

***Gastrodia elata* modulates amyloid precursor protein cleavage and cognitive functions in mice**

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Summary

Gastrodia elata (Tianma) is a traditional Chinese medicine often used for the treatment of headache, convulsions, hypertension, and cardiovascular diseases. The vasodilatory actions of Tianma led us to investigate its specific effects on memory and learning as well as on Alzheimer's disease (AD)-related signaling. We conducted a radial arm water maze analysis and the novel object recognition test to assess the cognitive functions of Tianma-treated mice. Our data show that Tianma enhances cognitive functions in mice. Further investigations revealed that Tianma enhances the α -secretase-mediated proteolytic processing of the amyloid precursor protein (App) that precludes the amyloid- β peptide production and supports the non-amyloidogenic processing of App which is favorable in AD treatment. We hypothesize that Tianma promotes cognitive functions and neuronal survival by inhibiting β -site App-cleaving enzyme 1 activity and promoting the neuroprotective α -secretase activity.

Keywords: Alzheimer's disease, β -Amyloid precursor protein, Kambo, Neurodegeneration, traditional Chinese medicine

1. Introduction

As the occurrence of dementia and cardiovascular disease increase with age, there has been a growing interest in developing novel protective agents because biological aging represents also the major risk factor with respect to the development of Alzheimer's disease (AD), vascular dementia (VD), and other cardiovascular diseases (CD). The number of patients suffering from AD, VD, and CD is a significant threat to the aging people all over the world. However, despite advances in technology and understanding of biological systems, drug discovery for these and other diseases is still a lengthy and expensive process. Traditional herbal medicine is especially attractive for disease prevention, health maintenance, and

sicknesses that are non-responsive to current Western medicine and thus has potential benefits that attract worldwide attention and interests. The use of medicinal herbs has a long history in Asia and is commonly used to treat various neurological diseases including stroke, epilepsy and VD (1,2). A total of 365 plants including several orchids are listed in the earliest known Chinese Materia Medica (Shennon bencaojing (~ 100AD) or Divine Husbandman's Classic of the Materia Medica). In Bencao gangmu (Compendium of Materia Medica), the most renowned herbal text in China, three orchids that have been extensively studied and widely used as herbal medicines are *Dendrobium nobile* (Shi Hu/Shifu), *Gastrodia elata* Blume (Tianma, Orchidaceae), and *Bletilla striata*. However, current Western methodologies need to take into consideration the complex mixture of chemicals and how they are to be used in human. The scientific proof and clinical validation of these herbal formulations require a rigorous approach that includes chemical standardization, biological assays, animal models, and clinical trials (3,4).

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In our project, we investigated the effect of the medicinal herb Tianma on physiological and pathogenic processes related to AD by using various cellular approaches and *in vivo* mice models. Specifically, we studied the effect of Tianma on the processing of the amyloid precursor protein (App) by the α -, β -, and γ -secretases (Adam, Bace, and Psen-1/2) and cognitive functions using the radial arm water maze (RAWM) and the novel object recognition tests. Our research approach aims at gaining more insight into the potential therapeutic application of medicinal herbs, such as Tianma, to explore new potential avenues for the treatment of neurocardiovascular diseases such as AD (5,6).

2. Materials and Methods

2.1. Reagents

Unless indicated, all reagents used for biochemical methods were purchased from Sigma-Aldrich (St. Louis, MO, USA). Huperzine-A was purchased from Sigma as well.

2.2. Animal material

Experimental procedures, including the killing of animals, were in accordance with the International Guiding Principles for Animal Research (WHO) and were approved by the local Institutional Animal Care & Use Committee (NTU-IACUC). One-year-old mice were obtained from the laboratory animals centre (National University of Singapore). Mice (C57BL/6J) were randomly assigned to control and Tianma-treated groups (10 each, 5 male, 5 female). According to previous reports and our own recent studies, the average daily dose of Tianma per mouse was 2.5 g/kg body weight (7-10). Feeding was done orally with a blunt needle syringe by dispensing the Tianma solution drop by drop for the period of two months. Control mice were treated with the same volume of the solvent only. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Herb preparation

