEM). Then, we demonstrated the neuroprotective effects of SBE against H2O2-induced toxicity in human neuroblastoma cells (SH-SY5Y) and motor neuron hybridoma cells (VSC 4.1) by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Materials and methods

Reagents
Salvianic acid A and borneol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), with Standard Medical Compound Certifications issued by the State Food and Drug Administration (SFDA), China. SBE (>95% purity) was kindly provided by Prof. Xiaohui Zheng. The Aβ1–40 peptide was purchased from Bachem AG (Bubendorf, Switzerland). ThT was obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). All reagents used in this study were of analytical grade.

Preparation and destabilization of Aβ oligomers
Aβ oligomers were prepared according to the method published previously (Klunk et al., 1999), with slight modifications. Aβ1–40 peptide (1mg) was dissolved in 2mL 0.01 M phosphate-buffered saline (PBS, pH=7.4) containing 1 mM EDTA. The solution was incubated at 37°C for 72 h with shaking. The resulting Aβ1–40 oligomers were stored at −80°C and were diluted 5-fold to give a concentration of 0.1mg/mL prior to assay.

The reaction mixtures containing aliquots of Aβ oligomers with/without a test agent were incubated at 37°C for 30 min with shaking. The agents used in this experiment included SBE, salvianic acid A, borneol at a final concentration of 10 µg/mL, and a mixture of salvianic acid A and borneol at 1:1 (5 µg/mL, respectively). Further, the incubation of Aβ oligomers with SBE was performed at various final concentrations of SBE (0.31–40 µg/mL) and hours (0–6 h) (10 µg/mL of SBE).

ThT fluorescence assay
ThT-induced fluorescence changes were measured to quantify Aβ oligomers by using a RF-5301PC Spectrofluorometer (Shimadzu, Japan) according to the method described by Fujitani et al. (2006). The mixtures containing Aβ oligomers with/without a 10 µg/mL test agent were added to PBS (0.01 M, pH=7.4) containing 3 µM ThT (Sigma). Each assay was run in triplicate.

Figure 1. A synthesis sketch map of salvianic borneol ester (SBE).
Excitation and emission wavelengths were set at 445 and 485 nm, respectively. The fluorescence intensity of 3 µM ThT was measured as the background. The fluorescence intensity of 3 µM ThT and Aβ oligomers without test agent was considered to be 100% as control. The amount of Aβ oligomers were calculated as a percentage of control.

Transmission electron microscopy
Aβ1–40 oligomers were incubated with/without SBE at concentrations 2.5, 20, and 40 µg/mL for 30 min at 37°C with shaking. Following incubation, 5 µL of samples were placed on copper grids for 60 sec; excess solutions were removed by using filter papers to touch the edge of the grids. The grids were washed with distilled water. After air-drying, the specimens were examined under H800 transmission electron microscope (TEM) (Hitachi, Japan) at an instrumental magnification of 100,000×. The length of Aβ1–40 oligomers was measured.

Cell culture and neurotoxicity assay
SH-SY5Y cells and VSC 4.1 cells (obtained from Peking University Health Science Center) were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium with 10% fetal bovine serum (Gibco, Carlsbad, CA) in 5% CO₂ humidified atmosphere at 37°C. The culture medium was changed every 48h.

Our preliminary test found the cytotoxicity was significant at 24 h after 200 µM H₂O₂ was added to cultured SH-SY5Y and VSC 4.1 (data not shown). SH-SY5Y cells and VSC 4.1 cells were separately plated in 96-well plates at a density of 1.67 × 10⁴ cells/mL. Cells were pre-incubated with various concentrations of SBE in culture medium for 24 h. The final concentrations of SBE ranged between 0.3 and 10 µM. After that, cells were exposed to 200 µM H₂O₂ for another 24 h. After washing with PBS, 10 µL MTT (5 mg/mL) solution was added and incubated for 4 h. Then, formazan crystals were dissolved with sodium dodecyl sulfate solution and measured by VICTOR3™V (PerkinElmer, Boston, MA) multilabel counter at a wavelength of 570 nm. The cell viability was expressed at percentage of viable cells in wells with SBE as compared with cells in control wells.

Data analysis
Each EC₅₀ value was calculated by the Bliss method using the Lan Zhou Pharmaceutical software series for EC₅₀ data processing, version 1.01 (Lan Zhou Inc., Sheng Yang, China). Statistical comparisons were made by one-way ANOVA analysis with Dunnett’s post hoc test using SPSS software (version 13.0). *P<0.05, **P<0.001. All values were represented as mean ± SD.

