

Neuroprotective effect of the Chinese medicine Tiantai No. 1 and its molecular mechanism in the senescence-accelerated mouse prone 8

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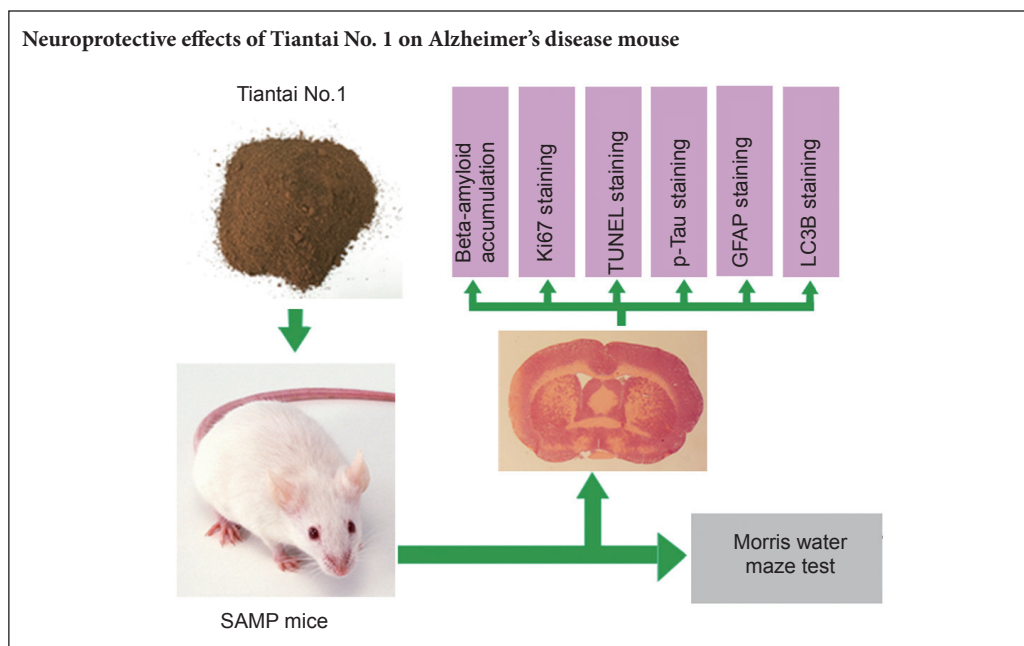
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Graphical Abstract



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Abstract

Tiantai No. 1, a Chinese medicine predominantly composed of powdered *Rhizoma Gastrodiae*, *Radix Ginseng*, and Ginkgo leaf at a ratio of 2:1:2 and dissolved in pure water, is neuroprotective in animal models of various cognitive disorders, but its molecular mechanism remains unclear. We administered Tiantai No. 1 intragastrically to senescence-accelerated mouse prone 8 (SAMP8) mice (a model of Alzheimer's disease) at doses of 50, 100 or 150 mg/kg per day for 8 weeks and evaluated their behavior in the Morris water maze and expression of Alzheimer's disease-related proteins in the brain. Tiantai No. 1 shortened the escape latency in the water maze training trials, and increased swimming time in the target quadrant during the spatial probe test, indicating that Tiantai No. 1 improved learning and memory in SAMP8 mice. Immunohistochemistry revealed that Tiantai No. 1 restored the proliferation potential of Ki67-positive cells in the hippocampus. In addition, mice that had received Tiantai No. 1 had fewer astrocytes, and less accumulation of amyloid-beta and phosphorylated tau. These results suggest that Tiantai No. 1 is neuroprotective in the SAMP8 mouse model of Alzheimer's disease and acts by restoring neuronal number and proliferation potential in the hippocampus, decreasing astrocyte infiltration, and reducing the accumulation of amyloid-beta and phosphorylated tau.