Gouteng (*Uncaria sinensis* or *Ramulus cum uncis Uncariae*) was obtained from Eu Yan Sang (Singapore). The rhizome of *Gastrodia elata* (Tianma) was collected from Zhaotong City, China and provided by Dr. Jun Zhou (Kunming Institute of Botany, Chinese Academy of Science, Yunnan, China). The species was identified and chemically analyzed as reported previously (11). In this study, whole dried tubers of the Tianma were hammered into smaller pieces and subsequently ground to fine powder. Seven point five grams of Tianma powder was mixed with 100 mL

sterilized Milli-Q water and boiled for 1 h at 100°C. The solution was centrifuged at 5,000× g for 10 min at room temperature. The supernatant was filtered with a whatman filter paper (GE Healthcare, Chalfont St Giles, UK), yielding approximately 85 mL. The Tianma solution was concentrated at 60°C under vacuum and the final volume was reduced to 10 mL for further applications. Gouteng was prepared accordingly. In addition, gastrodin (Kunming Pharmaceutical Corp, State New and High Technology Development Zone, Kunming, Yunnan, P.R. China) was pounded into powder, dissolved in sterile deionized water, ultrasonicated for 2 min and filtered through a 0.45 μ m Acrodisc® membrane (Pall Corporation, Singapore). For all cell-based assays, Tianma and Gouteng were applied at 1 mg/mL while gastrodin was administered at a concentration of 500 μ g/mL (12,13).

2.4. Cell culture

Mouse N2a cells (kindly provided by Prof. Zhiwei Feng, SBS, NTU, Singapore) were propagated at 37°C in humidified 5% CO₂/95% air, in Dulbecco's Modified Eagle's Medium (DMEM, GlutaMax™; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen), non-essential amino acids (Invitrogen), and antibiotic-antimycotic (Invitrogen). Rat TAF-PC12 cells were cultured as described previously (14).

2.5. Cell proliferation assays

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) and the fluorescein isothiocyanate (FITC) 5-bromo-2'-deoxyuridine (BrdU) Flow Kit (BD Biosciences, Franklin Lakes, NJ, USA) were used to analyze cell proliferation as described previously (15). Briefly, the CellTiter-Glo® Luminescent Cell Viability Assay generates a luminescent signal based on the quantity of adenosine triphosphate (ATP) present in viable cells, which is proportional to the number of metabolically active cells. N2a cells were cultured for three passages and thereafter plated at a density of 20,000 cells/well on an opaque 96-well plate in 100 μ L of complete N2a cell culture media. Tianma was added (as indicated in the text) and cells were exposed to it for 72 h. One volume of CellTiter-Glo® reagent was added and luminescence was recorded 20 min later using a plate reader luminometer (Fluoroskan Ascent FL; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Additionally, to quantify proliferating N2a cells, the FITC BrdU Flow Kit was used where 4 × 10⁶ N2a cells were obtained and labelled with 3 mM of BrdU for 72 h. This was performed for the control cells and Tianma-treated cells. BrdU-labeled N2a cells were then stained with the FITC-conjugated anti-BrdU antibody (1:50) according

to the manufacturer's protocol and 10,000 cells were analyzed by flow cytometry (BD FACSCalibur, BD Biosciences) using the FL1 detector for BrdU-positive cells and the FL3 detector for 7-amino-actinomycin D (7-AAD)-positive cells to determine the percentage of proliferating cells (BrdU and 7-AAD-positive) among the total cell population analyzed. Results shown represent quadruplicated measurements, where each measurement consisted of three repetitions.

2.6. Cell lysis, protein extraction, cell culture supernatant collection

For cell lysis, adherent cells were washed in the dish using ice-cold PBS (–/–), collected using a disposable cell scraper (Greiner Bio-One GmbH, Frickenhausen, Germany) and lysed by adding a specific lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 1% (v/v) Nonidet P-40, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 ng/mL of aprotinin and EDTA-containing protease inhibitor (Roche, Mannheim, Germany) as well as phosphatase inhibitor cocktails 1 and 2) followed by incubation on ice for 5-10 min. Lysed cells were centrifuged at 10,000 × g at 4°C for 10 min. The supernatant containing the protein extract (lysate) was either immediately used for further Western blot analyses or stored at –80°C. The cell culture supernatant of the adherent cells was used for enzyme-linked immunosorbent assay (ELISA).

2.7. SDS-PAGE and Western blot analysis

Twenty micrograms of cell lysates were resolved by 8-12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 0.02 A of constant current and transferred to a polyvinylidene fluoride (PVDF) membrane (0.22 µm; Amersham) using the 'semi-dry' transfer method (BioRad, Singapore) for 60 min at 0.12 A in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% (w/v) SDS. The membrane was blocked with 5% BSA (BioRad) in phosphate-buffered saline (PBS) plus 0.1% Tween-20 (PBS-T) for 2 h at RT, washed three times in PBS-T for 10 min each, and incubated with primary antibody (diluted in 2% BSA in PBS-T) for overnight at 4°C. The membranes were washed as described above, incubated with HRP-conjugated secondary antibody for 1 h at RT, and developed using the ECL plus Western blot detection reagent (Amersham, Piscataway, NJ, USA). X-ray films (Konica Minolta Inc., Tokyo, Japan) were exposed to the membranes before film development in a Kodak X-OMAT 2000 processor (Kodak, Ontario, Canada). For equal sample loading, protein quantification was performed using a '2D Quant' kit (Amersham) with at least two independent replicates. BSA was used

