World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 5.088

TETRAMETHYLPYRAZINE DIMER ALLEVIATES APP PROCESSING AND TAU HYPERPHOSPHORYLATION IN NEURO2A/APPSWE CELLS

Xiaoyi Lin[1,2], Xifei Yang[2]* and Xianfeng Huang[1,2]

[1]College of Pharmaceutical Engineering and Life Sciences, Changzhou University, Changzhou 213000, China.
[2]Key Laboratory of Modern Toxicology of Shenzhen, Shenzhen Center for Disease Control and Prevention, Shenzhen 518055, China.

***Corresponding Author: Xiaoyi Lin**
College of Pharmaceutical Engineering and Life Sciences, Changzhou University, Changzhou 213000, China.

Article Received on 13/03/2019 **Article Revised on 03/04/2019** **Article Accepted on 24/04/2019**

ABSTRACT

Alzheimer's disease (AD) is a common neurodegenerative disease whose two major pathological features are β-amyloid (Aβ) aggregation and tau hyperphosphorylation. Tetramethylpyrazine is an alkaloid monomer extracted from Chuanxiong, which has specific pharmacological effects such as improving learning and memory and protecting nerves. Here, we evaluated the neuroprotective effect of a newly synthesized tetramethylpyrazine dimer (DTMP) using the murine neuron-like cells (N2a) transfected with the human "Swedish" mutant amyloid precursor protein (N2aAPP). the half-inhibitory concentration (IC50) of DTMP against N2a/APPswe cells was 12.37 μM as measured by cell proliferation-toxicity assay (CCK-8). Western blot analysis showed that DTMP treatment significantly reduced the expression of BACE1, PS1 and t-APP in the APP pathway and attenuated the phosphorylation levels of Ser396, Ser262 and Thr231 in Tau protein. The modulation of dysregulated proteins implicated in AD pathogenesis implies the pharmacological mechanisms of DTMP and its potential as a novel therapeutic choice in AD.

KEYWORDS: Alzheimer's disease; DTMP; N2a/APPswe cells; APP processing; Tau hyperphosphorylation.

1. INTRODUCTION

Alzheimer disease (AD) is a central nervous system disorder and is the most common type of senile dementia.[1] The pathological lesions mainly include senile plaques formed by β-amyloid accumulation and neurofibrillary tangles formed by hyperphosphorylation of Tau protein and neuronal loss.[2,3] According to the 2016 World Alzheimer's Disease Report, approximately 50 million people worldwide suffer from Alzheimer's disease.[4] The number of AD patients is expected to double in the next 20 years, and the number of AD patients will reach 74.7 million by 2030 and reach 131.5 million by 2050.[5,6] However, effective treatment strategies are limited.

Ligustrazine is a novel calcium ion antagonist that extracts an alkaloid monomer from Chuanxiong.[7] According to reports, TMP not only improves learning and memory, but also reduces choline dysfunction in mice with D-galactose damage.[8] In the whole brain and focal cerebral ischemia model, TMP can also exhibit neuroprotective effects in whole brain and focal cerebral ischemic injury models.[9] One such compound present in garlic is diallyl disulfide (DADS), which has neuroprotective effects.[10,11] Garlic extract containing DADS and other ingredients was found to prevent APP processing and tau phosphorylation in the Alzheimer's disease transgenic model Tg2576.[12-14] Therefore, two tetramethylpyrazine were spliced together by DADS to obtain a compound tetramethylpyrazine dimer (DTMP) (Fig. 1A). In order to study the neuroprotective effect of DTMP on N2a/APPswe cells, this study explored the key proteins involved in APP processing and tau phosphorylation by Western blot analysis. The experimental results show that DTMP can play a neuroprotective role by regulating key molecules in the Aβ and Tau pathways.