Key Words: nerve regeneration; neuroprotective effects; Alzheimer's disease; Tiantai No. 1; SAMP8; amyloid-beta; autophagy-lysosome pathway; ubiquitin proteasome pathway; tau phosphorylation; neuronal apoptosis; astrocytosis; neural regeneration

Introduction

There are approximately 35.6 million people with dementia worldwide and this number is predicted to increase to more than 115 million by 2050 (Morley et al., 2012). The loss of neurons in the central nervous system in Alzheimer's disease (AD) results in profound pathological changes causing many behavioral and psychological symptoms (Zhang et al., 2013). The pathological hallmarks of AD include extracellular senile plaques, which are mainly composed of 40- to 42-amino-acid amyloid-beta ($A\beta$) peptides, and intracellular neurofibrillary tangles (Gold and El Khoury, 2015; Gouras et al., 2015). These hallmarks occur mostly in the cortex and the hippocampus of the brain (Selkoe and Hardy, 2016). Moreover, $A\beta$ acts by triggering a series of reactions known as the amyloid cascade, which eventually leads to dementia (Hardy and Selkoe, 2002; Huang and Mucke, 2012; Galimberti and Scarpini, 2016). However, the detailed etiology and pathogenesis of AD remain largely unknown.

Autophagic dysfunction in neurodegenerative diseases such as AD is associated with protein misprocessing and over-accumulation (Klionsky and Emr, 2000). Under normal conditions, the autophagic/lysosomal pathway makes efficient use of recycled organelles and substrate proteins (Bernard and Klionsky, 2013), whereas attenuation of autophagy under pathological conditions leads to the accumulation of proteins and lysosomal particles (Platt et al., 2012). These particles contain toxic lysosomal hydrolases, which enhance the formation of $A\beta$ (Komatsu et al., 2006; Jamasbi et al., 2016). Thus, autophagy is strongly associated with $A\beta$ accumulation and the development of AD.

The Chinese medicine Tiantai No. 1 has been used for the prevention and treatment of AD for many years (Wu et al., 2010). Previous pharmacodynamics studies have indicated that Tiantai No. 1 enhances learning and memory in various models of agnosia, improves neuronal ultrastructure and synaptic plasticity in models of AD, and increases the regeneration of cholinergic fibers (Wu et al., 2008, 2010a). Moreover, Tiantai No. 1 induces the differentiation of neuron-like cells from mesenchymal stem cells (Wu et al., 2010b), and has an antagonistic effect on tunicamycin-induced endoplasmic reticulum stress in mesenchymal stem cells. In addition, Tiantai No. 1 protects neurons against $A\beta$ toxicity and regulates the expression of nuclear factor kappa B and cyclic adenosine monophosphate response element binding protein (Li et al., 2015). Few studies have investigated the mechanism underlying the neuroprotective effect of Tiantai No. 1 in AD.

To this end, we explored the neuroprotective effects and possible molecular mechanism of Tiantai No. 1 in the senescence-accelerated mouse prone 8 (SAMP8) model of AD. The results suggest that Tiantai No. 1 acts by restoring the number and proliferation potential of neurons in the hippocampus, decreasing infiltration of astrocytes, and reducing accumulation of $A\beta$ and phosphorylated (p-) tau.

Materials and Methods

Animals

We used male specific-pathogen-free, 9- and 15-week-old

SAMP8 mice, weighing 20–24 g, as an animal model of AD. SAMP8 mice are used in dementia research because of their characteristic learning and memory deficits in old age (Flood and Morley, 1998; Qiu et al., 2010). Age-matched senescence-accelerated-resistant mice (SAMR1) were used as controls. The mice were obtained from the First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, China, and housed at $24 \pm 1^\circ\text{C}$, $50 \pm 5\%$ humidity, and under a 12-hour light/dark cycle, with free access to food and water. All experiments were carried out in accordance with the Chinese guidelines for animal care and use and were approved by the Animal Ethics Committee of the Shenzhen Institute of Integrated Traditional and Western Medicine, China.

Drugs

Tiantai No. 1 is predominantly composed of *Rhizoma Gastrodiae*, *Radix Ginseng*, and Ginkgo leaf. These herbs were purchased individually from Beijing Tongrentang (Group) Co., Ltd., China, and tested and approved by the Shenzhen Municipal Drug Inspection. The three herbs were mixed at a ratio of 2:1:2 (China Pharmacopoeia Committee, 2010). An extract was obtained by water extraction and alcohol precipitation, freeze-dried at -40°C and refrigerated. Pure water was used to dissolve the extracts to the required concentration, and the prepared medicine was stored in a refrigerator until use.