as a standard for protein quantification. To re-probe the same membrane with another primary antibody, Pierce's (Pierce Biotechnology, Inc., Rockford, IL, USA) 'stripping solution' was used to strip the membranes. In addition, equal sample loading was confirmed using Gapdh (Glyceraldehyde 3-phosphate dehydrogenase) as a reference protein. Western blot experiments were performed at least four times for statistical quantification and analyses ($n = 4$), and representative blots are shown. Values (= relative protein expression) represent the ratio of densitometric scores (GS-800 Calibrated Densitometer and Quantity One quantification analysis software version 4.5.2; BioRad) for the respective Western-blot products (mean ± S.D. (standard deviation)) using the Gapdh bands as a reference.

2.8. Quantification of soluble App-alpha (sApp-α) by ELISA

The various cell culture supernatants collected were centrifuged at 5,000 × g for 10 min. Concentrations of sApp-α in the supernatants were measured by solid phase sandwich ELISA using the mouse/rat sApp-α-specific assay kit (#27415; ARP American Research Products Inc., Belmont, MA, USA). To measure the concentration of human soluble Tumor necrosis factor receptor superfamily member 1B (hsTNFRSF1B), cleaved specifically extracellularly by α-secretase and resembling sApp-α from TAF-PC12 cells (14), the ELISA technique was employed and analyzed according to the manufacturer's protocol (Quantikine® DRT200; R&D Systems, Minneapolis, MN, USA). Absorbance measurements were taken at 450 nm using a Safire2™ microplate reader (Tecan Group Ltd., Männedorf, Switzerland) and analysis was done using the Magellan™ V5.01 (Tecan) software. Experiments were performed four times in triplicates. Data are presented as sApp-α levels in pg/mL compared to controls. Statistical evaluation of results was performed using analysis of variance (ANOVA) and statistical error was indicated as mean ± S.D.

2.9. Antibodies

Anti-β-site App-cleaving enzyme (Bace1) (1:1,000, mouse monoclonal; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (1:5,000, mouse monoclonal; Santa Cruz) and anti-App (1:10,000, rabbit polyclonal; Sigma-Aldrich).

2.10. RAWM test to analyze cognitive functions in Tianma-treated mice

Behavioral testing of mouse models is essential for establishing the progression of mnemonic deficits as

mice age. One of the most elegant, efficient and simple analysis is the Morris water maze (MWM) which is a simple spatial task based on visual cues in which a rodent swims in a tank to find a hidden platform. There are an increasing number of memory tasks including the MWM, the Y-maze, fear conditioning, conditioned food avoidance and object recognition testing. However, the recently found RAWM seems to be the most reliable task for detecting memory deficits in mice models and to study more efficiently learning performance while the test robustly discriminates between mice that learn well and those that learn poorly (16).

The RAWM is a hybrid of the MWM and a radial arm maze, which takes advantage of the simple motivation provided by immersion into water, together with the benefits of scoring errors (rather than time or proximity to platform location) associated with the radial arm maze. The RAWM contains six swim paths (arms) extending out of an open central area, with an escape platform located at the end of one arm (the goal arm). The goal arm location remains constant for a given mouse. The RAWM has the spatial complexity and performance measurement simplicity of the dry radial arm maze combined with the rapid learning and strong motivation observed in the MWM without requiring foot shock or food deprivation as motivating factors. Our mice were treated for two months with Tianma (~ 2.5 g/kg/day) before the RAWM test was performed. For RAWM, on day 1, mice were trained for 20 trials (spaced over 3 h), with trials alternating between visible and hidden platform. On day 2, mice were recorded for 20 trials with the hidden platform. Entry into an incorrect arm was scored as an error. If a mouse did not leave its start arm within 15 sec then it was also counted as an error. Each indicated block of trials consists of 4 trials. The data shown were obtained from Tianma-treated mice (squares, $n = 10$) and untreated littermates (circles, $n = 10$). The data shown were collected independently on two separate days with new mice each time. Statistical analysis was based on univariate and multivariate one-factor ANOVA, and between-group comparisons were made by Tukey's test.

