Neurochemistry International 99 (2016) 194–205

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Neuroprotection against glutamate-induced excitotoxicity and induction of neurite outgrowth by T-006, a novel multifunctional derivative of tetramethylpyrazine in neuronal cell models

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ARTICLE INFO

Article history:
Received 9 March 2016
Received in revised form
5 July 2016
Accepted 13 July 2016
Available online 18 July 2016

Keywords:
Alzheimer's disease
Tetramethylpyrazine derivative
Excitotoxicity
Neuroprotection
Neurite outgrowth

ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disorder, characterized by irreversible impairment of memory and cognitive function. The exact causes of Alzheimer's disease still remain unclear and current single target drugs could only offer limited therapeutic effect to the patients. We have previously reported that T-006, a promising anti-Alzheimer's compound derived from Chinese medicinal component tetramethylpyrazine, might protect neurons through inhibiting the overproduction of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS). In this study, we further investigated the neuroprotective effects, as well as the molecular pathways involved, of T-006 against glutamate-induced excitotoxicity in rat cerebellar granule neurons (CGNs). T-006 was also found to promote neuronal differentiation in both PC12 cells and primary cultured rat cortical neurons. The results showed that the pretreatment of T-006 (0.01–1 μM) might prevent glutamate-induced neuronal loss in a concentration-dependent manner. T-006 is found to inhibit the over-activation of NMDAR and ensued calcium overload caused by glutamate. The following activation of phosphorylated extracellular signal-regulated kinase (ERK) were also abolished. Moreover, T-006 concurrently prevented the suppression of phosphorylated protein kinase B (Akt) and glycogen synthase kinase 3β (GSK3β). T-006 was also found to promote neurite outgrowth in PC12 cells and primary cortical neurons. In our study, T-006 (0.1–3 μM) dose-dependently stimulated neurite outgrowth in PC12 cells and the efficacy was comparable to nerve growth factor (NGF). Moreover, co-treatment of T-006 and NGF revealed that T-006 could robustly potentiate the NGF-induced neuritogenesis. Further signal transduction studies indicated that T-006 rapidly up-regulated phosphorylation of ERK but did not activate tyrosine kinase receptor A (Trk A). These findings offer deeper understanding of the anti-neurodegenerative activity of T-006 and provide insight into its possible therapeutic potential for AD treatment in light of the multipotent nature of T-006.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative brain disorder characterized by irreversible impairment of memory and cognitive function, in association with a large number of neuronal loss (Huang and Mucke, 2012). In aged societies, AD has been one of the most serious threats to human health and life

Abbreviations: TMP, tetramethylpyrazine; ROS, reactive oxygen species; RNS, reactive nitrogen species; CGN, cerebellar granule neuron; ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase 3β; NGF, nerve growth factor; dbcAMP, Dibutyryl-cAMP; Trk A, tyrosine kinase receptor A; NMDAR, *N*-Methyl-$_D$-aspartate receptor.

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http://dx.doi.org/10.1016/j.neuint.2016.07.006
0197-0186/

quality. The exact causes of AD are yet to be elucidated. A convincing body of evidence indicates that glutamate-induced neurotoxicity is one of the most important contributors to neuronal loss in AD (Bliss and Collingridge, 1993). Glutamate is an amino acid that serves as one of the main excitatory neurotransmitters and metabolite in the CNS. Normally, glutamate activates the post-synaptic *N*-Methyl-$_D$-aspartate receptor (NMDAR) and leads to the open of channel gate that allow the influx of Na^+ and Ca^{2+} (Olsen and Sonnewald, 2014). Under pathological stimuli, excessive release of glutamate triggers over activation of NMDAR, resulting in an augmented intracellular Ca^{2+} influx, which is the key mediator of glutamate-induced neural excitotoxicity. The disrupted Ca^{2+} homeostasis initiates a cascade of signaling pathways, leading to up-regulation of nNOS (Hu et al., 2013), dysfunction of mitochondria, ER stress and so on (Kritis et al., 2015). MAPK/ERK pathway (Jiang et al., 2000; Li et al., 2005) and PI3-K/Akt/GSK3β pathway (Pi et al., 2004) are also reported to be closely involved in the glutamate-induced excitotoxicity.

