Depicting the Effects of Astragaloside IV on AD-Like Phenotypes: A Systematic and Experimental Investigation

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1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by cognitive decline and behavioral impairment. The incidence of AD is increasing as the world population ages. According to the World Alzheimer Report 2018 [1], there are more than 50 million people suffering from AD worldwide and it is predicted that by 2050, the number of AD patients will increase to 152 million. Currently, the pathogenesis and etiology of AD have not been fully elucidated, and there is no effective treatment for AD [2]. Remarkable efforts are made in developing strategies to resist mechanisms that lead to neuronal damage, synaptic deficits, neuroinflammation, and cognitive impairment [3–5]. Especially, amyloid-β (1-42) oligomers (AβO) accumulating in AD brains are linked to synaptic failure, neuroinflammation, and memory deficit [2, 6, 7].

Astragaloside IV (AS-IV), one of the major effective components purified from Astragalus membranaceus, has been documented in the treatment of diabetes and diabetic nephropathy [8, 9]. AS-IV has been reported to play a variety of beneficial roles in the prevention and treatment of neurodegenerative diseases with cognitive impairment [10]. Especially, AS-IV, as a selective natural PPARγ agonist,
inhibited BACE1 activity by increasing PPARγ expression and subsequently reduced Aβ levels in APP/PST1 mice [11]. In addition, other studies pointed out that AS-IV could inhibit Aβ1-42−induced mitochondrial permeability transition pore opening, oxidative stress, and apoptosis [12, 13]. PPARγ activation regulates the response of microglia to amyloid deposition, thereby increasing phagocytosis of Aβ and reducing cytokine release [14, 15]. In addition, PPARγ agonists are able to improve the memory deficits in AD models [16, 17], which are further confirmed in clinical trials [18, 19]. In a previous study, we reported that AS-IV prevented AβO-induced hippocampal neuronal apoptosis, probably by promoting the PPARγ/BDNF signaling pathway [20]. However, the findings were limited in the in vitro experiments, and systemic mechanisms have not been clearly disclosed.

In this study, we adopted a systematic study of the multi-scale mechanism to investigate the treatment effect of AS-IV for AD, which combined the drug prediction, network pharmacology, functional bioinformatics analyses, and molecular docking. Subsequently, experiments were carried out to validate the potential mechanisms from the target of PPARγ. This study would provide important implications for the treatment of AD.

2. Materials and Methods

2.1. Target Prediction. To obtain the molecular targets of AS-IV, a computer developed model SysDT based on random forest (RF) and support vector machine (SVM) algorithms [21], which integrates large-scale information on genomics, chemistry, and pharmacology was proposed to predict the potential targets with RF score ≥ 0.8 and SVM ≥ 0.7 as threshold. In addition, we also combined pharmacophore model [22] and structural similarity prediction methods to predict the targets of AS-IV [23].

2.2. Network Construction. To visualize and analyze the relationship between the targets of AS-IV and their related biological functions, we screened the relevant function corresponding to the targets, introduced them into Cytoscape, and constructed the network. In this section, three networks including compound-target (C-T), compound-target-function (C-T-F), and protein-protein interaction (PPI) [24] were structured to unclose the multitarget and multifunction therapeutic effect of AS-IV in combating AD (Figure 1).

2.3. Gene Ontology (GO) Enrichment Analysis. Presently, to further investigate the vital biological process connected with the AS-IV-related targets, we mapped these targets to DAVID 1 for analyzing targets’ biological meaning. The GO terms of biological process were utilized to symbolize generic function. Finally, those GO terms with BP ≤ 0.05 and FDR ≤ 0.05 were selected in subsequent research.

2.4. Molecular Docking. To validate the C-T network, AS-IV was docked to its predicted targets (PPARγ, caspase-1, GSK3β, PSEN1, and TRPV1) by the AutoDock software version 4.1 package with default settings based on a powerful genetic algorithm method [25]. The X-ray crystal structures of targets (5GTN, 5IRX, 6IYC, 6PZP, and 6GN1) were taken from the RCSB Protein Data Bank. Each protein was prepared using methods such as adding polar hydrogens, partial charges, and defining the rotatable bonds. Finally, the results were analyzed in the AutoDock Tools.

2.5. Drugs and Reagents. Astragaloside IV (purity: HPLC > 98%), GW9662, and Aβ1-42 were purchased from Sigma-Aldrich. ELISA kits for Aβ1-42, IL-1β, IL-6, and TNF-α were obtained from Shanghai Jianglai Biotechnology. Antibodies against microtubule-associated protein tau (tau), p-tau, PPARγ, postsynaptic density 95 (PSD95), synaptophysin (SYN), growth-associated protein 43 (GAP43), glial fibrillary acidic protein (GFAP), NOD-like receptor protein 3 (NLRP3), cleaved IL-1β, cleaved caspase-1, and GAPDH were obtained from Cell Signaling Technology. The antibody against activity-regulated cytoskeleton-associated protein (ARC) was obtained from Synaptic System. Antibodies against BDNF and microtubule-associated protein 2 (MAP-2) were obtained from Novus Biologicals. Alexa 488 or 594-labeled fluorescent secondary antibodies for immunofluorescence and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Thermo Fisher Scientific. Prestained Protein Ladder was obtained from Thermo Fermentas. SuperSignal chemiluminescence reagents were obtained from Pierce.