2.11. Novel object recognition test on Tianma-treated mice

Experiments were performed as described previously (17-19). In the one-trial object recognition task, animals were exposed to two different objects which they have to identify as novel or familiar based on the memory of an earlier experience with one of the two objects they encountered in the same open-field. Memory involved in one-trial object recognition is that of an episodic type in the life of these animals. The one-trial object recognition task is limited to memory

of an object, the location of an object and the context in which an object was encountered. It only provides measure of memory of when such encounter with an object, a place and/or a context took place (episodic memory), if a significant long temporal delayed dimension of an episode (e.g. 24 h as in our study) has been incorporated. If an object is completely novel, it will always attract attention and induce more exploration even when presented alongside a preferred object. Attention and motivation processes are likely to prioritize novelty detection. Anything new is alerting and needs to be examined. However, such level of exploration induced by object novelty can be observed within a short time window after which it would decline in favor of more exploration of a familiar preferred object. The apparent unconditioned preference for a novel object is considered as an indication that a representation of the familiar object exists in memory. Briefly, in our study all mice were given a habituation session which allowed each of them to explore the empty apparatus for 5 min. On the training session, each mouse was placed in the middle of the box with two identical objects (A1 and A2) located at both ends. Each mouse was allowed to explore for 3 min. The mouse was then removed and returned to its home cage. After one hour, the test began. Each mouse was placed into the box where an identical object and a new object (A1 and B) were introduced at two sides of the box. The mouse could explore for 3 min. The time spent to explore the novel object and the time spent to explore both objects and the box was measured. This is to test the short-term memory. After 24 h, the mouse was placed into the box again, with the identical object A1 and another novel object C. The time spent to explore the novel object and the total exploring time was measured as the day before. The parameters analyzed were percentage of time that the animals explored identical objects during the training and the percentage of time that the animals explored the novel object at 1 h (working memory or short term memory) and 24 h (episodic memory or long term memory) after training. We considered this last parameter also as an index of memory retention. Results are expressed as percentage of novel object recognition time.

2.12. Statistical evaluation

The data obtained in this investigation are illustrated as mean \pm S.D. Differences between the groups were established using an unpaired Student's *t*-test while within-group comparisons were performed using the paired Student's *t*-test. SPSS (Statistical Products and Service Solutions) for Windows Version 19 was used to perform ANOVA followed by Fisher's Protected Least Significant Difference (PLSD) *post hoc* tests, when warranted. To be considered statistically significant,

we required a probability value to be at least < 0.05 (95% confidence limit, * $p < 0.05$).

3. Results

In the present study, we sought to find a lead in the potential use of Tianma as a therapeutic agent for the prevention of AD. Using neurocellular *in vitro* experiments and *in vivo* animal models, the effect of Tianma on neural signaling cascades and on cognitive functions have been investigated.

3.1. Effect of Tianma on cognitive function in mice using RAWM test

To determine the effect of Tianma on cognitive functions, we applied the recently established RAWM test to thoroughly discriminate between mice that learn well and those that learn poorly in a complex memory and learning task (16). Interestingly, after the treatment of the mice over a period of two months, Tianma produced a significant improvement of the learning task (Figure 1).

3.2. Effect of Tianma on novel object recognition test

To further validate our observation, we performed the novel object recognition test. In this test, when exposed to a familiar object alongside a novel object, young and adult mice usually approach frequently and spend more time exploring the novel than the familiar object. However, there are instances where preference for novelty is supplanted by preference for familiarity. For instance, there is a growing experimental evidence showing preference for familiarity in young and old infants (20,21). A reduced preference for a novel

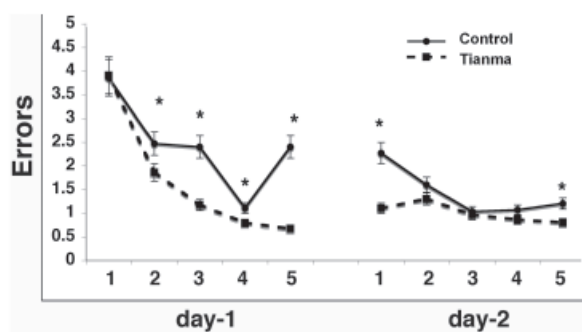


Figure 1. Tianma improves cognitive functions in mice. RAWM testing of Tianma-treated mice. Average error committed by one-year-old Tianma-treated (for 2 months) mice (squares, $n = 10$) and control littermates (circles, $n = 10$). Experiments were performed as described in Materials and Methods. Each block (1-5) consists of four trials. The data shown were obtained from Tianma-treated mice (squares, $n = 10$) and untreated littermates (circles, $n = 10$). The data shown were collected independently on two separate days with new mice each time. Data are presented as the mean \pm S.D. (by ANOVA) (* $p < 0.05$ compared with controls).

object can be accounted for by the physical properties of the novel object which may not require too much attention or by some affective attributes invested into the familiar object. For these kinds of reasons, a possibility has always been envisaged where a familiar object can be preferred to a novel object (22). Besides, the level of exploration induced by object novelty can be observed within a very short time window after which it would decline in favor of more exploration of a familiar preferred object. Novelty-preference is short-lived and lasts for the time necessary for mice to encode the physical properties of the stimulus induced by the novel object. Thus, it seems that Tianma-treated mice learned faster and were more quickly able to explore the new object than control mice and afterwards spent longer time with the familiar objects (Figure 2) – therefore, confirming the learning-task results obtained from the RAWM experiments (19).

