Inhibitory Effect of Piperlonguminine/Dihydropiperlonguminine on the Production of Amyloid β and APP in SK-N-SH Cells

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Abstract

The study was undertaken to explore whether piperlonguminine/dihydropiperlonguminine could inhibit the production of amyloid β (Aβ) in human neuroblastoma cells (SK-N-SH) and to examine the underlying mechanism of this effect. Piperlonguminine/dihydropiperlonguminine components (1:0.8) were extracted from Futokadsura stem, and then used to treat SK-N-SH cells at three different concentrations: 3.13 µg/ml, 6.25 µg/ml and 12.50 µg/ml. Subsequently, the production of Aβ42 and Aβ40 were measured by Western blot analysis and enzyme linked immunosorbent assay (ELISA). On the other hand, the expressions of amyloid precursor protein (APP), Notch1 (Notch intracellular domain) and β-site amyloid precursor protein cleavage enzyme (BACE-1) were also examined by Western blot assay. The activities of β-secretase and γ-secretase were detected at the same time. Furthermore, Aβ42 level was detected by immunocytochemistry staining. We demonstrated that the treatment of piperlonguminine/dihydropiperlonguminine could significantly decrease the levels of APP, Aβ42 and Aβ40 peptide in SK-N-SH cells, despite the fact that the activities of β-secretase and γ-secretase were not affected significantly. These data suggest that piperlonguminine/dihydropiperlonguminine components could significantly inhibit the level of APP, Aβ42 and Aβ40 peptide without affecting the activity of β-secretase and γ-secretase in SK-N-SH cells.

Key Words: Alzheimer disease, amyloid precursor protein, amyloid β, β-secretase, γ-secretase, piperlonguminine, dihydropiperlonguminine, futokadsura stem

Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disease characterized by progressive dementia and deterioration of cognitive function (12). It is the most common neurodegenerative disorder, affecting more than 20 million people worldwide, and as many as 50% of individuals who are older than 85 (3, 21).

The histopathological hallmarks of AD are the presence of extracellular deposits of amyloid β (Aβ) peptide in senile plaques of cerebral cortex and the intracellular aggregation of tau protein in neurofibrillary tangles (7, 12). Aβ is a toxic 40-42 residue peptide derived from proteolysis of the amyloid precursor protein (APP) (1, 16), which is a...
type I transmembrane glycoprotein (4, 13), and encoded by the APP gene located on chromosome 21q21 (17, 18). Certain mutations of APP gene may cause excessive cleavage of the protein by β-secretase and γ-secretase, leading to the excessive aggregation of neurotoxic Aβ peptide.

The proteolytic processing of APP is known to occur through two alternative pathways: the amyloidogenic pathway and the anti-amyloidogenic pathway. In the amyloidogenic pathway, APP is cleaved by a β-site amyloid precursor protein cleavage enzyme (BACE-1), a type of protease β-secretase. The proteolytic cleavage is taken place at the N-terminus of the Aβ domain and results in the secreted sAPPβ and a C-terminal 99 amino acid fragment of APP (C99). C99 is processed at its transmembrane domain by γ-secretase, leading to the secretion of Aβ42/Aβ40 and the APP intracellular domain (AICD) (21). The accumulation of Aβ leads to neurotoxicity in AD patients and contributes to the formation of senile plaques in cerebral cortex, which is the pathological characteristic of AD. In the anti-amyloidogenic pathway, APP is cleaved by α-secretase and γ-secretase to generate soluble fragments (10, 24). Both β-secretase and γ-secretase are key enzymes in the APP proteolytic processing pathway from APP to Aβ, regulating the production of Aβ. The proteolytic process of APP is very similar to the proteolytic processing of Notch signaling in terms of the activity of γ-secretase (14, 25, 28). Notch1 level is often used to monitor the activity of γ-secretase indirectly and its side effects.

