

Salvianolic acid B inhibits fibril formation and neurotoxicity of amyloid beta-protein *in vitro*¹

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KEY WORDS salvianolic acid-B; amyloid beta-protein; cell culture; PC12 cells

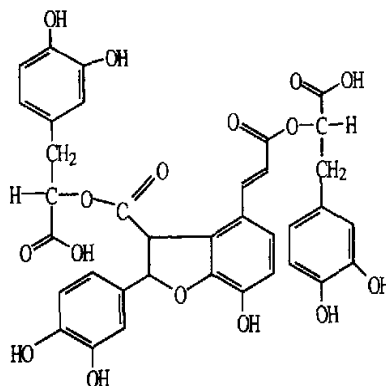
ABSTRACT

AIM: To observe the effect of salvianolic acid-B (SalB) on amyloid beta-protein (A-beta) fibril formation and its toxicity towards PC12 cells. **METHODS:** A-beta (1-40) was incubated with or without SalB at 25 °C for 30 h, 48 h, and 100 h. Fibril formation was then viewed under an electron microscope. Toxicity of the A-beta (1-40) towards PC12 cells was measured with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). A-beta (25-35) was aged by incubating at 25 °C for 7 d, then the peptide was incubated with PC12 cells with or without SalB. Toxicity of A-beta (25-35) towards PC12 cells was observed with MTT. **RESULTS:** Following incubation at 25 °C for 30 h, A-beta (1-40) (100 mg/L) aggregated and formed fibrils. SalB 10-100 nmol/L completely prevented the fibril formation within 30 h. Extension of amyloid fibrils increased with prolonging the incubation time. SalB inhibited the fibril formation process during this period. In the MTT assay A-beta (1-40) incubated with SalB manifested significantly lower toxicity to PC12 cells compared with that without SalB. Besides, SalB 1 μmol/L significantly attenuated the toxicity of aged A-beta (25-35) to PC12 cells. **CONCLUSION:** SalB could inhibit A-beta aggregation and fibril formation, as well as directly inhibit the cellular toxicity of aged A-beta towards PC12 cells.

INTRODUCTION

Amyloid beta-protein (A-beta) is the major protein

component of Alzheimer's plaques^[1]. One of the major pathological features of Alzheimer's disease is the deposition of A-beta. Cellular toxicity has been shown to be associated with fibrillar forms of A-beta^[2,3]. Inhibition of the fibril formation is therefore viewed as a possible method of slowing disease progression in Alzheimer's disease. Recently a series of benzofuran derivatives have been identified as inhibitors of fibril formation of the beta-amyloid peptide^[4,5]. Emerging evidence suggests that reactive oxygen species (ROS) mediate amyloid peptide-induced neurotoxicity. Salvianolic acid-B (SalB) is one of the water-soluble components isolated from the traditional Chinese drug — *Salvia miltiorrhiza*. Our previous studies have demonstrated that SalB possesses a very strong antioxidant activity^[6]. It has also been observed to alleviate ischemic damage in rats^[7]. The present study was designed to see whether SalB has an effect on A-beta fibril formation and on its toxicity towards PC12 cells.



Salvianolic acid B
C₃₆H₃₀O₁₆ M_r 718

MATERIALS AND METHODS

Drugs and reagents SalB (> 90 %) was supplied by the Department of Phytochemistry of our Insti-

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tute. A-beta (1-40), A-beta (25-35) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma.

Cell culture PC12 cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were routinely cultured at 37 °C, 5 % CO₂ with Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum, and 5 % horse serum.