3.3. Effect of Tianma on App cleavage in neuronal N2a cells

To determine a potential link between the improved cognitive functions caused by the application of Tianma and prevention of dementia such as AD, we studied a possible molecular mechanism responsible for this finding. Since App is the pivotal protein involved in the molecular cascade ultimately leading to AD (23,24), we characterized the effect of Tianma on the expression of the App protein. However, no changes in full length App expression could be detected though a slight increase in the C-terminal fragments (CTFs) could be observed (Figure 3A),

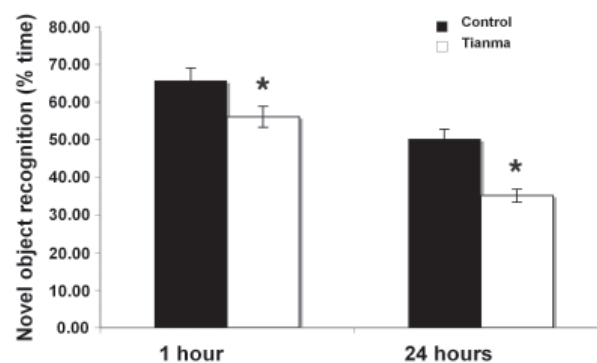


Figure 2. Effect of Tianma on memory performance in an object recognition test. Experimental procedure was carried out according to Materials and Methods. Mice were familiarized with various objects for a specific time period before again confronted with known and new objects after a time interval of 1 h and 24 h, respectively. Compared with the control group, Tianma-treated mice showed remarkable differences for the time spent to explore the novel and the familiar object, suggesting that the Tianma-treated mice have a better learning performance in recognizing novel objects faster and remembering the familiar object at both after 1 h and 24 h, respectively. Data presented are mean (percentage time = $t_{\text{novel}} / (t_{\text{novel}} + t_{\text{familiar}}) \times 100$) \pm S.D. (* $p < 0.05$).

probably initiated through an enhanced α -secretase-mediated App-cleavage. Therefore, we studied the proteolytic cleavage of App in neuronal N2a cells and the results obtained revealed that Tianma increased the sApp- α release in our *in vitro* cell culture system while the control herb Gouteng did not induce any significant changes (Figure 3B). To test whether this increased sApp- α cleavage was eventually due to an enhanced App protein expression or an increase in cell proliferation, we investigated both of it and found that neither App expression nor N2a proliferation was affected by Tianma (Figures 3C and 3D).

3.4. Effect of Tianma on α -secretase-mediated App cleavage in TAF-PC12 cells

To further elaborate on this interesting observation, we shifted to our previously established novel cellular system that is suitable to study App signaling as a classical ligand-receptor system and is particularly useful for the study of α -secretase-mediated App cleavage (14). We used TAF-PC12 cells to study specifically the effect of Tianma on App processing by the α -secretase. We found that also in this system Tianma enhanced sApp- α production, thus indicating