2.6. Animals and Treatments. Male C57BL/6 mice (5-6 weeks old, 20-25 g) were obtained from the Beijing Weishang Lituo Technology Co., Ltd (SCXK (Beijing) 2016-0009). The mice were housed in groups of six per cage with controlled room temperature and humidity, under a 12 h light/dark cycle, with free access to food and water. The mice were adapted for one week before administration. All protocols were approved by the Animal Ethics Committee of Anhui University of Chinese Medicine (approval No. AHUCM-mouse-2019015), and the procedures involving animal research were in compliance with the Animal Ethics Procedures and Guidelines of the People’s Republic of China.

The mice were randomly divided into the following groups (N = 8 per group) (Figure 1): a sham group, an AβO group, AβO plus AS-IV (10, 20, and 40 mg/kg/day, i.g.) groups, an AβO plus donepezil (5 mg/kg/day, i.g.) group, and an AβO plus AS-IV (20 mg/kg/day, i.g.) with GW9662 (1 mg/kg/day, i.p.) group. Drugs were administered once per day for one week followed by intrahippocampal infusion of AβO and continuously received AS-IV once per day for another four weeks. The dose of AS-IV and GW9662 was selected and modified based upon a previous study [11].

2.7. Preparation and Infusion of AβO. AβO were prepared from synthetic Aβ1-42 and incubated at 37°C for 1 week in a stock solution of 10 μg/μL, then routinely characterized by size-exclusion chromatography, as previously described [26, 27], and stored at -80°C until use after subpackaging. AβO were perfused at a final concentration of 2.5 μg/μL in aCSF.
For intrahippocampal infusion of AβO, mice were anesthetized with 5% isoflurane using a vaporizer system (RWD life Science Co., Ltd, Shenzhen, China) and maintained at 1% during the injection procedure, as previously described [26, 28]. AβO (5 μg per site) were bilaterally delivered into the hippocampal CA1 region (stereotaxical coordinates relative to bregma: 2.3 mm anteroposterior, ±1.8 mm mediolateral, and 2.0 mm dorsoventral). Injections were performed in a volume of 2 μL infused over 5 min, and the needle was left in place for 1 min to prevent backflow. Then, the mice were treated with penicillin to prevent infection. After the operation, the mice were kept under standard conditions with eating and drinking freely. Mice that showed signs of misplaced injections or any sign of hemorrhage were excluded from further analysis. Seven days before the AβO infusions, AS-IV (10, 20, and 40 mg/kg, once/day) was administrated intragastrically in mice. Behavioral and pathological studies were performed 4 weeks postinjections of AβO.

2.8. Fear Conditioning. FC was evaluated as previously described [29]. On adaption day, mice were allowed to freely explore the conditioning chamber (UgoBasile, Gemonio, Italy) with a camera that was connected to the ANY-Maze™ software (Stoelting, NJ, USA, RRID:SCR_014289) for 5 min. On conditioning day, mice were placed into the same test chamber, and then, an 80 dB audi tone (conditioned stimulus: CS) was presented for 30 s with a cotermi nating 1.0 mA, 2 s long foot shock (unconditioned stimulus: US) three times at a 73 s interval. Then, mice were
removed from the cage. The next day (contextual test), mice were put back into the conditioning chamber for 5 min, but without any auditory or foot shock. On day 4 (cued test), the cover of the back and side chamber walls was removed. The mice were returned to the chamber followed by three CS (without a foot shock) that were presented for 30 s each. The freezing time was recorded for each test using the software.

2.9. Preparation of Hippocampal Tissue. Twenty-four hours after behavioral tests, some mice were anesthetized with 5% isoflurane and decapitated, and the hippocampi were then rapidly dissected on ice and snap-frozen in liquid nitrogen before storing at -80°C for biochemical tests. Others received transcardial perfusion with 4% paraformaldehyde (PFA), and then, the hippocampi were rapidly dissected and postfixed with 4% PFA overnight at 4°C followed by immersions in a solution containing 30% sucrose at 4°C for graded dehydration. Parts of the hippocampi were then cut into serial coronal frozen slices (20 μm) for immunofluorescence assay, and other hippocampus samples were sliced into 4 μm thick coronal slices for histopathological analysis.

2.10. Hematoxylin and Eosin (HE) Staining. After fixed in 4% paraformaldehyde for 24 h at room temperature, the hippocampal tissues were embedded in paraffin and coronally cut into 4 μm thick slices (three slices per mouse). The tissues were dewaxed and successively rehydrated with alcohol (70%, 85%, 95%, and 100%), and then, the slices were stained with hematoxylin solution for 3 min followed by eosin solution for 2 min at room temperature. The slices were finally mounted by following dehydration with gradient alcohol and hyaline with xylenes and sealed with neutral gum. Representative photographs were captured by a light microscope with the DP70 software.