Group assignment

All mice were acclimatized for one week before the experiment. SAMP8 mice were then randomly allocated to six groups at 10 and 16 weeks of age, to receive the following treatments ($n = 6/\text{group}$): 0.5 mL/mouse/d of distilled water, intragastrically (AD model control; SAMP8); 50, 100, or 150 mg/kg/d Tiantai No. 1, intragastrically (SAMP8-L, SAMP8-M, and SAMP8-H, respectively); 1 g/kg/d 4-phenylbutyric acid, orally (SAMP8-PBA); 1 mg/kg/d rapamycin, orally (SAMP8-RAPA). SAMP8 mice that received 4-PBA or RAPA were positive controls (Wu et al., 2008). Age-matched SAMR1 mice served as normally aging reference mice and were given an equivalent amount (0.5 mL/mouse/d) of distilled water intragastrically. In all groups, treatments were given orally every day for 8 weeks (Figure 1A).

Morris water maze test

We performed the test after 8 weeks of treatment. The procedure was similar to that described by Morris (1984), with minor modifications. A circular pool (diameter 100 cm, height 40 cm), half-filled with opaque water at $25\text{--}27^\circ\text{C}$, was placed in the center of the room. All external visual cues were kept constant for the spatial orientation of the mice. A black platform (diameter 6 cm, height 25 cm) was placed in the center of one of the arbitrarily designated left, trained, right, and opposite quadrants and provided the only escape from the water. The task consisted of visible-platform acquisition training, hidden-platform training, and a probe trial. For the first two days, the platform was clearly marked with a flag located 1 cm above the surface of the water in the southwest quadrant of the pool (visible-platform training).

The Morris water maze was performed as described previously (Wang et al., 2013).

Immunofluorescence and immunohistochemistry

Mouse brain tissue was fixed with 4% formaldehyde and cut into 8–10 μm -thick frozen sections. The sections were incubated with rabbit anti-light chain-3B (LC3B), anti-A β , and anti-p-tau (Ser³⁹⁶) polyclonal antibodies (1:150; Jiaka Life Science, Shenzhen, China). Next, the sections were incubated with CY3/fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit antibody (1:200; Beyotime Institute of Biotechnology, Jiangsu, China) at room temperature for 1 hour in the dark, followed by incubation with 4',6-diamidino-2-phenylindole (DAPI) (1:1,000; Biosharp Biotech, Hefei, China) for 5 minutes. They were then washed three times with phosphate-buffered saline (PBS) to remove excess staining solution. For immunohistochemistry, the same primary antibodies were used, with a horseradish peroxidase-conjugated secondary antibody (1:200; Beyotime Institute of Biotechnology). Subsequently, the proteins were visualized with 3,3'-diaminobenzidine (0.2 mL; Beyotime Institute of Biotechnology). The sections were viewed under a laser scanning confocal microscope (FV1000S-SIM/IX81; Olympus, Tokyo, Japan). Eight to 12 sections from each of the six mice per group were examined, and representative images from all analyzed tissue sections were presented. For the Ki67 tissue section staining assay, 8 microscope views per section were randomly selected and the cell positive for Ki67 was counted, and 8–10 sections per sample were analyzed, and representative image was shown. All analyses were performed manually.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

Brain sections were rinsed twice with PBS-Tween 20, for 2 minutes each time. Sections were incubated with 3% H₂O₂ in PBS for 10 minutes to block endogenous peroxidase activity, rinsed in PBS-Tween 20 for 3 \times 2 minutes, and incubated in TdT Reaction Buffer (Roche, Basle, Switzerland) for 10 minutes. Afterwards, sections were incubated with TdT Reaction Mixture (Roche) for 1–2 hours at 37–40°C in a humidified chamber, and rinsed in Stop/Wash Buffer (Roche) for 10 minutes followed by PBS-Tween 20 for 3 \times 2 minutes. They were then incubated with streptavidin-horseradish peroxidase (Roche) in PBS for 20 minutes at room temperature, rinsed in PBS-Tween 20 for 3 \times 2 minutes, incubated with 3,3'-diaminobenzidine (Roche) for 1–2 minutes, rinsed in tap water, counterstained with Gill's hematoxylin for 30 seconds, and rinsed under running tap water for 5 minutes. All sections were dehydrated through 95% ethanol for 5 minutes, 100% ethanol for 3 \times 2 minutes, permeabilized in xylene for 2 \times 5 minutes, and mounted with xylene-based mounting medium.