2. MATERIALS AND METHODS

2.1 Cell culture

N2a/WT and Na/APPswe cells were supported by Professor Wang Jianzhi (Huazhong University of Science and Technology, Wuhan, China). The cells were cultured in an equal volume of Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM containing 5% fetal calf serum in 5% CO2 at 37℃, and 0.2 g/L of G418 (Thermo Fisher Scientific) was added to screen stably transfected cells.

2.2 Cell Viability Assay

$1x10^4$ / mL N2a/APPswe cell suspensions were placed in 96-well plates (100 uL per well). After the cells were cultured for 18 h, the medium was removed and a cell culture medium containing DTMP or vehicle (0.5% DMSO) (Thermo Fisher Scientific, Waltham, MA, USA) was added. After treatment with 0-1000 μM DTMP for 24 h, the medium was removed and 10% CCK-8 solution (Dojindo Laboratories, Shanghai, China) was added. After incubation at 37°C for 1.5 h, the absorbance of the samples was measured at 450 nm using a microplate reader (Tecan M1000, Männedorf, Switzerland). Cell viability (%) = (infected group - blank control) / (control group - blank control) * 100%.

2.3 Western-Blot

After 24 h after of DTMP treatment, cells were washed twice with cold 1 x PBS and 200 μL IP lysis buffer was added to each 25cm2 flask for 30 min on ice. Cells were scraped from the flask and collected into a 1.5 mL tube, and centrifuged at 20000g at 4℃ for 30 min. The total protein concentration was measured by a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Protein samples were combined with 5 x SDS loading buffer (Thermo Fisher Scientific) and heated at 96°C for 8 min, then separated on 8%-12% SDS-PAGE and transferred to a 0.45 μM PVDF membrane. After blocking for 1 h at room temperature, it was incubated with primary antibody in 1 x TBST overnight at 4°C (see Table 1). The membranes were washed with TBST and incubated with anti-rabbit or anti-mouse IgG HRP secondary antibody for 1 h. Thereafter, the membrane was washed with TBST three times and visualized using the chemiluminescent reagent from ECL kit (Thermo Scientific Pierce ECL, USA) color. Quantitative densitometry analysis was performed using quantity one software.

2.4 Statistical Analysis

Table 1: The primary antibodies used in this study.

Antibody	Cat.	Type	Dilution	Source
t-APP	ab32136	Rabbit	1:3000	Abcam
sAPPα	11088	Mouse	1:50	Immuno-Biological
sAPPβ	10321	Mouse	1:50	Immuno-Biological
BACE1	#5606	Rabbit	1:1000	Cell Signaling
PS1	#5643	Rabbit	1:1000	Cell Signaling
pS396	ab109390	Rabbit	1:20000	Abcam
pS404	sc-12952	Goat	1:3000	Santa cruz
pT231	355200	Mouse	1:1000	Thermo Fisher
pS262	44750G	Rabbit	1:1000	Thermo Fisher
pS202	44736G	Rabbit	1:1000	Thermo Fisher
Tau 1	MAB3420	Mouse	1:200000	Millipore
Tau 5	ab80579	Mouse	1:3000	Abcam
β-actin	sc-47778	Mouse	1:3000	Santa cruz

Results are expressed as mean ± SEM. One-way analysis of variance was used for statistical analysis (GraphPad Prism 7.0, http://www.graphpad.com/). P< 0.05 were considered statistically significant.

3. RESULTS

3.1 DTMP has low cytotoxicity for N2a/APPswe cells

DTMP is a compound in which two tetramethylpyrazines are spliced by DADS. We measured the cytotoxicity of DTMP on N2a/APPswe cells using CCK-8. CCK-8 is a highly sensitive, non-radioactive colorimetric assay for the determination of cell proliferation or toxicity. As the concentration of DTMP increased, the growth of N2a/APPswe cells was also increased (Fig. 1B). The IC50 of N2a/APPswe cells was calculated to be 12.37 μmol/L. Therefore, 24 h treatment with a maximum concentration of 10.0 μM DTMP was used in the following study.