Results
We compared the effects of SBE, salvianic acid A, borneol, and a mixture of salvianic acid A and borneol on the destabilization of Aβ1–40 oligomers. As shown in Figure 2, Aβ1–40 oligomers was significantly reduced in the presence of SBE and the mixture (salvianic acid A and borneol, 1:1), but the destabilizing activity of salvianic acid A or borneol alone was not significant. Though the mixture of salvianic acid A and borneol was effective, SBE showed stronger destabilizing effect.

SBE destabilized preformed Aβ1–40 oligomers. Significant destabilizing effects of SBE were observed dose-dependently (Figure 3A) and time-dependently (Figure 3B). The EC₅₀ value was 19.4 µg/mL. Furthermore, TEM was used to confirm the form of the aggregates and the effect of SBE on Aβ1–40 oligomers. Figure 4A showed the Aβ1–40 oligomers without treatment, and the mean length of oligomers was 85.2 ± 6.3 nm. As is shown in Figure 4, in the presence of SBE, less Aβ1–40 oligomers was observed visually by TEM. The Aβ1–40 oligomers were destabilized by SBE dose-dependently.

We then examined the neuroprotective effects of SBE on SH-SY5Y cells and VSC4.1 cells. We pre-incubated SH-SY5Y and VSC 4.1 cells with various doses of SBE for 24 h, then exposed cells to 200 µM H₂O₂ solution for another 24 h and cell viability was finally tested. As shown in Figure 5, H₂O₂-induced toxicity was attenuated after cells were incubated with SBE in concentration-dependent manner. Compared with control, SBE at 1, 3, and 10 µM significantly increased the cell viability to 47.0 ± 7.0%, 54.7 ± 5.4%, and 62.2 ± 3.6%, respectively, in SH-SY5Y cells (Figure 5A), to 39.7 ± 6.0%, 59.8 ± 7.0%, and 65.9 ± 3.6%, respectively, in VSC4.1 cells (Figure 5B). The protective effects could also be confirmed by the morphological observation (Figure 5C–5H). After exposure to H₂O₂, neurite degeneration of cells was evident and a TMV-positive signal was observed in control cells (Figure 5C). In contrast, SH-SY5Y cells treated with SBE (10 µg/mL) showed a lower TMV-positive signal than control cells (Figure 5C). However, SH-SY5Y cells exposed to SBE (10 µg/mL) and H₂O₂ showed a higher TMV-positive signal than control cells (Figure 5C). The data suggested that SBE had a protective effect on SH-SY5Y cells against H₂O₂-induced toxicity.

Figure 2. The destabilization of Aβ1–40 oligomers measured by the thioflavin T fluorescence assay. Preformed Aβ1–40 oligomers were incubated in the presence of salvianic borneol ester (SBE) (10 µg/mL), borneol (10 µg/mL), salvianic acid A (10 µg/mL), or the mixture of borneol and salvianic acid A (1:1, both 5 µg/mL) for 30 min. Values are reported as mean ± SD (n=3). *P<0.05, **P<0.001 versus control (Aβ1–40 oligomers without treatment) (one-way ANOVA analysis with Dunnett’s post hoc test).

Figure 3. Aβ1–40 oligomers destabilized by SBE in a concentration-dependent manner (one-way ANOVA analysis with Dunnett’s post hoc test).
A conventional idea for searching a new lead compound from traditional Chinese medicine (TCM) is to separate active ingredients from a single herb. We adopt a new idea to explore new bioactive compounds, which are combined by two compounds of TCM. SBE is a compound formed as a product of synthesis of salvianic acid A and borneol, based on classical SM formulas that are widely used in TCM. This study provides the first evidence that SBE destabilizes preformed Aβ oligomers and protects SH-SY5Y cells and VSC4.1 cell against H2O2-induced cytotoxicity. According to the data, the destabilizing activity of salvianic acid A or borneol alone was not significant. Though the mixture of salvianic acid A and borneol could destabilize

Figure 3. Effects of salvianic borneol ester (SBE) on Aβ1-40 oligomers destabilization measured by the thioflavin T fluorescence assay. SBE destabilized Aβ1-40 oligomers (A) in dose-dependent manner (incubation for 30 min) and in time-dependent manner (SBE at 10 µg/mL) (B). Values are reported as mean ± SD (n=3). Significant destabilizing activities of SBE were observed dose-dependently at concentration above 5 µg/mL (P<0.001 vs. control) and time-dependently at and above 1 h (P<0.001 vs. control) (one-way ANOVA analysis with Dunnett’s post hoc test).