2.11. Enzyme-Linked Immunosorbent Assay. Hippocampal tissues were collected and homogenized with ice-colded saline, supplemented with protease and phosphatase inhibitor cocktails. The supernatants were collected for further analysis. The levels of endogenous Aβ 1-42, IL-1β, IL-6, and TNF-α were determined using ELISA kits according to the manufacturer’s instructions. The absorbance was recorded at 450 nm using a microplate reader (SpectraMax M2/M2e; Molecular Devices, Sunnyvale, CA, USA), and the concentrations of Aβ 1-42, IL-1β, IL-6, and TNF-α were calculated from standard curves. Results were expressed as picograms per milliliter. Data were generated from 6-8 mice per group.

2.12. Immunofluorescence. Mice were sacrificed, and the hippocampi were snap-frozen in optimal cutting temperature (OCT) compound (Sakura Finetechical, Japan). For immunofluorescence staining, the OCT-embedded hippocampi were cut into serial coronal 20 μm thick slices and mounted on adhesive microscope slides. The slices were fixed with ice-cold acetone for 10 min and then blocked in 10% goat serum (containing 0.04% Triton X-100) for 90 min at room temperature. Subsequently, the slices were incubated with primary antibodies to MAP-2 (1:200), PSD95 (1:200), SYN (1:400), GAP43 (1:200), and GFAP (1:200) overnight at 4°C followed by incubation with Alexa-conjugated secondary antibodies (Thermo Fisher Scientific) for 2 h at room temperature. After counterstained with DAPI solution in the dark, the fluorescent images of slices were acquired using a confocal scanning microscope (FV1000, Olympus, Japan). At least six representative images were taken from each mouse for analysis by the Image J software (NIH, USA, RRID:SCR_003070).

2.13. Immunohistochemistry. Hippocampal slices were deparaffinized and rehydrated as described above. After antigen retrieval, slices were incubated with 3% H2O2 for 15 min and blocked in goat serum (containing 0.1% Triton X-100) for 30 min followed by incubation overnight at 4°C with primary antibodies to PPARγ (1:200) and BDNF (1:200). Then, the slices were washed three times with PBS and incubated with the horseradish peroxidase (HRP) conjugated goat anti-rabbit or anti-mouse IgG (1:100) secondary antibody for 2 h at room temperature followed by incubation with 50 μL 3,3′-diaminobenzidine (DAB) substrate (DAKO, Denmark) at room temperature for 10 min. The number of immunoreactive cells in the hippocampus was assessed using light microscopy (DP70; Olympus, Japan). At least three different fields (200 × 200 μm) per slice were randomly selected for visualization. The mean optical density in the hippocampus region was calculated and used to determine PPARγ and BDNF expression levels.

2.14. Golgi-Cox Staining. Golgi-Cox staining was performed to assess changes in dendrites and dendritic spines within hippocampal neurons using the FD Rapid GolgiStain™ Kit (FD NeuroTechnologies, USA) according to the manufacturer’s instructions. Briefly, mice were anesthetized with 5% isoflurane and decapitated, and the brains were rapidly removed and immersed in the impregnation solution (A : B = 1 : 1, total 2 mL/mouse) at room temperature in the dark and then replaced with new impregnation solution after 2 days. Two weeks later, brains were transferred into solution C and stored at 4°C for three days and then rinsed 3 times with PBST (containing 0.3% Triton X-100). Brains were then cut serially into 100 μm coronal slices on a vibration microtome, and each slice was transferred to a gelatin-coated slide with solution C and then dried at room temperature at dark for up to 3 days. Then, the slices were placed in a mixture consisting of solution D, solution E, and distilled water (1 : 1 : 2) for 15 min followed by a dehydration series consisting of 50%, 70%, 85%, 95%, and 100% ethanol, for 3 applications at 5 min each. The slices were then transparentized with xylenes and sealed with neutral gum for light microscopic observation. At least 3-5 dendritic segments of apical dendrites per neuron were randomly selected in each slice, and 5 pyramidal neurons were analyzed per mouse. For each group, the number of spines per dendritic segment of at least 3 mice was analyzed with using the Image J software (NIH, USA, RRID:SCR_003070). Results are expressed as the mean number of spines per 10 μm.

2.15. Transmission Electron Microscopy. The hippocampi were rapidly dissected and placed in 2.5% glutaraldehyde
Figure 2: Continued.
at 4°C for 4 h followed by fixation with 1% osmium tetroxide for 1.5 h. After a series of gradient ethanol dehydrations, the tissues were immersed in propylene oxide for 30 min and then infiltrated with a mixture of propylene oxide and epoxy resin overnight. Then, the tissues were embedded in epoxy resin and placed in oven at 60°C for 48 h and then cut into serial ultrathin slices (70 nm thickness) and stained with 4% uranyl acetate for 20 min followed by 0.5% lead citrate for 5 min. The synaptic ultrastructures were observed under TEM (HT7700; Hitachi, Tokyo, Japan). In this study, at least 10 micrographs were randomly taken from each mouse and analysis of synaptic density was performed using the Image J software (NIH, USA, RRID:SCR_003070).