Statistical analysis

Data are expressed as the mean \pm SEM. Statistical analysis was performed in GraphPad Prism 5.01 software (GraphPad Software, Inc., CA, USA) using one-way analysis of variance followed by Dunnett's *t* test. $P < 0.05$ was considered statistically significant.

Results

Tiantai No. 1 attenuated memory deficit in SAMP8 mice

In the Morris water maze, there was no significant difference in the mean escape latency between 10-week-old SAMR1 and SAMP8 mice (Figure 1B). However, the escape latency was significantly greater in 24-week-old SAMP8 mice than in SAMR1 mice at the same age ($P < 0.01$). SAMP8-PBA and SAMP8-RAPA mice had shorter escape latencies than SAMP8 mice ($P < 0.05$). Importantly, SAMP8 mice that received Tiantai No. 1 also had significantly shorter escape latencies during the visible-platform training trial ($P < 0.05$; Figure 1C). A probe test was carried out to further evaluate the effect of Tiantai No. 1 on the cognitive impairment of SAMP8 mice. This showed that SAMR1 mice and SAMP8-PBA mice searched preferentially in the target quadrant, where the platform had been during the training trials ($n = 6$, $P < 0.01$), whereas untreated SAMP8 mice showed no significant preference for that quadrant. SAMP8 mice that received Tiantai No. 1 also preferentially searched in the target quadrant ($n = 6$, $P < 0.05$; Figure 1D). These results indicate that Tiantai No. 1 attenuated the cognitive impairment observed in the AD mouse models.

Tiantai No. 1 reduced A β accumulation and restored the proliferation of cells in the hippocampus

Amyloid plaques were rarely detected in SAMR1 mice, with significantly more observed in SAMP8 control mice. SAMP8-PBA and SAMP8-RAPA mice showed markedly less amyloid plaque accumulation than SAMP8 controls. A dose-dependent decrease in the number of amyloid plaques was observed in SAMP8 mice that had received Tiantai No. 1 ($P < 0.05$) (Figure 2A, C).

There was a significant correlation between the hippocampal levels of Ki67 and working memory errors. Ki67 protein expression was detected in the hippocampus of 24-week-old aged SAMR1 and the different groups of SAMP8 mice. The hippocampal levels of Ki67 were significantly attenuated in SAMP8 mice, but appeared restored in SAMP8-PBA and SAMP8-RAPA mice ($P < 0.01$). Tiantai No. 1-treated mice showed a dose-dependent increase in Ki67 expression ($P < 0.05$ vs. SAMP8; Figure 2B, D).

Together, these data suggest that Tiantai No. 1 attenuated amyloid accumulation and restored Ki67 expression in the hippocampus in AD mouse models.

Tiantai No. 1 attenuated neuronal death, tau phosphorylation, and astrogliosis in the brain in SAMP8 mice

TUNEL assay signals were lower in SAMP8-PBA and SAMP8-RAPA mice, and in Tiantai No. 1-treated SAMP8 mice, than in SAMP8 controls (Figure 3A). We examined p-tau-immunopositive neurons in the hippocampus of the brain; they were markedly fewer in SAMR1, SAMP8-PBA and SAMP8-RAPA mice than in SAMP8 control mice. In the Tiantai No. 1-treated SAMP8 groups, there were also significantly fewer p-tau-immunopositive cells in the hippocampus than in the SAMP8 control mice, and the reduction in number was dose-dependent (Figure 3B).