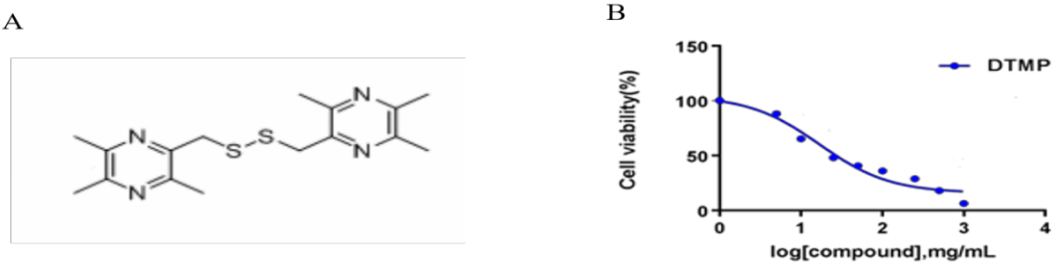

Figure 1: DTMP has low cytotoxicity to N2a/APPswe cells. (A) Chemical structure of DTMP. (B) Cell viability of N2a/APPswe with DTMP treatment.

3.2 DTMP inhibited APP processing in N2a/APPswe cells

We explored the key proteins involved in APP processing by using Western blot analysis. The experimental results (Fig. 2 A, B) showed that the levels of t-APP, PS1 and BACE1 proteins were significantly increased in N2a/APPswe cells compared with N2a/WT cells. Compared with N2a/APPswe, the DTMP-treated group significantly reduced t-APP, PS1, and BACE1 protein levels at low doses. Western blot results showed that DTMP inhibited APP processing.

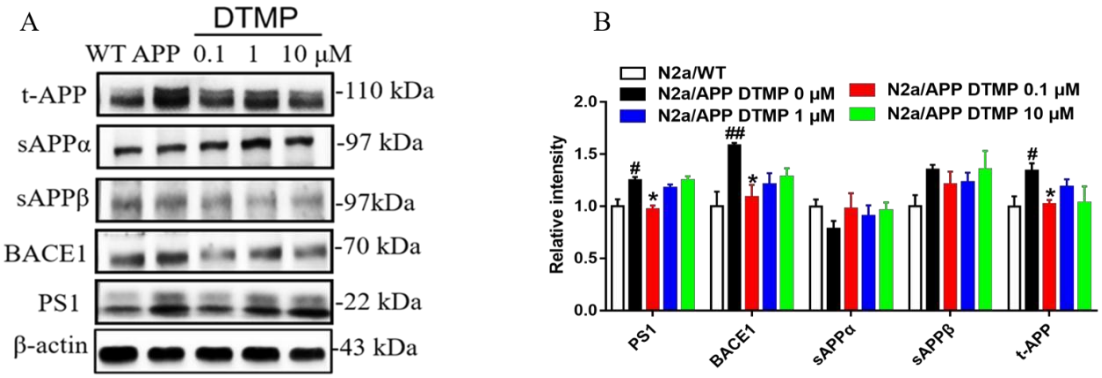

Figure 2: DTMP inhibited APP processing.

Levels of t-APP, sAPPα, sAPPβ, BACE1, and PS1 were determined by Western blot analysis (A, B). β-actin was used as a loading control. N = 3. Data show the mean± SEM. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. N2a/WT cells, $^{*}P<0.05$, $^{**}P<0.01$ vs. N2a/APPswe cells.

3.3 DTMP attenuated Tau phosphorylation in N2a/APPswe cells

In this study, the effect of DTMP on Tau protein phosphorylation was detected by Western blot. The experimental results (Fig. 3 A, B) showed that the phosphorylation level of Tau protein in the Ser396, Ser404 and Ser262 sites in N2a/APPswe cells was significantly higher than that in N2a/WT cells. Compared with N2a/APPswe, the DTMP-treated group significantly reduced Tau protein phosphorylation at the Ser396, Thr231 and Ser262 sites at low doses. These results indicate that DTMP can slow the phosphorylation of Tau protein in N2a/APPswe cells.