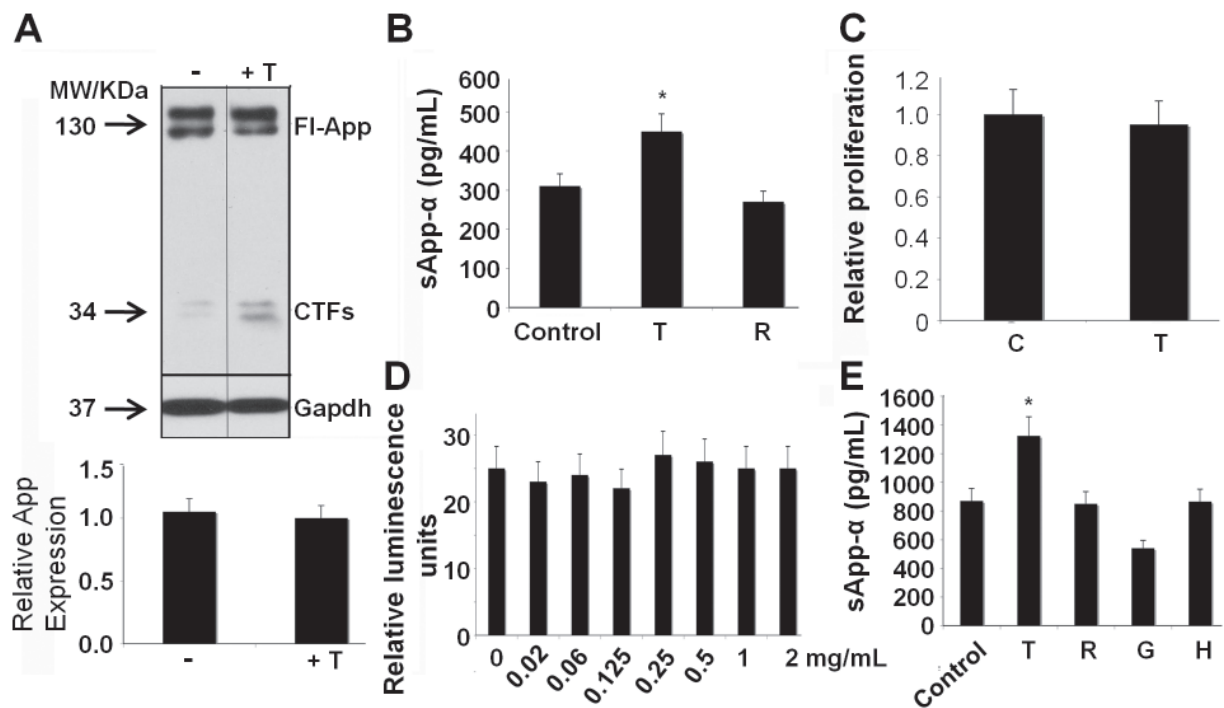


Figure 3. Effect of Tianma and Gouteng on App expression, soluble App release and cell proliferation. (A) Western blot analysis (A, top) of Tianma-treated N2a cells reveals no changes in App protein expression levels. N2a cells were grown and treated with Tianma (1 mg/mL) for 24 h as described in "Materials and Methods". Gapdh was used as the reference protein. FI-App = full length App, CTFs = C-terminal fragments of full-length App. Western blot experiments were performed at least four times for statistical quantification and analyses ($n = 4$). Values (= relative protein expression) represent the ratio of densitometric scores for the respective Western blot products (mean \pm S.D.) using the Gapdh bands as reference. No significant difference in full length App expression levels could be observed between control and Tianma-treated N2a cells (A, bottom). The slight increase in the C-terminal fragments (CTFs) was probably initiated through an enhanced α -secretase-mediated App-cleavage (A, top). (B) Estimation of sApp- α levels in the cell culture supernatant of neuronal N2a cells treated with Tianma and measured by ELISA. N2a cells were grown and treated with Tianma (1 mg/mL) or Gouteng (R) (1 mg/mL) for 24 h as described in Materials and Methods. Experiments were performed four times in triplicates. Data are presented as sApp- α levels in pg/mL compared to controls. Statistical evaluation of results was performed using ANOVA and statistical error was indicated as mean \pm S.D. (* $p < 0.05$). Only Tianma-treated cells showed a significant elevation of sApp- α levels. (C) The effect of Tianma on BrdU-positive cell proliferation. N2a cells were cultured for three passages before BrdU analysis of proliferation was performed in the presence of Tianma. Quantification of BrdU incorporation is represented as the mean (\pm S.D.) of the percentage of BrdU-positive cells in the experimental population obtained from quadruplicated measurements, where each measurement consisted of three repetitions. No significant difference could be observed between control and Tianma-treated cells. (D) Dose-dependent proliferation analysis of N2a cells treated with Tianma. In the proliferation assay based on ATP-consumption, control N2a cells were cultured in N2a cell culture media as mentioned in "Materials and Methods", while experimental samples were exposed to Tianma for 72 h at various concentrations as indicated. Values represent mean luminescent units (\pm S.D.) obtained from quadruplicated measurements, where each measurement consisted of three repetitions. No significant difference could be observed between control and Tianma-treated cells. (E) Estimation of sApp- α levels in the cell culture supernatant of neuronal TAF-PC12 cells treated with Tianma and measured by ELISA. TAF-PC12 cells were grown and treated with Tianma (T) (1 mg/mL), Gouteng (R) (1 mg/mL), gastrodin (G, 500 μ g/mL), or hyperzine-A (H, 10 μ M) for 24 h as described in Materials and Methods. Experiments were performed four times in triplicates. Data are presented as sApp- α levels in pg/mL compared to controls. Statistical evaluation of results was performed using ANOVA and statistical error was indicated as mean \pm S.D. (* $p < 0.05$). Only Tianma-treated cells showed a significant increase of sApp- α levels. Gastrodin alone had rather an inhibitory effect.

enhanced cleavage of App by α -secretase. As control, the other herb Gouteng, gastrodin (one active component of Tianma) and huperzine-A (a bioactive compound of *huperzia serrata* known to act as an acetylcholinesterase inhibitor and also described to affect App proteolytic processing (25-28), were included in this study, but had no significant effect on sApp- α production (Figure 3E).

3.5. The potential mechanism of Tianma-mediated App cleavage in neuronal N2a cells

To investigate the effect of Tianma on App receptor signaling, N2a cells were subjected to Tianma treatment for a period of 24 h. While sApp- α levels were increased as described in Figure 3, we examined the potential mechanism involved in this process by checking the expression levels of various secretases (α -, β -, and γ - such as Adam10, Adam17, Bace1/2, and Psen1/2) known to be involved in the processing of App (23). Accordingly, the expression of the β -secretase (Bace1) was inhibited by Tianma (Figure 4). The expression levels of the other secretases were unchanged (data not shown) – though their activity still could be affected.