Futokadsura stem is the petiole of piper plant Kadsura, and it is used to treat inflammatory diseases such as rheumatism in Chinese traditional medicine. Experimental studies show that Futokadsura Stem is an efficient anti-inflammatory and anti-platelet drug (9, 29). In previous experiments, Futokadsura stem in shown to have selective inhibition on the expression of APP (5, 6). Subsequently, we demonstrated that such inhibition on APP gene expression in human neuroblastoma cells (SK-N-SH) was mediated through the piperlonguminine/dihydropriperlonguminine components extracted from Futokadsura stem (29).

The aggregation of Aβ plays an important role in the pathological pathway of AD due to its cytotoxicity to neuronal cells (19, 26, 27). Although piperlonguminine/dihydropriperlonguminine could efficiently inhibit the expression of APP at the protein level, whether they also affect the activities of Aβ and Aβ-generating enzymes (such as β-secretase and γ-secretase) remains unclear. To answer this question, we investigated the effects of piperlonguminine/dihydropriperlonguminine on the production and proteolytic processing of Aβ.

## Materials and Methods

### Reagents

Amyloidβ<sub>42</sub> antibody was obtained from Upstate Biotechnology, Lake Placid, NY, USA. Amyloidβ<sub>1-40</sub> antibody and Notch1 polyclonal antibody were obtained from Millipore Corporation, Bedford, MA, USA. Human APP695 antibody, BACE-1 antibody, β-secretase activity kit and γ-secretase activity kit were obtained from R&D Systems, Inc, Minneapolis, MN, USA. Human β-actin antibody was obtained from Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA. Aβ<sub>42</sub> colorimetric ELISA kit and Aβ<sub>40</sub> colorimetric ELISA kit were obtained from BioSource International, Inc. Camarillo, CA, USA. CytoTox-ONE Homogeneous Membrane Integrity assay kit was bought from Promega Corp. Madison, WI, USA. All other reagents and solvents were of analytical grade.

### Preparation of Piperlonguminine/ Dihydropiperlonguminine (1:0.8) from Futokadsura

Piperlonguminine/dihydropriperlonguminine components were separated from futokadsura stem according to a previously described method (29). Futokadsura stem samples were first extracted with water, and consecutively extracted with petroleum ether, ethyl acetate and normal butyl alcohol. The ethyl acetate extract phase was dissolved by 95% ethanol, then subject to silica gel column chromatography, chloroform-acetone gradient elution. The chloroform-acetone (9:1) elution phase Fraction 31 was repeatedly crystallized by petroleum ether-acetone to obtain crystal HFT-1. According to the data of 1HNMR and 13CNMR spectra, the crystal HFT-1 was composed of piperlonguminine (A) and dihydropiperlonguminine (B) with the ratio of A to B being 1:0.8. The chemical structures of piperlonguminine and dihydropiperlonguminine are shown in Fig. 1. (See Fig. 1).

Based on previous experimental results (5, 6, 29), 9.3 µg piperlonguminine/dihydropriperlonguminine components were dissolved in dimethyl sulphoxide (DMSO) to the following concentrations: 3.13 µg/ml, 6.25 µg/ml and 12.50 µg/ml. Finally, one ml of DMSO solution was added to 1 ml of culture medium. Hence, the final concentrations of piperlonguminine/dihydropriperlonguminine components in the culture medium were 3.13 µg/ml, 6.25 µg/ml, and 12.50 µg/ml.

### Cell Line and Cell Culture

Human neuroblastoma cells (SK-N-SH) were
obtained from the Cell Institute of the Chinese Science Academy (Shanghai, PRC). The cells were cultured in Eagle’s Minimum Essential Medium (EMEM), containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Invitrogen Biotechnology Co., Carlsbad, CA, USA). In all experiments, SK-N-SH cells were incubated at 37°C in a humid 5% CO2/95% air incubator.