2.16. Immunoblotting. Hippocampi were collected and homogenized in RIPA buffer containing protease and phosphatase inhibitor cocktails, and the protein concentration was determined by bicinchoninic acid method (Pierce Biotechnology, Inc., USA). Then, 25 μg total protein from each sample was resolved by 8-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis at room temperature and electroblotted onto nitrocellulose membrane (GE Healthcare, USA) at 4°C. After incubation with secondary anti-mouse or anti-rabbit IgGs (1:10000 in TBST) at room temperature for 90 min, membranes were washed in TBST buffer, developed with SuperSignal chemiluminescence substrate (Thermo Fisher Scientific, MA) and imaged with a chemiluminescence detector (FluorChem FC3; ProteinSimple, USA). The protein expression was quantified with the Quantity One software (Bio-Rad, Hercules, CA, USA, RRID:SCR_014280), and the densitometric plots of the results were normalized to the intensity of the GAPDH.

2.17. Statistical Analysis. All analyses were performed with the GraphPad Prism 5.0 software (GraphPad Prism, San Diego, CA, USA, RRID: SCR_002798), and data were expressed as mean ± standard deviation (SD). The statistical significance of difference between groups was evaluated using one-way ANOVA followed by Tukey test. P values of <0.05 were considered statistically significant.

3. Results

3.1. C-T Network. In this study, we used a comprehensive method to screen AS-IV targets. Figure 2(a) shows that there are 64 targets with the combining capacity to AS-IV. Among the targets, octagons with different colors represent the nervous system, inflammatory, cell proliferation, apoptosis, pyroptosis, calcium ion, and steroid overlapped targets, respectively. The circles in the middle are the nervous system, inflammatory, cell proliferation, apoptosis, calcium ion, and steroid overlapped targets. Node size is proportional to its degree. (c) The PPI network of AS-IV. The color and size of the node are proportional to the degree, and the color and thickness of the connecting line are proportional to betweenness centrality. (d) Gene Ontology analysis of AS-IV target genes. (e) Distribution of AS-IV target proteins in the underlying pathways involved in AD. (f) Distribution of AS-IV target proteins in chromosome.
Figure 3: Continued.
constructed the C-T-F network. Figure 2(b) depicts the global view of the C-T-F network, in which the diamond, circle, and hexagon nodes represent AS-IV, targets, and the corresponding function of the targets, respectively. Further observation of this network shows that these 64 targets are related to 7 functions, including inflammation, nervous system, cell proliferation, apoptosis, pyroptosis, calcium ion, and steroid.

3.3. PPI Network. Proteins do not exert their functions independently of each other but interact together in the PPI network [30]. It is very helpful to understand the functions of proteins through analyzing the topological characteristics of proteins in PPI networks. Here, we constructed the PPI network of the 64 target proteins obtained from AS-IV and calculated the degree of each node. As shown in Figure 2(c), the degree of ADRA2A, ADRA2B, ADRA2C, CHRM2, S1PR5, S1PR2, DRD3, and HRH3 was the highest (degree = 7), followed by APH1B, PSENEN, PSEN1, PSEN2, and NCSTN (degree = 4), demonstrating that these proteins are hub targets and may be responsible for bridging other proteins in the PPI network.

3.4. GO Enrichment Analysis. Through the GO enrichment analysis (Figures 2(d)–2(f)), the targets were related to following biological processes, including G-protein coupled acetylcholine receptor signaling pathway (count = 2,3,6), protein kinase B activity (count = 1), Notch receptor processing (count = 5), protein processing (count = 7), and inflammatory response (count = 4). These processes were usually related to cell proliferation, gene transcription, differentiation, and development.

3.5. Molecular Docking. Figures 3(a)–3(l) depict the binding interactions of AS-IV with caspase-1, GSK3B, PSEN1, and TRPV1 after docking simulations. The results showed that hydrophobic and H-bond interactions influenced the binding affinity of AS-IV to their target proteins (Figures 3(i)–3(l)). AS-IV was anchored into a hydrophobic pocket in caspase-1, GSK3B, PSEN1, and TRPV1. In detail,
for the binding pocket of caspase-1 with its ligand, there were large hydrophobic interactions formed by residues Trp340, Pro343, and Ala284; with respect to GSK3β, the hydrophobic interactions were formed by residues Val110, Leu188, Ala83, Leu132, Val70, Phe67, and Ile62. Additionally, in PSEN1, it was formed by residues Phe14, Ile408, Ile135, Phe6, Trp404, Leu142, and Ala98. Also, in TRPV1, it was formed by residues Phe543, Phe522, Met547, Val518, Leu515, Ile573, Ala566, Leu553, and Ile569.