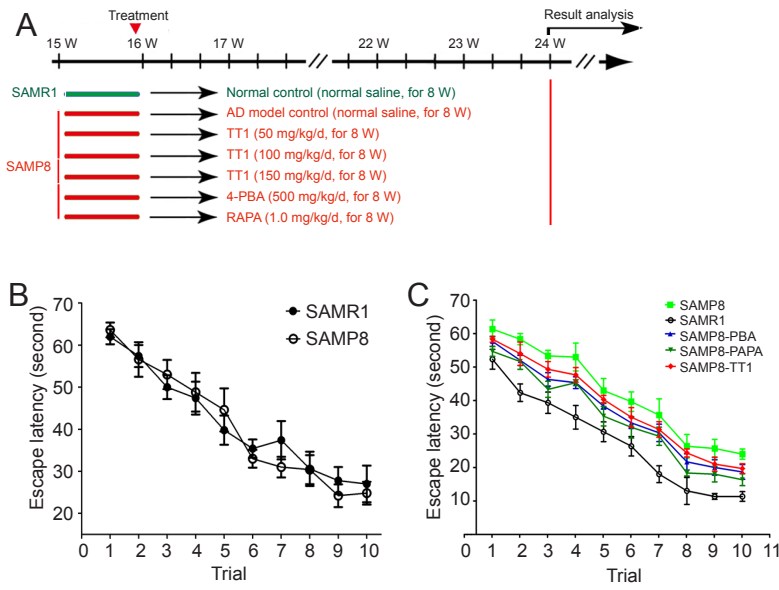


Figure 1 Tiantai No. 1 attenuated memory deficit in SAMP8 mice.

(A) Group assignment and treatment protocol. (B, C) Escape latency (Morris water maze). There was no significant difference in mean escape latency between 10-week-old SAMR1 and SAMP8 mice (B); but in 24-week-old mice, mean escape latency was significantly greater in SAMP8 mice than in SAMR1 mice ($P < 0.01$), and this difference was significantly smaller after treatment with 4-PBA (1 g/kg) and RAPA (1 mg/kg) daily for 8 weeks ($P < 0.05$); Tiantai No. 1 also significantly decreased escape latency in SAMP8 mice during the training trial ($P < 0.05$) (C). (D) Spatial memory of platform location was assessed after reference memory training. In the transfer test, the SAMR1 and SAMP8-PBA mice searched preferentially in the trained quadrant ($P < 0.01$), whereas SAMP8 mice did not. Tiantai No. 1-treated SAMP8 mice also searched preferentially in the trained quadrant ($P < 0.05$). Data are expressed as the mean \pm SEM ($n = 6$; one-way analysis of variance followed by Dunnett's t test). * $P < 0.05$, ** $P < 0.01$, vs. trained quadrant. TT1: Tiantai No. 1; 4-PBA: 4-phenylbutyric acid; RAPA: rapamycin; SAMP8: senescence-accelerated mouse prone 8; SAMR1: senescence-accelerated-resistant mice; W: weeks.