SK-N-SH cells were divided into five groups: group 1 (control group) was treated with culture medium; group 2 was treated with 0.1% DMSO in culture medium; group 3 was treated with 3.13 µg/ml piperlonguminine/dihydropiperlonguminine in culture medium; group 4 was treated with 6.25 µg/ml piperlonguminine/dihydropiperlonguminine in culture medium; and group 5 was treated with 12.50 µg/ml piperlonguminine/dihydropiperlonguminine in culture medium.

Cell Viability Assay

Cell viability was assayed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) method and the lactate dehydrogenase (LDH) release method.

SK-N-SH cells were seeded at 5 x 10^5/ml in 96 well plates. When cells covered 80% of each well, piperlonguminine/dihydropiperlonguminine was added at different concentrations. After incubation for 22 hours, MTT (Promega, Madison, WI, USA) stock solution (5 mg/ml) was added to each well at 10% of the original culture volume. After a further 4 hours of incubation, the medium was removed and the reduced dye was dissolved from the adherent cells with DMSO. The optical density was measured at 570 nm with a Multiscan MK3 microtiter plate reader (Labsystem Co., Finland).

LDH release was measured using the CytoTox-ONE Homogeneous Membrane Integrity assay according to the manufacturer’s instructions (Promega Corp. Madison, WI, USA). In brief, cells were treated with piperlonguminine/dihydropiperlonguminine for 22 h in 96 well plates, after which the culture medium was removed from the cells and equilibrated to 22°C. Next, an equal volume of Cyto-Tox-ONE reagent was added for 10 min. Fluorescence (excitation, 560 nm; emission, 590 nm) was measured using a fluorescent plate reader (Infinite M200, Tcanc, Austria).

Fig. 1. Chemical structures of piperlonguminine and dihydropiperlonguminine. A: piperlonguminine, B: dihydropiperlonguminine.

APP, BACE-1, Notch1, Aβ40 and Aβ42 Detected by Western Blot

SK-N-SH cells were seeded in 50 ml culture flasks and treated with piperlonguminine/dihydropiperlonguminine at different concentrations. After 22h incubation, the cells were collected and lysed in 100 µl of cell lysis buffer solution (50 mM Tris-HCl, pH 7.4, NaCl 0.88%, NaN3 0.02%, SDS 0.1%, NP-40 1%). Protein concentrations were determined by the BCA-100 Protein Quantitative Analysis Kit (Sangon Biological Engineering Technology & Services Co., Shanghai, PRC). Total cell lysates (40 µg of protein) were separated by SDS-PAGE (8%) and transferred to PVDF membranes (Millipore Co. Bedford, MA, USA). The membranes were blocked for 1 h at 24°C with 5% fat-free dry milk in TBST (25 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20). The membranes were incubated with primary antibody at 37°C for 1 h, and then incubated with secondary antibody for 45 min at 24°C. Immunodetection was performed with appropriate antibody using an enhanced chemiluminescence system (Pierce Biotechnology, Inc., Rockford, IL, USA). The membranes were exposed to X-ray film for 20 min and the fluorography was recorded. The film was scanned and analyzed with an image analyzer (Alpha Innotech, San Leandro, CA, USA). The level of β-actin was used as a control for equal loading of protein.

Aβ42 and Aβ40 Levels Detected by ELISA

The concentration of Aβ42 and Aβ40 in the cell culture medium was detected by ELISA. SK-N-SH cells were seeded in 50 ml culture flasks and treated with piperlonguminine/dihydropiperlonguminine at different concentrations for 22 h. The culture medium was collected in 15 ml conical tubes and centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was collected and stored at -70°C. The detection of Aβ42 and Aβ40 by ELISA was performed according to the manufacturers’ instructions. The optical density was measured at 450 nm by a Multiscan MK3 plate reader (Labsystem Co., Helsinki Finland).
Inhibitory Effect of Piperlonguminine on Abeta

Immunocytochemistry Staining of Aβ

SK-N-SH cells were grown on 13 mm diameter cover-slips and then treated with piperlonguminine/dihydropiperlonguminine components at different concentrations for 22 h. Next, the cells were fixed in 4% paraformaldehyde (PFA), permeabilized in 0.2% Triton X-100 and unspecific binding sites were blocked in 3% H2O2 for 15 min, followed by 5% bovine serum albumin for 30 min. Subsequently, cells were incubated with Aβ42 antibody at 4°C overnight. Then, the secondary antibody was used for 30 min at 24°C. After washed in 0.1 M PBS, DAB (3,3'-diaminobenzidine, Sigma-Aldrich) staining was performed. Finally, the cells were stained with hematoxyllin solution. The integral optical density (IOD) of positive staining (brown color) was recorded and analyzed.