Discussion

A conventional idea for searching a new lead compound from traditional Chinese medicine (TCM) is to separate active ingredients from a single herb. We adopt a new idea to explore new bioactive compounds, which are combined by two compounds of TCM. SBE is a compound formed as a product of synthesis of salvianic acid A and borneol, based on classical SM formulas that are widely used in TCM. This study provides the first evidence that SBE destabilizes preformed Aβ oligomers and protects SH-SY5Y cells and VSC4.1 cell against H2O2-induced cytotoxicity. According to the data, the destabilizing activity of salvianic acid A or borneol alone was not significant. Though the mixture of salvianic acid A and borneol could destabilize

Aβ oligomers, the effect of SBE was more significant. SBE destabilized Aβ1-40 oligomers in dose- and time-dependent manners. We also found SBE could destabilize Aβ1-42 oligomers in a higher concentration and time-dependently (data not shown). In addition, it has been reported that cryptotanshinone extracted from SM strongly attenuated amyloid plaque deposition in transgenic mice and decreased Aβ generation in vitro (Yu et al., 2007; Mei et al., 2009). We found that SBE destabilized Aβ1-40 oligomers at a similar level with cryptotanshinone in ThT fluorescence assay (data not shown). Based on these findings, it is reasonable to conclude that SBE is a novel alternative template for developing therapeutic agents of AD and research for new compounds through combination of chemical components from TCM is one of viable strategies of drug discovery.

Accumulating evidence suggests that Aβ oligomers are the most neurotoxic forms among Aβ aggregates (Dahlgren et al., 2002). It was reported that oligomers inhibit neuronal viability 10-fold more than fibrils and ~40-fold more than unaggregated peptides. Protofibrils, one kind of oligomers, are <200 nm in length and bind ThT with increased fluorescence intensity (Jan et al., 2010). Fibrils have been described as >1 μm long and Aβ monomers do not change the fluorescence intensity of ThT (Jan et al., 2010). In our study, the Aβ aggregates are about 85 nm in length and bind ThT. These results suggest that the Aβ aggregates formed in our experiment are just the protofibrils possessing strong neurotoxicity. SBE could destabilize oligomers, the most toxic Aβ aggregates, which enhances the value of SBE to be a potential AD drug.

Like most other compounds destabilizing Aβ fibrils or oligomers, SBE has aromatic group and phenolic hydroxy groups that have been considered as an important

Figure 4. Electron micrographs of Aβ1-40 oligomers. Aβ1-40 oligomers were incubated in the absence (A) or presence of salvianic borneol ester (SBE) 2.5 µg/mL (B), 20 µg/mL (C), or 40 µg/mL (D). Magnification: 100,000x; scale bar = 170 nm.
it was reported that some polyphenolic compounds preventing amyloid accumulation have two aromatic end groups and the length and flexibility of the linker region are related to inhibitory activity (Reinke and Gestwicki, 2007). On the other hand, some nonsteroidal anti-inflammatory drugs (such as ibuprofen) that have an aromatic-based hydrophobic structure with some fused ring structures and methyl and/or carboxyl groups can also destabilize Aβ aggregates (Hirohata et al., 2008). Therefore, the structures of amyloid-destabilizing compounds are various and the exact destabilizing mechanisms are unclear. In the present study, salvianic acid A and borneol alone could not destabilize Aβ1–40 oligomers, may be because their sizes were too small to fully occupy the binding pocket of oligomers. When they exist simultaneously in the form of mixture or compound SBE, they occupied more binding sites in the binding pocket and destabilized Aβ1–40 oligomers. SBE might bind to oligomers more stably and showed stronger destabilizing effect.

SH-SY5Y cells are human neuroblastoma cells that are commonly used nerve cell line in the study of neurodegenerative disease. VSC4.1 cells are motor neuron hybridoma cells that have some characteristics of normal cells. We used these two kinds of cell lines as H2O2-injured cell models. SBE similarly protected SH-SY5Y and VSC 4.1 against H2O2-induced cytotoxicity. SBE at 0.3–10 μM was not cytotoxic, which was detected in our preliminary study (data not shown). In these H2O2-injured cell models, the damage induced by H2O2 was very serious (only about 20% cell viability was left). Cell viability of cells treated with 10 μM SBE reached about 60%, approximately three times the cell viability of the H2O2-injured cells without SBE. SBE showed strong antioxidative bioactivity in both cells.

Based on the bifunctional activities of anti-amyloid and neuroprotection, it is reasonable to suggest that SBE will be an alternative lead compound for the development of new drugs against AD. Additional studies are needed to elucidate the mechanisms underlying the anti-amyloid and antioxidative effects of SBE and to confirm its efficacy in an animal model of AD. Toxicological research of SBE is also quite necessary.

Conclusion
In this study, we showed SBE destabilized preformed Aβ oligomers and protected SH-SY5Y cells and VSC4.1 cells against H2O2-induced toxicity. These results suggest that SBE may have therapeutic potential for the treatment of AD.

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Declaration of interest

There is no conflict of interest to declare.

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