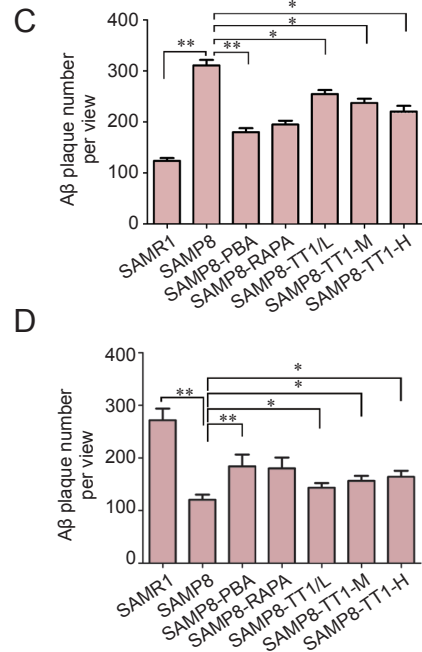
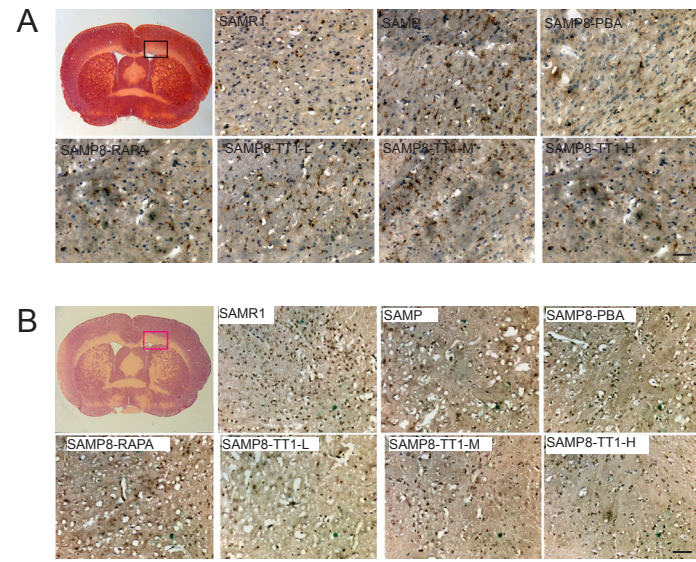
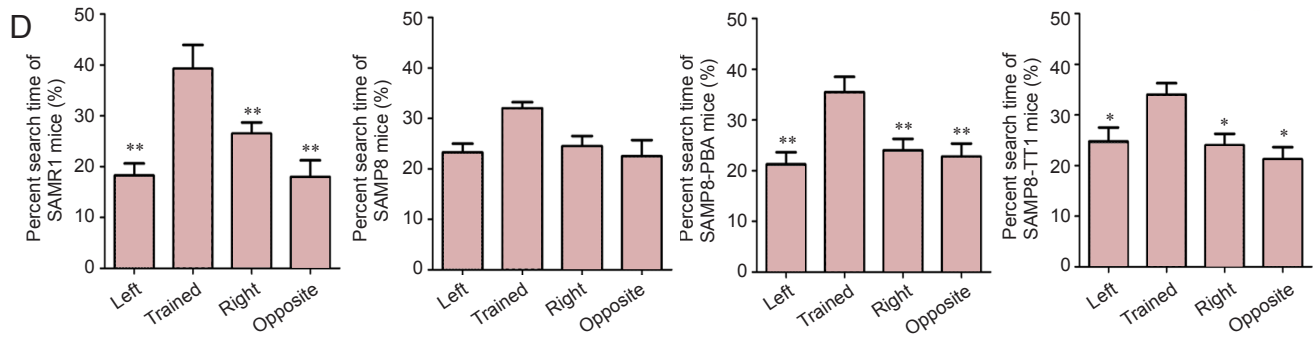


Figure 2 Tiantai No. 1 reduces amyloid-beta accumulation and restores proliferation of cells in the hippocampus (immunohistochemistry). (A) Amyloid plaques were rarely detected in SAMR1 mice; there was a dose-dependent decrease in amyloid plaques in SAMP8 mice treated with Tiantai No. 1, all groups showing fewer plaques than the control SAMP8 mice. Scale bar: 50 μ m. (B) There was an increase in Ki67 expression after administration of Tiantai No. 1. The increase in Ki67 expression was significantly correlated with the dose of Tiantai No. 1. Scale bar: 50 μ m. (C) Quantification of amyloid plaques. (D) Quantification of Ki67 expression. Data are expressed as the mean \pm SEM ($n = 6$; one-way analysis of variance followed by Dunnett's t test). * $P < 0.05$, ** $P < 0.01$. 4-PBA (1 g/kg) and RAPA (1 mg/kg) were administered daily for 8 weeks. TT1: Tiantai No. 1; 4-PBA: 4-phenylbutyric acid; RAPA: rapamycin; L, M, H: low, moderate, and high doses (50, 100 and 150 mg/kg per day), respectively; SAMP8: senescence-accelerated mouse prone 8; SAMR1: senescence-accelerated-resistant mice.