4. Discussion

Herbs with potential neuroregenerative activities that have been identified include *Curcuma longa*, *Zingiber*

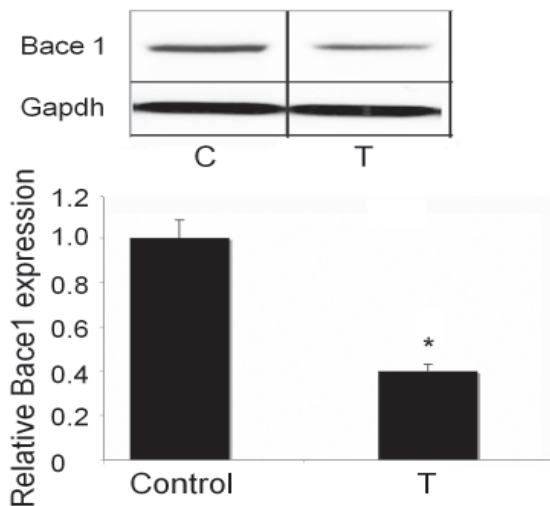


Figure 4. Relative Bace1 expression in N2a cells treated with Tianma and evaluated by Western blot analyses. N2a cells were grown and treated with Tianma (1 mg/mL) for 24 h as described in Materials and Methods. Top: representative Western blot results for Bace1 and Gapdh; bottom: statistical evaluation of the Western blots. Gapdh was used as the reference protein. Western blot experiments were performed at least four times for statistical quantification and analyses ($n = 4$). Values (= relative protein expression) represent the ratio of densitometric scores for the respective Western blot products (mean \pm S.D.) using the Gapdh bands as a reference. Tianma-treated cells clearly show a significant reduction of Bace1 expression (* $p < 0.05$).

officinale, *Huperzia serrata*, *Nigella sativa*, *Rhizoma acori graminei*, *Verbena officinalis* Linn, and Tianma (4,13,28-31). According to ancient Chinese medical literature, Tianma is an herbal medicine for the control of the internal movement of wind. The dry tuber of Tianma has long been officially listed in the Chinese Pharmacopoeia and is used in treating headaches, dizziness, tetanus, epilepsy, infantile convulsions, numbness of the limbs and also for the improvement of cognitive functions (4,31-36). From scientific investigations, Tianma possesses anti-oxidative and free radical scavenging (37), neuroprotective (38), and anti-depressant effects (7,39,40). Tianma, *huperzia serrata* and other herbs have recently also been discussed as a relevant source of potential therapeutic remedies that target AD symptoms or the primary pathogenic processes of AD especially as researchers are now exploring the possibility of a connection between AD, VD, diabetes mellitus (type 2, DMT2), and CD (5,41,42). The risk for dementia is particularly high when diabetes mellitus occurs together with severe systolic hypertension or heart diseases (43,44). The strong association of cardiovascular risk factors with AD and VD suggests that these diseases share some biological pathways in common and were discussed recently (5,45). Epidemiological and clinico-pathological data have indicated that antihypertensive drugs show protective effects in reducing the risk of dementia and data suggest overlaps between AD and cerebrovascular lesions that may magnify the effect of mild AD pathology and promote the progression of cognitive decline or may even precede neuronal damage and dementia (46). Therefore, the contribution of CD to AD and VD foreshadows that cardiovascular therapies might prove useful in treating or preventing AD and dementia (47-52). In traditional herbal medicine practice, synergistic and or antagonistic therapeutic efficacy among the herbs in any one prescription plays an important role in the treatment of illness. These observations may explain the medicinal effects related to AD and VD observed for Tianma and some other herbs (28-31,33-35,53-62). For instance, Gouteng has been traditionally used alongside with Tianma in treating high blood pressure (63). A recent study has illustrated that the use of Tianma and Gouteng with two other herbs has anti-hypertensive effects and shows an enhancing effect on cognitive functions and thus, taken our results into account, could be used against cognitive defects and dementia (8,9). However, the complexity and multi-actions of herbal bioactive compounds remain unclear. A variety of studies were carried out to purify the herbs' effective constituents and to investigate their biological functions. Data revealed that huperzine-A shows neuroprotective function and can ameliorate learning dysfunction in AD patients (27,28,53,54,62). Gastrodin and vanillin, derived from Tianma, were shown to exert sedative and anticonvulsive effects. Gastrodin seems to be a

