THE POTENT NEUROPROTECTIVE ACTIVITIES AND UNDERLYING MOLECULAR MECHANISMS BY T-006: A NOVEL ANTI-ALZHEIMER'S AGENT DERIVED FROM CHINESE MEDICINE

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The Potent Neuroprotective Activities and Underlying Molecular Mechanisms by T-006: A Novel Anti-Alzheimer's Agent Derived from Chinese Medicine

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CERTIFICATE OF ORIGINALITY

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XU Daping

Abstract

Backgrounds: Alzheimer's disease (AD) is a progressive neurodegenerative disorder, featured by progressive impairment of memory and cognitive function. The explicit pathogenesis mechanism underlies AD still remain unclear. Nevertheless, it is well accepted that increased oxidative stress, inappropriate neuronal apoptosis and impairment in neurogenesis and neuronal differentiation is intimately related with the occurrence and progression of AD. Current single target drugs exert only limited therapeutic effect to patients and the new paradigm of multi-functional compound development may open up new horizon on our way of defeating AD.

Here in this research, we jointly synthesized a Chinese medicinal component tetramethylpyrazine with J147, another multi-potent compound, and yield a novel agent with potential anti-AD activity named T-006.

Methodology: Various assessments and experiments were undertaken to investigate the pharmacological activity of T-006. In *in vitro* models, MTT and LDH assay were used to determine cell viability and FDA/PI/Hoechst staining were used to exhibit apoptosis and necrosis. Intracellular ROS, RNS and calcium were detected by fluorescent probes. Immunostaining is conducted to manifest neuronal differentiation. Various chemical inhibitors and western blot were employed to interrogate the signaling pathways

underlying T-006's protection. In *in vivo* model, APP/PS1 transgenic mice were used to probe the anti-AD effects of T-006.

Results: T-006 firstly showed free radical scavenging ability by suppressing the overproduction of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS). Then, T-006 protected rat cerebellar granule neurons (CGNs) against glutamate-induced excitotoxicity with a EC_{50} value of 59.4nM, which was much more potent than the clinical available NMDA receptor blocker memantine. Further study indicates that T-006 blocked the over-activation of NMDA receptor and ensued calcium influx. The protection of T-006 is also achieved through the concurrent regulation of ERK and PI3-K/Akt/GSK-3β pathway. The anti-AD effects of T-006 were then confirmed in huAPPswe/PS1ΔE9 transgenic AD mice model.

Summary and prospects: T-006 shows radical scavenging activity as well as excitotoxicity inhibition effect. The amelioration of learning and memory are also found in T-006-treated *in vivo* AD model. Above results 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. To develop T-006 into an anti-AD candidate, further researches are needed to determine its bioavailability and pharmacodynamic features as well as the more detailed mechanisms underlying its neuroprotection.

(386 words)

List of publications

1. **Xu DP**, Chen HY, Mak SH, Hu SQ, Tsim WK, Hu YJ, Sun YW, Zhang GX, Wang YQ, Zhang ZJ, Han YF. Neuroprotection against glutamate-induced excitotoxicity and induction of neurite outgrowth by T-006, a novel multifunctional derivative of tetramethylpyrazine in neuronal cell models *Neurochemistry International, 2016 Oct;99:194-205*

2. Chen HY *, **Xu DP** *, Tan GL, Cai W, Zhang GX, Cui W, Wang JZ, Long C, Sun YW, Yu P, Tsim KW, Zhang ZJ, Han YF, Wang YQ.A Potent Multi-functional Neuroprotective Derivative of Tetramethylpyrazine. *J Mol Neurosci.* 2015 56: 977. doi:10.1007/s12031-015-0566-x *(*equal contribution)*

3. **Xu DP**, Duan H, Zhang Z, Cui W, Wang L, Sun Y, Lang M, Hoi PM, Han YF, Wang Y, Lee MY. The novel tetramethylpyrazine bis-nitrone (TN-2) protects against MPTP/MPP+-induced neurotoxicity via inhibition of mitochondrial-dependent apoptosis. *J Neuroimmune Pharmacol. 2014 Mar;9(2):245-58.*

4. Guo B *, **Xu DP***，Duan H, Du J, Zhang Z, Lee MY, Wang Y. Therapeutic effects of multifunctional tetramethylpyrazine nitrone on models of Parkinson‘s disease in vitro and in vivo. *Biol Pharm Bull. 2014;37(2):274-85. Epub 2013 Dec 3. (*equal contribution)*