Fibril formation A-beta (1-40) was dissolved in sterilized double deionized water and filtered through a 0.22- μ m filter (Millipore, USA). The peptide solution was diluted to a concentration of 250 mg/L and dispensed into Eppendorf tubes (64 μ L/tube). SalB (dissolved in sterilized double deionized water) 16 μ L was added to tubes containing A-beta (1-40) peptide. Then the solution was mixed with an equal volume of 0.2 mol/L sodium phosphate buffer (pH 7.4). The final concentration of A-beta (1-40) peptide was 100 mg/L. The final concentration of SalB was 10 nmol/L and 100 nmol/L respectively. The peptide solution was incubated at 25 °C. The peptide solution was removed at different time intervals. Peptide solution 30 μ L was used for measurement of fibril formation, the rest was used for cytotoxicity studies. For fibril formation, the peptide solution was absorbed onto 200 mesh Formvar-coated copper grids, dried at 25 °C for 1 min, and negatively stained with 2 % uranyl acetate. The samples were viewed for fibrils under a JEM-101B electron microscope (JEOL, Japan).

Cytotoxicity Cytotoxic effect of A-beta was assessed by measuring cellular redox activity with MTT. PC12 cells were plated at a density of 5000 cells per 100 μ L per well of fresh medium in 96-well tissue culture plates. For A-beta (1-40), the solution mentioned above in fibril formation was added to the well to a final concentration of 10 μ mol/L in triplicate. Following a 48-h incubation, MTT was added to a final concentration of 0.5 g/L, and then was incubated for a further 4 h. Cell lysis buffer (100 μ L per well; 20 % SDS/50 % N, N'-dimethylformamide, pH 4.7) was then added. Following incubation at 37 °C for 20 h, colorimetric determination of MTT reduction was made at 570 nm. The values obtained upon addition of vehicle were taken as 100 %, that obtained following the addition of 0.1 % Triton-X100 to lyse the cells taken as 0 %. For A-beta (25-35), the peptide was dissolved in sterilized 0.1 mol/L sodium phosphate buffer (pH 7.4) to a concentration of 1 g/L and then incubated at 37 °C for 7 d. After 1 h incu-

bation with SalB, A-beta (25-35) solution was added to the cells in 96 plates to a final concentration of 10 μ mol/L. Following 48 h incubation at 37 °C, MTT reduction was measured as described above.

RESULTS

After being incubated for 30 h at 25 °C, A-beta (1-40) (100 mg/L) aggregated and formed fibrils. With an increase of incubation time, an extension of amyloid fibrils was observed. After a 100-h incubation more aggregated peptide granules adhered onto the fibrils. SalB 10-100 nmol/L prevented the fibril formation completely in 30 h. At 48 h and 100 h SalB also significantly inhibited the fibril formation (Fig 1).

After 30 h aging at 25 °C, A-beta (1-40) 10 μ mol/L significantly inhibited the MTT reduction of PC12 cells. The inhibitory effect became more apparent with an increase in the aging time, and this toxic effect was significantly reduced when the protein was aged in the presence of SalB (Fig 2).

Aged A-beta (25-35) 10 μ mol/L significantly inhibited MTT reduction of PC12 cells which was 85 % of control following a 48-h incubation. SalB 1 μ mol/L significantly inhibited the toxicity induced by A-beta (25-35). SalB 10 nmol/L and 100 nmol/L also inhibited the toxic effect of A-beta, although no significant difference was observed (Tab 1).

DISCUSSION

SalB manifested a significant effect of inhibiting A-beta fibril formation in this study. However SalB did not act similar to the benzofuran derivatives, among which a series of compounds have been found to act as inhibitors of fibril formation of the beta-amyloid peptide. As a non benzofuran derivative inhibitor, it is not known whether SalB binds to the same site on the beta-amyloid molecule as the benzofurans, or there is a novel site by which SalB inhibits fibril formation.

Beta-amyloid peptides are deposited in an aggregated fibrillar form in both diffused and senile plaques in the brains of patients with Alzheimer's disease. The neurotoxicity of A-beta in neurons is dependent on its aggregation^[8]. Lorenzo and Yankner demonstrated that A-beta neurotoxicity required fibril formation and suggested that a common cytopathic effect of amyloid fibrils may contribute to the pathogenesis of Alzheimer disease and other