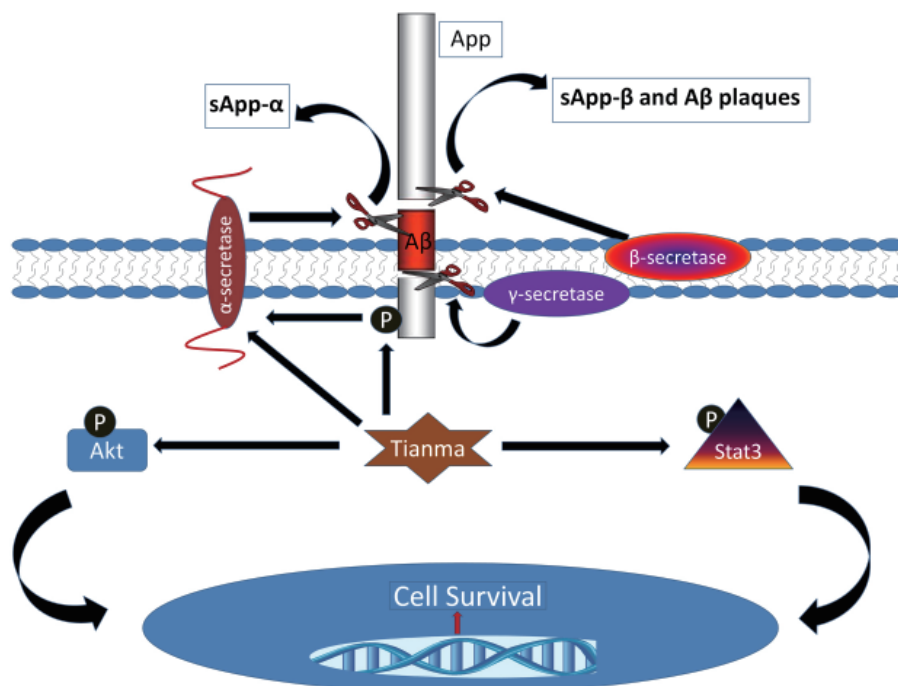


Figure 5. Schematic illustration of the action of Tianma. Tianma may become a potential therapeutic agent for the treatment of AD. Tianma's abilities in enhancing the α -secretase' activity and possible phosphorylation of App greatly encourage the proteolytic processing of App towards the non-amyloidogenic pathway that results in increased production of the neuroprotective sApp- α (68). Tianma's inhibitory effect on the β -secretase precludes amyloid- β peptide generation and prevents AD. It may also enhance the phosphorylation of Akt and Stat3 that aid in cell survival and retards AD pathogenesis.

safe and effective drug for treating anxiety, insomnia, neurasthenia, and mental hyper-excitation while vanillin is presumably an anti-convulsive agent (4,31). In addition to gastrodin and vanillin, other bioactive components of Tianma include vanillyl alcohol, 4-hydroxybenzylaldehyde, and 4-hydroxybenzyl alcohol (64) with gastrodin being the primary active ingredient (65). The importance of other secondary metabolites such as flavones and anthocyanins has been largely neglected. According to the research, gastrodin exhibits also an anti-coagulant effect (66) and protects cerebral cortical and hippocampal cells against amyloid- β peptide-induced neurotoxicity (67) which suggests that gastrodin could play a pivotal role in AD treatment. Though, in our study, another component besides gastrodin seems to be responsible for the specific effect on enhanced sApp- α cleavage (Figure 5).

As herbs contain multiple compounds with potentially versatile modes of action, the modernization and the acceptance of traditional herbal medicine into mainstream medicine in the past is restricted by the problems of unauthenticated raw material, unknown mechanisms of action, unknown bioactive compounds, non-standardization of herbal products with respect to active ingredients and lack of toxicology and safety data. However, the recent introduction of good manufacturing practice has resulted in herbs with standardized bioactive compounds. The pharmacodynamics, pharmacokinetics, safety, and efficacy of bioactive compounds can be examined in animal models according to the US-FDA' published guidance for industry on the policy for

botanical drug product development which enables the use of fingerprint to show lot-to-lot consistency so that there is no need to identify the function of each individual component. If a botanical drug has been used historically to treat a disease, then the combination of Phase-I and -II for a drug development is allowed. Thus, our current study used a standardized Tianma (11) and provides an additional interesting insight into the molecular and cellular mechanisms of herbal medicine that may guide us to the further identification of the bioactive components (by phytochemistry) that may contribute to Tianma's potential cognitive function-enhancing activities. However, a more systemic biology study is necessary to understand the neurovascular and neuroprotective functions of herbs such as Tianma and to unravel the medically active components in Tianma (4,69,70).

Acknowledgement

This study was supported by the Institute of Advanced Studies, Nanyang Technological University, Singapore.

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(Received April 3, 2011; Revised April 23, 2011; Accepted April 26, 2011)