AS-IV interacted with many residues in the active sites of caspase-1, and three H-bond networks were formed (Figure 3). AS-IV forms H-bond networks with GSK3β in Lys85, Val135, Lys60, Tyr134, Arg141, and Asn64. AS-IV forms H-bond interactions with PSEN1 in Ala139, while forms with TRPV1 in Asn551, Thr550, Arg557, and Ser512 (Figure 3). AS-IV is well suited to the receptor binding pocket as the binding of AS-IV to amino acids was tight and deep into the cavity. The binding free energy of AS-IV with caspase-1, GSK3β, PSEN1, and TRPV1 was -5.30 Kcal/mol, -4.85 Kcal/mol, -6.41 Kcal/mol, and -6.07 Kcal/mol, respectively. These results indicated that AS-IV showed high binding affinities to its targets.

3.6. Interaction of PPARγ with Caspase-1, GSK3β, PSEN1, and TRPV1. Figures 4(a)–4(c) depict the binding interactions of AS-IV with PPARγ after docking simulations. For the target PPARγ, AS-IV is directed toward the binding site and stabilized by the hydrogen-bonding interactions with Gln343, Cys285, and Ser289. Five critical proteins in the network, including PPARγ, caspase-1, GSK3β, PSEN1, and TRPV1, were selected to further validate the PPI. As shown in Figure 4(d), these five proteins showed a close interaction.

3.7. Effect of AS-IV on AβO-Induced Memory Impairment and Pathological Changes. FC task was further performed by the intensity of freezing to context and auditory cue to assess the effects of AS-IV on fear memory in AβO-infused mice. During the adaptation session, there was no difference in freezing time among experimental groups (data not shown). By exposure to the context and auditory cue, freezing response was both higher in sham mice than AβO-infused mice (Figures 5(a) and 5(b)). The freezing time was lower in AβO-infused mice after administration of AS-IV (10, 20, and 40 mg/kg) or donepezil, a positive control.
Figure 5: Continued.
drug. These results suggested that AS-IV prevented AβO-induced contextual and cued fear memory impairments.

HE staining showed that the pyramidal cells in CA1 region of the hippocampus of sham mice had intact cell body and round nuclei with tight arrangement, and no cell loss was found. However, the pyramidal layer was disintegrated, and neuronal loss was observed in the CA1 region. Additionally, neurons with shrunken or irregular shape of cell bodies and degeneration of nuclei were also found in the hippocampus of AβO-infused mice (Figure 5(c)). It is worth mentioning that AS-IV (10, 20, and 40 mg/kg) administration attenuated the structural damage and loss of neurons to some extent relative to AβO-infused, which indicated a neuroprotective effect of AS-IV.

Next, the level of Aβ1-42 and phosphorylated tau expression was measured in the hippocampus. Results showed that there was no difference in the hippocampal Aβ1-42 level among experimental groups (Figure 5(d)). Compared with sham mice, the phosphorylated tau expression was increased significantly in AβO-infused mice. Compared with AβO-infused mice, AS-IV treatment reduced the hippocampal phosphorylated tau expression (Figure 5(e)).

We also observed MAP-2 expression in the hippocampus of mice by immunofluorescence assay. Results showed that there were a large number of MAP-2+ cells, with regular arrangement of neurons, obvious neurites arranged in bundles in the hippocampus of sham mice. Compared with sham mice, the numbers of MAP-2+ cells were remarkably reduced, the arrangement of dendrites was disordered, and the length of the neurites was significantly shortened in the hippocampus of AβO-infused mice. In contrast, AS-IV (20 mg/kg) administration reversed the inhibitory effects of AβO on the growth of MAP-2+ neurites (Figure 5(f)). Based on these findings, AS-IV administration alleviated AβO-induced neuronal injury and reduced tau phosphorylation in the hippocampus, but had no effect on endogenous Aβ1-42 level in AβO-infused mice.

3.8. AS-IV Suppresses AβO-Induced Synaptic Deficit in the Hippocampus. The effects of AS-IV on synaptic protein expression were investigated through determining the expression of PSD95, SYN, GAP43, and ARC. Results from immunofluorescence assays showed that the synaptic proteins PSD95, SYN, and GAP43 were all significantly reduced in hippocampal regions after AβO infusion when compared with sham mice. In contrast, AS-IV administration increased the immunoreactivity of PSD95, SYN, and GAP43 as compared to AβO-infused mice (Figures 6(a) and 6(b)).

The results from immunoblotting assays also showed that there was a significant decrease in the expression of PSD95, SYN, and GAP43 in response to AβO infusion, while AS-IV administration significantly ameliorated AβO-induced downregulation of these synaptic protein expressions in the hippocampus (Figures 6(c) and 6(d)). By contrast, there was no difference in these groups of mice regarding ARC expression (Figures 6(c) and 6(d)).

We next detected the density of dendritic spines in hippocampal neurons among experimental groups by Golgi-Cox staining assay. Results showed that the density of dendritic spines in hippocampal neurons of AβO-infused mice was significantly lower than that in sham mice, but these AβO infusion-induced changes in dendritic spine densities were significantly ameliorated by AS-IV (20 mg/kg) administration (Figures 6(e) and 6(f)).