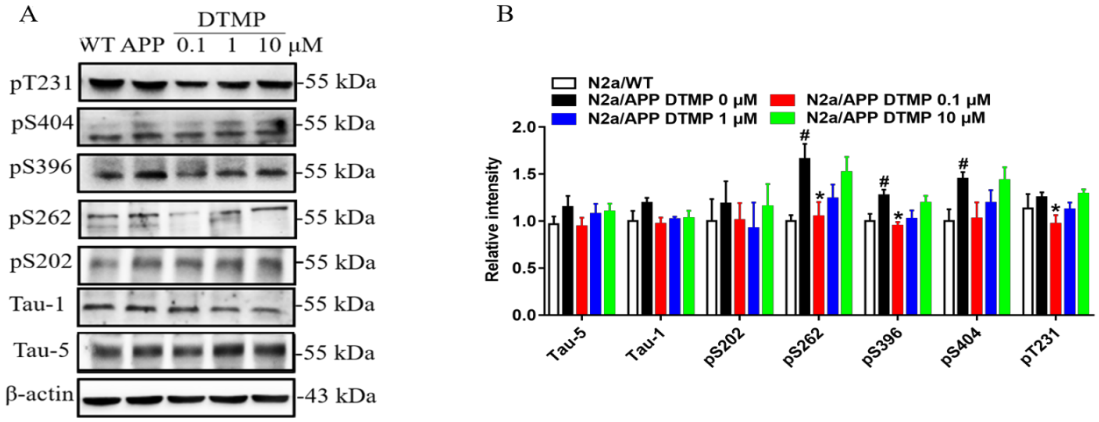

Figure 3: DTMP attenuated tau phosphorylation in both N2a/APPswe cells.

Shown are the levels of phosphorylated tau and total tau in N2a/APPswe cells (A, B). β-actin was used as a loading control. N = 3. $^{\#}P<0.05$ vs. N2a/WT cells, $^{*}P<0.05$ vs. N2a/APPswe cells.

4. DISCUSSION

In this study, our methods of Western blot research DTMP neuroprotective effect on neuroblastoma N2a cells in AD- related lesions, using human Swedish mutant amyloid precursor gene transfected mouse neural element N2a cells as an AD model.[15] This result indicates that the neuroprotective effect of DTMP on neuroblastoma N2a cells is mainly involved by inhibiting the processing of APP and slowing the phosphorylation of Tau protein.

The amyloid precursor protein (APP) is cleaved by β-, γ-secretase to produce β-amyloid (Aβ) metabolic abnormalities are the main cause of AD formation.[16] The APP is cleaved by α-secretase to

produce an APP (sAPPα) secreted at the N-terminus.[17] In the hydrolysis pathway of APP, APP is cleaved by BACE1 (β-secretase) at the N-terminus of the Aβ sequence to cleave a short fragment called APPβ.[18,19] The protein expression of BACE1, sAPPα, sAPPβ and PS1 in APP pathway was detected by Western blot. It was found that DTMP can decrease the expression of BACE1 and PS1. Therefore, it can be judged that DTMP may affect APP pathway through BACE1 and PS1, and then affect Aβ.