Detections of β-Secretase Activity and γ-Secretase Activity

SK-N-SH cells were seeded in 50 ml culture flasks and treated with piperlonguminine/dihydropiperlonguminine components at different concentrations. After 22 h incubation, the activity of β-secretase and γ-secretase was detected according to the protocols of β-secretase activity kit and γ-secretase activity kit (R&D systems, Inc., Minneapolis, MN, USA). Fluorescence (excitation, 345 nm; emission, 500 nm) was measured using a fluorescent plate reader (Infinite M200, Tecan, Austria).

Statistical Analysis

All data are expressed as means ± standard deviations (SD). The results of the MTT assay, enzyme activity assay and LDH release assay were analyzed as percentage relative to the control. Western blot and ELISA values are presented as means ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by LSD post hoc tests. A value of P < 0.05 was considered to be statistically significant.

Results

Influence of Piperlonguminine/Dihydropiperlonguminine on the Cell Viability

The results from MTT assays showed that different concentrations of piperlonguminine/dihydropiperlonguminine (3.13 µg/ml, 6.25 µg/ml and 12.50 µg/ml) as well as 0.1% DMSO did not significantly affect the viability and proliferation of SK-N-SH cells as compared with those of the control group after 22 h treatment (P > 0.05). Similarly, there was no significant difference in LDH release between the treated and untreated SK-N-SH cells (P > 0.05). These results together indicated that piperlonguminine/dihydropiperlonguminine were not toxic to SK-N-SH cells at the tested concentrations (Fig. 2).

Inhibition on the Levels of APP, Aβ42 and Aβ40 by Piperlonguminine/Dihydropiperlonguminine

APP, Aβ40, Aβ42 and β-actin was detected by Western Blot as band of 130 kD, 4 kD, 4.3 kD and 42 kD, respectively. The levels of APP, Aβ40 and Aβ42 significantly decreased in piperlonguminine/dihydropiperlonguminine treated groups, as compared with those of the control group (P < 0.05). However,
the levels of APP, Aβ40 and Aβ42 were not significantly influenced in the DMSO mock treatment group, as compared with those of the control group (P > 0.05, Fig. 3).

By ELISA, the concentrations of Aβ42 and Aβ40 of SK-N-SH cells were detected to be significantly decreased in all three piperlonguminine/dihydropiperlonguminine treated groups, as compared with the control group (P < 0.05). However, treatment of 0.1% DMSO alone did not show any differences, as compared to the control group (Fig. 4).

On immunocytochemistry, SK-N-SH cells of all the groups showed positive staining for Aβ42. However, IOD analysis revealed that the levels of Aβ42 decreased in piperlonguminine/dihydropiperlonguminine treated cells, as compared with those of the control group (P < 0.05), but not between the DMSO-mock treated and the control groups (Fig. 5).

**Influence of Piperlonguminine/Dihydropiperlonguminine on the Level of BACE and Notch1**

On Western blot, BACE-1 protein was detected as the size of 70 kD, and Notch1 protein was detected as 100 kD. The expression of BACE-1 and Notch1 was not significantly different among the three piperlonguminine/dihydropiperlonguminine treated groups, the control group, and the DMSO-mock treated group (P > 0.05, Fig. 3).