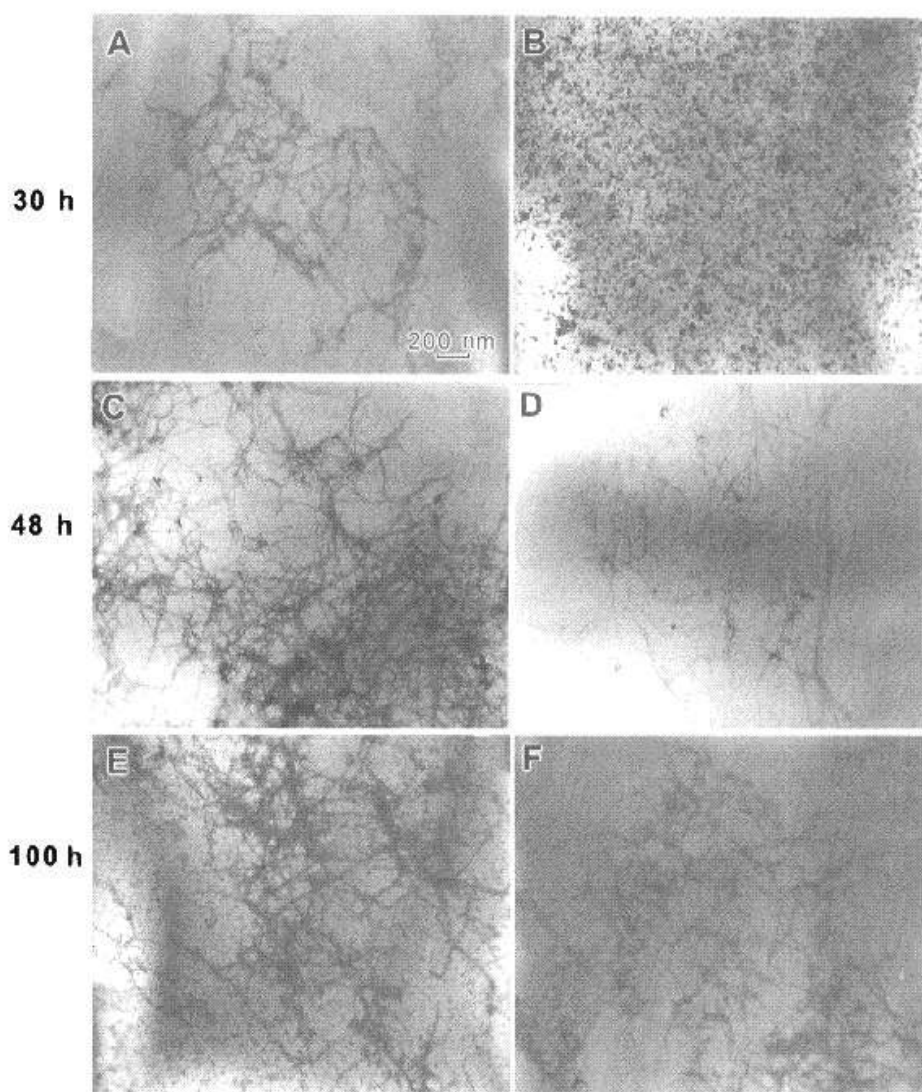


Fig 1. Effect of SalB (10 nmol/L) on the fibril formation of synthetic A-beta 1-40 peptide following 30 h, 48 h, and 100 h incubation at 25 °C. A, C, E; Control. B, D, F; SalB-treated.

Tab 1. Effect of A-beta (25-35) on MTT reduction of PC12 cells. $n=3$ experiments. $\bar{x} \pm s$. $P < 0.05$ vs A-beta (25-35) group.

Group/nmol·L ⁻¹	MTT reduction/%
Untreated cells	100
A-beta (25-35)	85.0±3.1
Sal B 10 + A-beta (25-35)	87.5±1.5
Sal B 100 + A-beta (25-35)	89.7±6.5
Sal B 1000 + A-beta (25-35)	92.1±1.8 ^b

amyloidoses^[2]. In our study, when incubated with SalB the fibril formation process of A-beta (1-40) was inhibited, and its neurotoxicity also diminished significantly. The results support the hypotheses that fibril formation is necessary for the neurotoxicity of A-beta.