We further used transmission electron microscopy to examine the synaptic ultrastructure of hippocampal

**Figure 5:** Effects of AS-IV on AβO-induced fear memory impairment and pathological changes in mice. (a) The freezing time of contextual memory. (b) The freezing time of cued memory. (c) Representative images of HE staining in the hippocampus (200x). Scale bar: 50 μm. (d) The content of Aβ1-42 in the hippocampus measured by ELISA assay. (e) The expression of p-tau protein in the hippocampus measured by western blotting. (f) MAP-2 expression in the hippocampus measured by IF. Scale bar: 200 μm.

**Table 1:**

| Drug          | Aβ1-42 Content (pg/mg protein) | p-tau Expression (%) | MAP-2 Expression (%) |
|---------------|-------------------------------|----------------------|----------------------|
| Sham          | 0.5                           | 10                   | 70                   |
| AβO           | 2.0                           | 15                   | 30                   |
| AS-IV 10 mg/kg| 1.5                           | 8                   | 65                   |
| AS-IV 20 mg/kg| 1.0                           | 5                   | 60                   |
| AS-IV 40 mg/kg| 0.8                           | 2                   | 50                   |
Figure 6: Continued.
neurons. Our data showed that AβO infusion resulted in a significant decrease of numbers of hippocampal synapses as compared to that of sham mice, whereas AS-IV (20 mg/kg) administration significantly ameliorated this synaptic loss (Figures 6(g) and 6(h)). Overall, the results indicate that AS-IV affords protection against AβO-induced synaptic deficits.

3.9. AS-IV Promotes AβO Infusion-Inhibited PPARγ Expression in the Hippocampus. The hippocampus was collected at four time points after AβO infusion (2 h, 1 d, 14 d, and 28 d). The expression of PPARγ significantly decreased at 2 h, 1 d, 14 d, and 28 d after AβO infusion (Figure 7(a)). By contrast, AS-IV attenuated the decrease of PPARγ in AβO-infused mice. A specific PPARγ antagonist, GW9662, was used to suppress PPARγ activation in AβO-infused mice. Interestingly, the effect of AS-IV was blocked by GW9662 in the hippocampus of AβO-infused mice (Figure 7(b)).

3.10. AS-IV Inhibits AβO-Induced BDNF Reduction via Promoting PPARγ Expression in Mouse Hippocampi. To further explore the underlying neuroprotective mechanism of AS-IV on AβO-infused mice, the levels of PPARγ and BDNF in hippocampus were detected by immunohistochemistry. Compared with sham group, PPARγ and BDNF immunoreactivity was decreased in the hippocampus of AβO-infused mice, whereas hippocampal immunoreactivity of PPARγ and BDNF was higher in AS-IV-treated mice compared to AβO-infused mice (Figures 8(a)–8(f)). Additionally, the effect of AS-IV on the expression of BDNF and PPARγ was blocked by GW9662 in the hippocampus of AβO-infused mice (Figures 8(a)–8(f)).

3.11. AS-IV Inhibits AβO-Induced Neuroinflammation via Promoting PPARγ Expression. Our data showed that there were significant differences among the experimental groups with regard to the number of astrogial in DG region of the hippocampus, as detected by immunofluorescence (Figure 9(a)). Infusion of AβO induced a remarkable activation of astrogial responses in the hippocampus of mice, which was prevented by AS-IV (20 mg/kg) administration. Consistently, infusion of AβO also increased GFAP expression as determined with
immunoblotting assay, while AS-IV (20 mg/kg) administration significantly suppressed GFAP expression in AβO-infused mice (Figure 9(b)). Furthermore, we asked whether PPARγ mediated the beneficial effect of AS-IV on anti-inflammatory response in AβO-infused mice. Interestingly, PPARγ inhibition by GW9662 blocked the inhibitory effects of AS-IV on GFAP immunoreactivity and expression in the hippocampus of AβO-infused mice (Figures 9(a) and 9(b)).

We measured the hippocampal IL-1β, IL-6, and TNF-α level in AβO-infused mice by ELISA. Results showed that AβO infusion led to an upregulation of IL-1β, IL-6, and TNF-α level in the hippocampus compared with sham mice, but AS-IV administration suppressed the upregulation of cytokines following AβO infusion. In line with the above findings, this effect of AS-IV was blocked by GW9662 (Figure 9(c)). These results suggest that AS-IV prevented the inflammatory response in the hippocampus via PPARγ.

3.12. AS-IV Inhibits AβO-Induced Pyroptotic Cell Death via Promoting PPARγ Expression. As shown in Figures 10(a)–10(c), the protein expression of NLRP3 and cleaved caspase-1 was significantly elevated in the hippocampus of AβO-infused mice compared with sham mice. In contrast, AS-IV (20 mg/kg) administration suppressed AβO-induced expression of NLRP3, as well as cleaved caspase-1 in the hippocampus of AβO-infused mice.