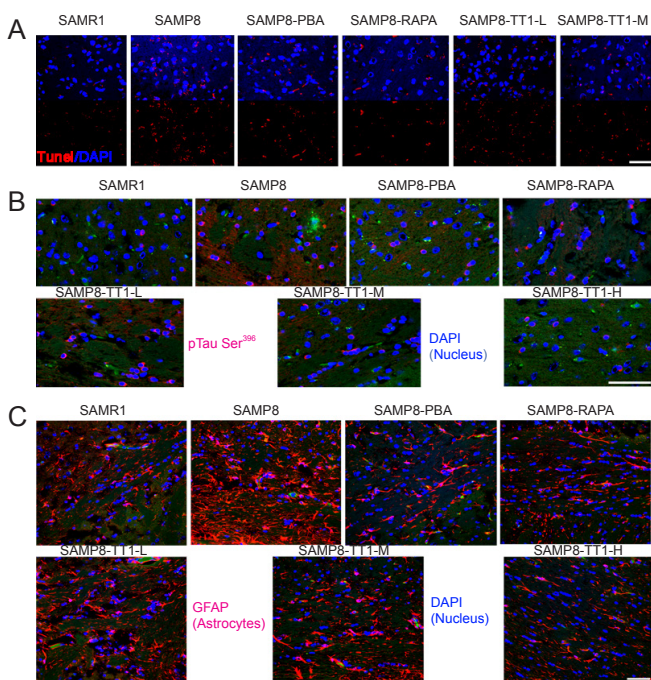


Figure 3 Tiantai No. 1 attenuates cell apoptosis, tau phosphorylation and astrocytosis in the brain of SAMP8 mice (immunofluorescence). (A) Neuronal apoptosis in the hippocampus was visualized using the TUNEL assay. (B) Immunofluorescence staining of p-tau-immunoreactive cells in the hippocampus. (C) Immunofluorescence staining of GFAP-immunoreactive cells in part of the hippocampus. Scale bar: 50 μ m. 4-PBA (1 g/kg) and RAPA (1 mg/kg) were administered daily for 8 weeks. TT1: Tiantai No. 1; L, M, H: low, moderate, and high doses (50, 100 and 150 mg/kg/d), respectively; GFAP: glial fibrillary acidic protein; 4-PBA: 4-phenylbutyric acid; RAPA: rapamycin; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; SAMP8: senescence-accelerated mouse prone 8; SAMR1: senescence-accelerated-resistant mice.

Brain inflammation, microgliosis and astrocytosis are the secondary hallmarks of AD pathology (Belbin et al., 2008; Lee and Landreth, 2010). In the present study, the area of GFAP staining (a marker of astrocytosis) in the hippocampus was significantly smaller in SAMR1, SAMP8-PBA and SAMP8-RAPA mice than in SAMP8 mice. Mice that received Tiantai No. 1 showed a dose-dependent decrease in GFAP-positive cells in the hippocampus (Figure 3C).

Tiantai No. 1 increased autophagy in the hippocampus in SAMP8 mice

To determine whether Tiantai No. 1 enhances the autophagic process in SAMP8 mice, double-staining for LC3B and A β was performed. Confocal microscopy revealed that LC3B and A β were largely co-localized. Further immunostaining analysis showed that Tiantai No. 1, like 4-PBA and RAPA, increased the number of LC3B-positive autophagosomes, and reduced the accumulation of A β (Figure 4). This result suggests that Tiantai No. 1 decreases A β accumulation by enhancing autophagy.

Discussion

Interest in the use of traditional Chinese medicines for the treatment of AD is increasing, because no currently available therapies (cholinesterase inhibitors or N-methyl-D-aspartate receptor antagonists) successfully halt the progression of the

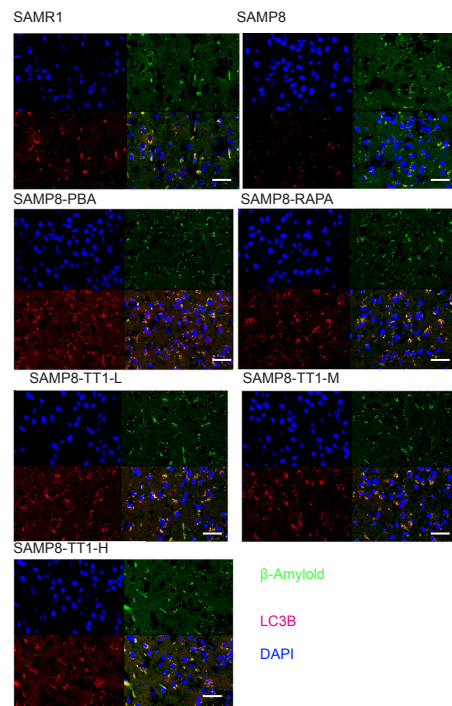


Figure 4 Tiantai No. 1 affects the lysosomal pathway in SAMP8 mice (immunofluorescence).