There is currently no approved causal treatment for AD. The chemical drug and antibody drug development pipelines seem to meet the bottle neck. Many researchers have put their attention on the natural and natural derived anti-AD agents, wishing to discover a disease-modifying drug. Focusing on the overall regulation of the pathophysiological condition of the body, traditional Chinese medicine has some advantage in comprehensive modulation of the multi-target diseases. Tetramethylpyrazine (TMP), the main active ingredient extracted from traditional Chinese medicinal herb Chuanxiong (*Ligusticum wallichii Franchat*), has been approved to treat ischemic cardiovascular and cerebrovascular diseases as a prescription drug in China for decades (Gao et al., 2015). It was also shown to have multiple functions, including prevention of oxidative stress-induced neuronal death, blockade of calcium channel, protection against kainite-induced excitotoxicity and enhancement of mitochondrial biosynthesis (Li et al., 2010; Liu et al., 2010; Tan, 2009) which all related with the pathogenesis of AD. In this study, our compound T-006 was designed by replacing the methoxyphenyl group of J147, an agent showed broad neuroprotective effects *in vitro* and *in vivo* (Cates et al., 2011; Chen et al., 2011; Prior et al., 2013), with TMP (see Fig. 1). Our previous study has shown that T-006 exhibited strong RNS and ROS scavenging activity and exerted protective effects in *in vivo* models of AD (Chen et al., 2015b). In this study, we evaluate the neuroprotective effects of T-006 against glutamate-induced excitotoxicity in CGNs as well as the underlying mechanisms.

2. Methods

2.1. Chemicals and reagents

Unless otherwise noted, all media and supplements used for cell cultures were purchased from Gibco (Carlsbad, CA, USA). PD98059 were obtained from Calbiochem (San Diego, CA, USA). SB415286, LY294002, wortmannin, K252a were obtained from Sigma Chemicals (St Louis, MO, USA). Antibodies against phospho-Ser473 Akt, phospho-Ser9 GSK3â, phospho-Thr202/Tyr204 p44/42 MAPK (ERK1/2), Bax and Bcl-2 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against â-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

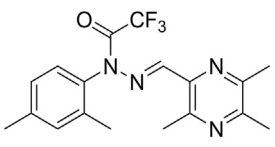

Fig. 1. Chemical structure of T-006 (Chen et al., 2015a).

2.2. Primary cell cultures

CGNs were prepared from 8-day-old Sprague–Dawley rats (The Hong Kong Polytechnic University) as described in our previous publication (Subramaniam et al., 2005). Briefly, neurons were seeded at a density of 2.7×10^5 cells/cm^2 in basal modified Eagle's medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, and penicillin (100 U/ml)/streptomycin (100 ìg/ml). Cytosine arabinoside (10 ìM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. With the use of this protocol, 95–99% of the cultured cells were granule neurons. All experiments were performed in CGNs at 8 days *in vitro* (DIV).

Fetal rat brains from Sprague-Dawley pregnatal rats (The Central Animal Facilities of Hongkong Polytechnic Univeristy) of 18 days of gestation were used for the primary culture of cortical neurons. The processing method is as previously described (Fu et al., 2006). Briefly, the freshly dissected brains were chopped and disassociated by incubation in 0.5% trypsin 37 °C for 15 min. The isolated neurons were resuspended in neurobasal medium containing 10% FBS, 0.25% glutamine and penicillin (100 U/ml)/streptomycin (100 ìg/ml). The cortical neurons were plated at a density of 4×10^5/ml cells. Half of the culturing medium were changed by Neuro-basal medium containing 1% B27, 2 mM glutamine and penicillin (100 U/ml)/streptomycin (100 ìg/ml) at the following day and half-changes of culture media were done twice weekly for maintenance of the neurons. At 3 days *in vitro* (DIV), cortical neurons were subjected to experiments. All experiments were conducted in accordance with the Hong Kong government Animals (Control of Experiments) Regulations and the protocols were approved by the animal subjects ethics sub-committee.

2.3. MTT reduction assay

Neurotoxicity was assessed using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide dye (MTT) assay. The assay was performed according to the specifications of the manufacturer (MTT Kit I; Boehringer Mannheim, Mannheim, Germany). Briefly, neurons were cultured in 96-well plates, 10 ìl of 5 mg/ml MTT labeling reagent was added to each well in 100 ìl of medium, and the plate was incubated for 4 h in a humidified incubator at 37 °C. After the incubation, 100 ìl of the solvating solution (0.01 N HCl in 10% sodium dodecyl sulfate (SDS) solution) was added to each well for 16–20 h. The absorbance of the samples was measured at a wavelength of 570 with 655 nm as a reference wavelength. Unless otherwise indicated, the extent of MTT conversion in cells exposed to different stresses is expressed as a percentage of the control.

2.4. Assay of neurite outgrowth in PC12 cells

The quantification of neurite-bearing cells was carried out as previously described (Hu et al., 2015). For the study of neuritogenesis-promoting activity of various compounds, PC12 cells were seeded onto 6-well plates at a density of 1×10^4 cells/well. 24 h after incubation, cells were switched to low-serum medium (DMEM supplemented with 0.5% FBS and 0.5% HS) containing various compounds that were renewed every 2 days. After treatment, neurite-bearing PC12 cells were observed and photographed using a light microscope equipped with a phase-contrast condenser, a 10 × objective lens and a digital camera. Cells were scored as positive for neurite outgrowth if at least one neurite was longer than the diameter of the cell body. The neurite