Tau protein hyperphosphorylation is one of the main pathological features of AD.[16] Tau protein is mainly divided into cytoplasmic normal tau protein (C-tau), hyperphosphorylated soluble tau protein (ADP-tau) and tau protein (PHF-tau) aggregated into PHF.[20] At present, 45 phosphorylation sites have been identified in PHF-tau, mainly phosphorylation of threonine and serine residues.[21] Phosphorylation of the Tau protein in the MBD region is critical for regulating the stability of the microtubules, particularly phosphorylation at the Ser262 and Ser356 sites, altering the conformation of the tau protein to the microtubule binding site, resulting in disintegration of the microtubule and destruction of the cytoskeleton.[22] In addition, phosphorylation sites outside the MBD region such as Ser214 and Thr231 are also involved in the regulation of cytoskeletal stability. At present, most studies select key sites such as Thr181, Thr231, Ser199, Ser396, and Ser404, which are considered to be reliable predictors of mild cognitive dysfunction to AD transition.[23,24] The protein expression of Tau-1, Tau-5, Ser396, Ser404, Thr231, Ser262 and Ser202 in Tau pathway was detected by Western blot. It was found that DTMP can decrease the expression of Ser396, Thr231 and Ser262, so it can be judged that DTMP can slow the hyperphosphorylation of Tau protein.

5. CONCLUSION

In summary, our current data indicate that DTMP is less toxic and neuroprotective against N2a/APPswe cells. Western blot analysis showed that DTMP treatment significantly decreased the expression of BACE1, PS1 and t-APP in N2a/APPswe cells and attenuated the phosphorylation levels of Ser396, Ser262 and Thr231 in Tau protein. Therefore, DTMP can regulate key molecules in the Aβ and Tau pathways, thereby protecting neurons.

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China (81673134, 81401570), Shenzhen Special Fund Project on Strategic Emerging Industry Development (JCYJ20160428143433768, JCYJ20 170818111012390, JCYJ20150529164656093, JCYJ20 150529153646078, JCYJ20160422143433757, JCYJ20 150529112551484 and JCYJ2017081016332 9510) and Sanming Project of Medicine in Shenzhen (SZSM201611090).

REFERENCES

1. Lane CA, Hardy J, Schott JM. Alzheimer's Disease. Eur J Neurol, 2018; 25(1): 59-70.
2. Nisbet RM, Gotz J. Amyloid-Beta And Tau In Alzheimer's Disease: Novel Pathomechanisms And Non- Pharmacological Treatment Strategies. J Alzheimers Dis., 2018; 64(S1): S517-S27.
3. Bloom GS. Amyloid-Beta And Tau: The Trigger And Bullet In Alzheimer Disease Pathogenesis. JAMA Neurol. 2014; 71(4): 505-8.
4. Alzheimer's A. 2016 Alzheimer's Disease Facts And Figures. Alzheimers Dement, 2016; 12(4): 459-509.
5. S JC. Biologic Therapeutic Drug Monitoring In Australia. J Gastroenterol Hepatol, 2016; 31 Suppl 1: 8-9.
6. Foloppe DA, Richard P, Yamaguchi T, Etcharry-Bouyx F, Allain P. The Potential Of Virtual Reality-Based Training To Enhance The Functional Autonomy Of Alzheimer's Disease Patients In Cooking Activities: A Single Case Study. Neuropsychol Rehabil, 2018; 28(5): 709-33.
7. Chen HY, Xu DP, Tan GL, Cai W, Zhang GX, Cui W, Et Al. A Potent Multi-Functional Neuroprotective Derivative Of Tetramethylpyrazine. J Mol Neurosci, 2015; 56(4): 977-87.
8. Zhang C, Wang SZ, Zuo PP, Cui X, Cai J. Protective Effect Of Tetramethylpyrazine On Learning And Memory Function In D-Galactose-Lesioned Mice. Chin Med Sci J., 2004; 19(3): 180-4.
9. Chang Y, Hsiao G, Chen SH, Chen YC, Lin JH, Lin KH, Et Al. Tetramethylpyrazine Suppresses HIF-1alpha, TNF- Alpha, And Activated Caspase-3 Expression In Middle Cerebral Artery Occlusion-Induced Brain Ischemia In Rats. Acta Pharmacol Sin, 2007; 28(3): 327-33.
10. Park HY, Kim ND, Kim GY, Hwang HJ, Kim BW, Kim WJ, Et Al. Inhibitory Effects Of Diallyl Disulfide On The Production Of Inflammatory Mediators And Cytokines In Lipopolysaccharide-Activated BV2 Microglia. Toxicol Appl Pharmacol, 2012; 262(2): 177-84.
11. Gupta VB, Indi SS, Rao KS. Garlic Extract Exhibits Antiamyloidogenic Activity On Amyloid-Beta Fibrillogenesis: Relevance To Alzheimer's Disease. Phytother Res., 2009; 23(1): 111-5.
12. Chauhan NB. Anti-Amyloidogenic Effect Of Allium Sativum In Alzheimer's Transgenic Model Tg2576. J Herb Pharmacother, 2003; 3(1): 95-107.
13. Chauhan NB. Effect Of Aged Garlic Extract On APP Processing And Tau Phosphorylation In Alzheimer's Transgenic Model Tg2576. J Ethnopharmacol, 2006; 108(3): 385-94.
14. Chauhan NB, Sandoval J. Amelioration Of Early Cognitive Deficits By Aged Garlic Extract In Alzheimer's Transgenic Mice. Phytother Res., 2007; 21(7): 629-40.
15. Yan Y, Gong K, Ma T, Zhang L, Zhao N, Zhang X, Et Al. Protective Effect Of Edaravone Against Alzheimer's Disease-Relevant Insults In Neuroblastoma N2a Cells. Neurosci Lett., 2012;