As shown in Figures 10(a)–10(d), AβO infusion significantly increased the levels of IL-1β in the hippocampus, which was inhibited by AS-IV administration. In order to further confirm the role of PPARγ in AS-IV-mediated suppression of AβO-induced pyroptosis, specific PPARγ antagonist, GW9662, was used to suppress PPARγ activation in AβO-infused mice. Interestingly, the effects of AS-IV against AβO-induced expression of NLRP3 and cleaved caspase-1 were blocked by GW9662. Moreover, the blockade of PPARγ was able to significantly reverse the effect of AS-IV on AβO-induced proinflammatory cytokine IL-1β overexpression (Figures 10(a)–10(d)).

4. Discussion

In this study, we applied systemic pharmacology strategies and in vivo experiments to probe the mechanism of AS-IV in treatment of AD. AS-IV could interact with 64 targets, and those targets had multipharmacological properties relevant to nervous system, inflammation, cell proliferation, apoptosis, pyroptosis, and steroid. Molecular docking suggested that AS-IV could regulate the AD-like phenotypes by binding with caspase-1, GSK3β, PSEN1, and TRPV1. Furthermore, in vivo experiments evidenced that AS-IV promoted the expression of PPARγ and BDNF in hippocampal neurons of mice infused with AβO and prevented synaptic deficits, inflammation, and memory impairments in AD-like mice. Consistent with the bioinformatics data, in vivo data also verified that AS-IV could suppress AβO infusion-induced neuronal pyroptosis. This systematic analysis provides new implications for the therapeutic of AD by AS-IV.

4.1. AS-IV Prevents AD Phenotypes through Multiple Mechanisms. In the present study, we screened 64 related targets of AS-IV and these targets together play important roles in the pathogenesis of AD, possibly through regulating cell proliferation, calcium dysregulation, inflammation, pyroptosis, and apoptosis [20, 31–33]. Specifically, the G-protein coupled acetylcholine receptor signaling pathway.
Figure 8: Continued.
and protein kinase B/GSK3B axis are involved in the processes of AD pathogenesis, resulting in cognitive dysfunction [34–36]. Besides, the decrease of response to hypoxia and dysregulation of vasoconstriction could effectively ameliorate vascular dementia [37, 38]. Furthermore, the neuroinflammation caused by the generation of caspase-1-mediated IL-1β and IL-18 is involved in the development and progression of AD [32]. GSK3B plays an important role in hyperphosphorylation of tau, which is one of the pathological features in AD [35]. PSEN1 mutation is a risk factor for AD [39]. Additionally, TRPV1, a nonselective cation channel, is involved in synaptic plasticity and memory [40]. Our molecular docking results demonstrate that AS-IV could integrate with caspase-1, GSK3B, PSEN1, and TRPV1. The binding affinity of AS-IV is mainly through electrostatic, H-bond, and hydrophobic interaction, suggesting the reliability of the docking model. Therefore, AS-IV may improve cognitive impairment by binding to AD-related gene, such as caspase-1, TRPV1, PSEN1, and GSK3B, reduce cell death, and ultimately inhibit AD-phenotypes.

4.2. AS-IV Reduces Tau Hyperphosphorylation in AD Model. AβO accumulate in the brains of AD patients and induce AD-like cognitive dysfunction [41]. Therefore, AβO-induced AD-like phenotypes may be a promising model to find treatments [41, 42]. In this study, we investigated the impact of AβO in the brains of mice and further confirmed the effect of AS-IV on memory formation in mice infused with AβO and to assess the mechanisms. Our results demonstrated that intrahippocampal infusion of AβO impaired both contextual and cued fear memory, which is consistent with previous study [43]. Conversely, AS-IV prevents AβO-induced contextual and cued fear memory impairment. Considering that hippocampus is an important brain region involved in the formation and expression of fear memory, our findings suggest that AβO infusion damaged the structure and function of hippocampus and subsequently blocked the formation of learning and memory, which can be prevented by AS-IV administration.

Similar to previous studies, our findings showed that AβO infusion induced neuronal loss, as well as increased tau phosphorylation, suggesting that the pathological changes of the hippocampus induced by AβO infusion may be the basis of AD-like behavioral changes [3, 44]. On the contrary, AS-IV inhibited the pathological changes of hippocampal neurons and tau phosphorylation induced by AβO infusion, which may contribute to memory improvement in AD-like mice. It is speculated that Aβ pathology in AD brain is earlier than those of tau, and neurofibrillary tangles develop downstream of toxicity induced by Aβ and eventually lead to neuronal death. Moreover, the mutual promotion between them accelerates the pathogenesis of AD, which is consistent with previous reports [44–46]. Certainly, we also note that AβO infusion has no effect on endogenous Aβ1-42 content in the hippocampus, suggesting it may not cause the increase and accumulation of Aβ and formation of amyloid plaque in the brain. Through bioinformatics prediction, AS-IV could integrate with GSK3B tightly. As GSK3B is practically responsible for the hyperphosphorylation of tau, the tight interaction of AS-IV with GSK3B might contribute to the effects of AS-IV on the reduction of tau hyperphosphorylation.