**Influence of Piperlonguminine/Dihydropiperlonguminine on the Activities of β-Secretase and γ-Secretase**

Compared with the control group, the activities of β-secretase and γ-secretase in SK-N-SH cells was not significantly affected by piperlonguminine/dihydropiperlonguminine at three different concentrations or by DMSO alone (P > 0.05, Fig. 6).
These results indicate that piperlonguminine/dihydropiperlonguminine did not significantly affect the activities of β-secretase and γ-secretase, nor the expression of BACE-1 and Notch1 in SK-N-SH cells.

**Discussion**

To test the effects of piperlonguminine/dihydropiperlonguminine components on the production of Aβ in SK-N-SH cells, piperlonguminine/dihydropiperlonguminine was used at 3.13 µg/ml to 12.50 µg/ml. These concentrations were chosen based on our former experiments and others research on Futokadsura stem (5, 6, 29). Western Blot showed that the productions of Aβ_{42} and Aβ_{40} significantly decreased in SK-N-SH cells by treatment of piperlonguminine/dihydropiperlonguminine. Similarly, the decrease in the amount of proteins secreted in the culture medium was also detected by ELISA, which was the most sensitive assay to detect extracellular level of Aβ_{42} and Aβ_{40}. These results indicate that piperlonguminine/dihydropiperlonguminine can reduce the levels of Aβ both intracellularly and extracellularly, and these effects were not related to the solvent (0.1% DMSO) used in this study. Although the components did not affect the enzymatic activity of either β-secretase or γ-secretase, nor the expression of BACE-1 and Notch1, a decreased APP expression may cause the observed decrease of Aβ in SK-N-SH cells. Consistent with the inhibitory effect of piperlonguminine/dihydropiperlonguminine on the APP gene, the decreased APP mRNA expression is likely the key mechanism to decrease the APP protein level and ultimately the production of Aβ.

The expression of the APP gene is mainly regulated via its promoter, especially in the proximal promoter region. This region is also considered to be a drug target for the treatment of AD (13). Whether piperlonguminine and dihydropiperlonguminine affect the DNA binding activity of the APP promoter and therefore regulate the expression of APP gene still remains to be studied.

Aβ exerts several functions both in vitro and in vivo, including activation of the inflammatory response, the release of neurotoxic cytokines (11), and the generation of free radicals. A combination of such effects could possibly result in many of the abnormalities in the AD brain, such as mitochondrial oxidative damage, Ca^{2+} dyshomeostasis, tau hyperphosphorylation, synaptic dysfunction and inflammatory changes (2, 8, 22). Therefore, the inhibition of Aβ production may be a preventive measure for neuronal damage.

Chronic inflammation has been linked to AD pathogenesis. For example, the expression of the inflammatory cytokine IL-1 has been shown to activate the promoter of APP, and ultimately increase the aggregation of Aβ (4, 23). Conversely, certain non-steroidal anti-inflammatory drugs could lower the level of Aβ_{42} specifically (26). Futokadsura stem is an anti-inflammatory drug in traditional medicine, but whether its extracts- piperlonguminine/dihydropiperlonguminine- also act as anti-inflammatory factors needs further analysis.

To date, four acetylcholinesterase inhibitors and one NMDA receptor antagonist have been applied to treat AD. Other strategies that may prevent AD including anti-amyloid therapies, neuroprotective approaches, anti-oxidative drugs, anti-inflammatory
agents, hormone replacement therapies, metal chelators, anti-inflammatory drugs, and cholesterol lowering drugs, are still in experimental research phases (2, 8, 15, 21, 22, 30). However, new drugs with enhanced efficacy aiming at multiple targets to treat AD are still highly desirable to delay or prevent dementia (20).

In conclusion, our present data showed that piperlonguminine/dihydropiperlonguminine significantly inhibit the expression of APP and the production of Aβ in SK-N-SH cells. This is the first time that the specific components from a Chinese traditional drug
is shown to decrease the production of Aβ. We hope our findings will contribute to the understanding of Alzheimer’s disease pathogenesis, and eventually to a novel treatment of the disease.

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References