531(2): 160-5.
16. Gotz J, Lim YA, Ke YD, Eckert A, Ittner LM. Dissecting Toxicity Of Tau And Beta-Amyloid. Neurodegener Dis., 2010; 7(1-3): 10-2.
17. Tomaselli S, Pagano K, D'Arrigo C, Molinari H, Ragona L. Evidence Of Molecular Interactions Of Abeta1-42 With N-Terminal Truncated Beta Amyloids By NMR. ACS Chem Neurosci, 2017; 8(4): 759-65.
18. Wang X, Zhou X, Li G, Zhang Y, Wu Y, Song W. Modifications And Trafficking Of APP In The Pathogenesis Of Alzheimer's Disease. Front Mol Neurosci, 2017; 10: 294.
19. Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Et Al. Beta-Secretase Cleavage Of Alzheimer's Amyloid Precursor Protein By The Transmembrane Aspartic Protease BACE. Science, 1999; 286(5440): 735-41.
20. Haense C, Buerger K, Kalbe E, Drzezga A, Teipel SJ, Markiewicz P, Et Al. CSF Total And Phosphorylated Tau Protein, Regional Glucose Metabolism And Dementia Severity In Alzheimer's Disease. Eur J Neurol, 2008; 15(11): 1155-62.
21. Hanger DP, Anderton BH, Noble W. Tau Phosphorylation: The Therapeutic Challenge For Neurodegenerative Disease. Trends Mol Med, 2009; 15(3): 112-9.
22. Buerger K, Ewers M, Pirttila T, Zinkowski R, Alafuzoff I, Teipel SJ, Et Al. CSF Phosphorylated Tau Protein Correlates With Neocortical Neurofibrillary Pathology In Alzheimer's Disease. Brain, 2006; 129(Pt 11): 3035-41.
23. Diniz BS, Pinto Junior JA, Forlenza OV. Do CSF Total Tau, Phosphorylated Tau, And Beta-Amyloid 42 Help To Predict Progression Of Mild Cognitive Impairment To Alzheimer's Disease? A Systematic Review And Meta- Analysis Of The Literature. World J Biol Psychiatry, 2008; 9(3): 172-82.
24. Ewers M, Buerger K, Teipel SJ, Scheltens P, Schroder J, Zinkowski RP, Et Al. Multicenter Assessment Of CSF- Phosphorylated Tau For The Prediction Of Conversion Of MCI. Neurology, 2007; 69(24): 2205-12.