Ghanta *et al* reported a peptide, which did not alter the apparent secondary structure of beta-amyloid nor prevent its aggregation, but could completely block beta-amyloid toxicity towards PC-12 cells^[9]. The authors suggest that complete disruption of amyloid fibril forma-

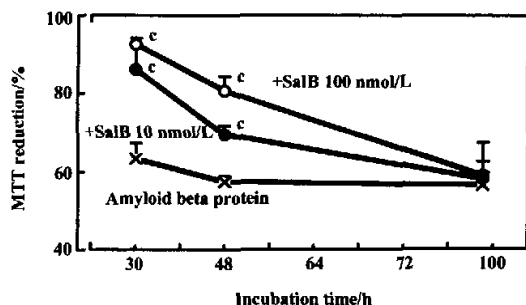


Fig 2. Effect of A-beta (1-40) on MTT reduction of PC12 cells. A-beta (1-40) 100 mg/L was aged at 25 °C for 30 h, 48 h, and 100 h with or without SalB. The peptide solution was then added to PC12 cells with a final concentration of 10 μmol/L. Following a 48-h incubation at 37 °C, MTT reduction was determined. $n=3$ experiments. $\bar{x} \pm s$. $^c P < 0.01$ vs A-beta control group with the same incubation time.

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tion is not necessary for abrogation of toxicity. Interestingly, we found that besides inhibiting the fibril formation, SalB could also directly inhibit the neurotoxicity of previously aged A-beta (25-35) towards PC12 cells. So there might exist more than one mechanisms for SalB to inhibit the neurotoxicity of A-beta to PC12 cells.

At present no satisfactory explanation has yet been proposed to explain the mechanisms of beta-amyloid aggregation and toxicity. Hensley *et al* reported that free radicals may be involved in the neurotoxicity of A-beta⁽¹⁰⁾. Bruce *et al* demonstrated that EUK-8, a synthetic catalytic superoxide and hydrogen peroxide scavenger could protect mature and immature hippocampal slice cultures from A-beta-induced neurotoxicity⁽¹¹⁾. In our previous study⁽⁶⁾ SalB was demonstrated to have a very potent effect in scavenging reactive oxygen species. In this study we found that SalB inhibited the toxicity of aged A-beta towards PC12 cells which not only supports a role for oxygen free radicals in A-beta toxicity but also highlights the therapeutic potential of free radical scavengers (such as SalB) in Alzheimer's disease.

丹酚酸 B 体外抑制淀粉样 β 蛋白的纤维形成及其细胞毒作用¹

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关键词 丹酚酸 B; 淀粉样 β 蛋白; 细胞培养; PC12 细胞

目的: 观察丹酚酸 B 对淀粉样 β 蛋白的纤维形成及其细胞毒作用的影响. **方法:** 将不同浓度丹酚酸 B 与淀粉样 β 蛋白(1-40)在 25 °C 共同孵育, 于不同时间取样品电镜观察纤维形成. 用 MTT 法观察此不同时间点淀粉样 β 蛋白(1-40)对 PC12 细胞的毒性作用. 另将淀粉样 β 蛋白(25-35)预先老化 7 d, 用

MTT 法观察此老化蛋白对 PC12 细胞的毒性及丹酚酸 B 的作用. **结果:** 丹酚酸 B 10-100 nmol/L 可以完全抑制淀粉样 β 蛋白(1-40) 25 °C 放置 30 h 的纤维形成, 对淀粉样 β 蛋白(1-40) 25 °C 放置 48 及 100 h 的纤维形成也有明显抑制作用. MTT 法显示, 经与丹酚酸 B 共同孵育的淀粉样 β 蛋白(1-40)明显较未与丹酚酸 B 孵育的淀粉样 β 蛋白对 PC12 细胞的毒性小. 丹酚酸 B 1 μmol/L 可明显抑制预先老化的淀粉样 β 蛋白(25-35)对 PC12 细胞的毒性作用. **结论:** 丹酚酸 B 可抑制淀粉样 β 蛋白的老化及纤维形成, 同时可直接抑制老化淀粉样 β 蛋白对 PC12 细胞的毒性作用.

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