4.3. AS-IV Prevents AβO-Induced Synaptic Deficit. Consistent with previous studies [47, 48], our findings demonstrated that AβO had neurotoxicity and synaptic toxicity before plaque formation in the brain, causing brain damage and eventually leading to AD-like behaviors. Given the mounting evidences that AβO caused synaptic deficits [3, 49, 50], elucidating the precise molecular pathways has important implications for treating and preventing the disease. Here, we demonstrate that AβO infusion reduced the immunoreactivity and expression levels of PSD95, GAP43, and SYN, which is similar to previous results [51, 52]. It
has been shown that the SYN immune response density in the brain of transgenic mice is negatively correlated with Aβ levels, but has nothing to do with plaque loading, indicating that Aβ has synaptic toxicity when plaques are not formed [6]. We further found that AS-IV increased the immunoreactivity and expression levels of PSD95, GAP43, and SYN in the hippocampus of AD-like mice. PSD95, GAP43, and SYN are important markers of synaptic plasticity, and they are positively correlated with hippocampal learning and memory function [14, 53]. Furthermore, ARC plays a key role in synaptic plasticity and memory consolidation [54, 55]. Surprisingly, we note that there is no significant difference in ARC expression among the experimental mice, which suggested that AβO infusion did not target ARC. Our results of Golgi-Cox and TEM further showed that AS-IV increased the density of dendritic spines and synapse number in hippocampal neurons, which suggested that AS-IV improved synaptic structure damage and alleviated synaptic toxicity in the hippocampus of mice infused with AβO.

In a previous study, we reported that AS-IV promoted PPARγ expression in cultured cells and activated the BDNF-TrkB signaling pathway [20]. Our in vivo findings further showed that PPARγ expression in the hippocampus of mice infused with AβO was significantly decreased along with the reduction of BDNF expression, while AS-IV significantly prevented AβO-induced inhibition of PPARγ and BDNF expression. Considering the important functions of BDNF-TrkB signaling pathway performed in synaptic function [29], those data further supported that AS-IV prevented AβO-induced synaptic deficit.

4.4. AS-IV Prevents AβO-Induced Neuroinflammation and Pyroptosis. Numerous studies have confirmed that neuroinflammation accelerates the pathogenesis of AD [46, 56, 57]. In this study, we found that AβO infusion increased the
immunoreactivity and expression of GFAP and upregulated IL-1β, IL-6, and TNF-α levels in the hippocampus, which were reversed by AS-IV. These results suggested that AS-IV prohibited AβO-induced neuroinflammation in the brain, which was beneficial for cognitive function improvement, which further confirmed the network screening.

PPARγ plays a neuroprotective role by reducing brain inflammation and Aβ production [58, 59]. Our findings showed that AβO infusion inhibited PPARγ expression in mice, implicating that PPARγ participated in inflammation response of AD-like mice. Furthermore, AS-IV blocked AβO-induced inhibition of PPARγ expression. Pyroptosis is an inflammatory form of programmed cell death that has been reported in neurological pathogenesis [60]. Reducing pyroptosis was shown to alleviate cognitive impairment in AD animal models [61] and the progression of Parkinson’s disease [62]. Interestingly, NLRP3 has been reported to initiate neuronal pyroptosis [63, 64]. Indeed, NLRP3 inhibition has been shown to exhibit neuroprotective effects through the suppression of pyroptosis [65] and improve neurological functions in a transgenic mouse model of AD [63]. In this study, we demonstrated that AS-IV could inhibit AβO-induced pyroptotic neuronal death, whereas PPARγ antagonist GW9662 blocked the beneficial effect of AS-IV. In the systematic analyses, we also found that AS-IV had a high binding capacity with caspase-1, which might indicate the potential function of AS-IV in the pyroptosis.

4.5. AS-IV Reduces Tau Hyperphosphorylation, Synaptic Deficit, Neuroinflammation, and Pyroptosis via Regulating PPARγ.

In this study, we disclosed that AβO administration could progressively reduce PPARγ expression in the hippocampus from 2 h to one day and kept the PPARγ level at a relative low level from one day to 28 days. These data suggested that PPARγ would be an initial event after AβO administration. AS-IV could prevent AβO-induced reduction of PPARγ. The effects of AS-IV on brain inflammation, pyroptosis as well as synaptic deficit in AβO-induced AD phenotypes might be PPARγ-dependent. On the one hand, PPARγ antagonist blocked the effects of AS-IV on PPARγ expression, brain inflammation, and pyroptosis as well as BDNF expression. On the other hand, PPI indicated that
PPARγ, caspase-1, GSK3β, PSEN1, and TRPV1 had a close interaction. The reduced expression of PPARγ induced by AβO administration contributes to deregulation of caspase-1, GSK3β, PSEN1, and TRPV1, which might lead to brain inflammation and pyroptosis as well as synaptic deficit.

5. Conclusions

In summary, our present study indicates that AS-IV could suppress tau hyperphosphorylation, synaptic deficits, neuroinflammation, and pyroptosis to prevent AD-like phenotypes, likely through interactions of PPARγ with caspase-1, GSK3β, PSEN1, and TRPV1. This study offers a novel and reliable strategy for studying traditional Chinese medicine monomers.

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