LC3B and A β were largely co-localized in all groups. Tiantai No. 1 increased the number of LC3B-positive autophagosomes, and reduced A β accumulation. Scale bar: 50 μ m. 4-PBA (1 g/kg) and RAPA (1 mg/kg) were administered daily for 8 weeks. TT1: Tiantai No. 1; L, M, H: low, moderate, and high doses (50, 100 and 150 mg/kg/day), respectively; PBA: 4-phenylbutyric acid; RAPA: rapamycin; LC3B: light chain-3B; A β : amyloid-beta; SAMP8: senescence-accelerated mouse prone 8; SAMR1: senescence-accelerated-resistant mice.

disease (Wu et al., 2011; Luo et al., 2016). Recently, researchers have isolated many active compounds from herbs, which have the potential to alleviate dementia and neurodegenerative syndrome, and have fewer side effects than conventional drugs (Wang et al., 2013, 2016). This makes traditional Chinese medicines promising candidates in the treatment of AD, but there have been few investigations to date of their cellular and molecular mechanisms. Here, we investigated the neuroprotective effects of Tiantai No. 1 and explored its possible mechanisms in SAMP8 mouse models of AD.

The memory deficit observed in SAMP8 mice in the Morris water maze, demonstrated by a longer escape latency in SAMP8 mice than in SAMR1 mice, was attenuated after 8 weeks of treatment with Tiantai No. 1. However, Tiantai No. 1 also decreased the escape latency in SAMP8 mice during the training trial, similarly to that in positive control mice (SAMP8-PBA and SAMP8 RAPA). These results indicate that Tiantai No. 1 attenuated cognitive impairment in AD mice, consistent with previous pharmacodynamics and molecular studies (Wu et al., 2008). This demonstrates that Tiantai No. 1 markedly improves cognitive impairment in SAMP8 mice, and provides further evidence that Tiantai No. 1 is a strong candidate for the treatment of AD.

There were a large number of amyloid plaques in the hippocampus of SAMP8 mice, compared with very few in

SAMR1 mice. Tiantai No. 1 treatment appeared to decrease amyloid plaque accumulation in a dose-dependent manner; this is an interesting and important result, as amyloid plaque formation is one of the most characteristic features of AD, indicating that Tiantai No. 1 could reduce or prevent AD progression. Consistent with this result, LC3B staining also showed that Tiantai No. 1 enhanced the autophagic process. Indeed, this may underlie the effect of Tiantai No. 1 on A β plaques, because amyloid degradation may depend on autophagy (Cavieres et al., 2015); this hypothesis needs further investigation, for example by using PC12 cells to explore the neuroprotection imparted by Tiantai No. 1.

Tiantai No. 1 increased cell proliferation in hippocampal tissue, shown by a dose-dependent increase in Ki76 expression levels in Tiantai No. 1-treated mice. This is also an interesting result, as it indicates another possible neuroprotective mechanism of Tiantai No. 1, in which it promotes nerve cell proliferation. Neuronal degeneration is the major cause of AD symptoms, so it would be of great clinical interest to further evaluate the role of Tiantai No. 1 in the promotion of neuronal proliferation both *in vivo* and *in vitro*.

In vivo and *in vitro* methods must be used to identify the pathways and mechanisms of cell death during AD.

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Author contributions: YHL was responsible for study design, fund-raising for the study and data collection. XSW was responsible for the research work, data collection, statistical analysis and paper writing. YJ and XLC were in charge of data collection and statistical analysis. HBC was in charge of study design. XQJ and YFZ supported the references and collected data. ZZW provided the critical revision of the paper for intellectual content, study conception and administrative support. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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