5. **Xu DP**, Zhang K, Sun Y, Guo B, Hoi PM, Han YF, Wang Y， Lee MY， Zhang Z. A novel tetramethylpyrazine bis-nitrone (TN-2) protects against 6-hydroxyldopamine-induced neurotoxicity via modulation of the NF-κB and the PKCα/PI3-K/Akt pathways. *Neurochemistry International 2014 Dec;78:76-85.*

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List of Abbreviations

Aβ	Amyloid β peptide
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	cholinesterase inhibitor
AD	Alzheimer's disease
ADDL	Aβ-derived diffusible ligand
ALS	Amyotrophic Lateral sclerosis
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Atr	Atropine
BBB	blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
cdk-5	cyclin-dependent protein kinase-5
CGN	Cerebellar granule neuron
ChAT	Choline acetyltransferase
CMA	Chaperon mediated autophagy
CNS	Central nervous system
dbcAMP	2'-O-dibutyryladenosine 3':5' cyclic

	monophosphate
DHE	Dihydro-β-erythroidine
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
E2020	Donepezil
EGCG	Epigallocatechin gallate
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FDA	Fluorescein diacetate
GABA	γ-aminobutyric acid
GAP-43	Growth associated protein 43
Glu	Glutamate
GSK3 β	Glycogen synthase kinase 3β
HD	Huntington's disease
ITI	Inter-trial interval
JNK	c-Jun N-terminal kinase
L-NMMA	N^G-monomethyl-L-arginine
LRRK2	leucine-rich repeat kinase 2
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MEM	Memantine

MK801	5H-dibenzo[a, d]cyclohepten-5, 10-imine
MLA	Methyllycaconitine
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide
MWM	Morris Water Maze
NFT	Neurofibrillary Tangle
NGF	Nerve growth factor
NMDA	*N*-methyl-$_{D}$-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NSAIDS	Non-steroidal anti-inflammatory drugs
NSC	Neural stem cell
PBS	Phosphate-buffered saline
PD	Parkinson’s disease
PDGF	Platelet-derived growth factor
PI3-K	Phosphatidylinositol 3-kinase
PS	Presenilin
PVDF	Polyvinylidene fluoride
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SOD1	Antioxidant superoxide dismutase 1
TMP	Tetramethylpyrazine
TrkA	Tropomyosin receptor kinase A
UCH-L1	Ubiquitin-C-terminal hydrolase-L1
UFO	Uncompetitive NMDA receptor antagonist with Fast Off-rate
UPS	Ubiquitin-proteasome system
V	Voltage
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2

Chapter 1
Introduction

Overview

Development of the modern society with the advent of medical care prolongs the life expectancy of human and brings about the aging of the population. The prevalence of the neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic Lateral sclerosis (ALS) considerably increased during the past decades. Currently, neurodegenerative diseases are still regarded to be fetal and all available treatments are symptom-oriented, unable to prevent, halt or cure the diseases. Between 2000 and 2013, deaths caused by AD increased by 71%, while the death number attributed to heart diseases (the No.1 killer) decreased 14%. Neurodegenerative disorders, albeit varied in manifestation, share similar pathological character of progressive neuronal loss in specific regions in the central nervous system (CNS). Although the pathogenesis of the disease is still elusive, oxidative stress is a vital common feature and a crucial contributor as substantiated by accumulating lines of evidences. Cumulative oxidative stress, produced by free radicals or other non-radical reactive agents, induce lipids oxidation, impairments of DNA repair system and mitochondria dysfunction, thus accelerates the aging process and development of neurodegenerative diseases. A convincing body of evidence indicates that glutamate-induced neurotoxicity is also an important contributing factor in

AD (Bliss et al. 1993; Olsen et al. 2014). Due to the over-activation of NMDA receptors and L-type voltage-sensitive calcium channels, a massive influx of calcium is triggered in the neurons and thus cause glutamate excitotoxicity. The calcium overload initiate a series of cytoplasmic, mitochondrial and nuclear pathological processes that promote neuronal cell apoptosis and death. Therefore, agents with anti-oxidative and neuroprotective properties may confer therapeutic benefit to the prevention and treatment of AD and other related neurodegenerative disorders.

In addition to the neuroprotection strategy, renewal of the damaged neurons by induction of neurogenesis is considered to be another therapeutic approach with great potential. Neurotrophic factors, such as nerve growth factor (NGF)(Pardridge 2002), brain-derived nerve neurotrophic factor (BDNF)(Mattson 2008) and fibroblast growth factor-2 (FGF-2)(Sarchielli et al. 2014) are found to stimulate neurogenesis and maintain neuronal functions. Decrease of neurotrophic factor levels are found in AD brains and replenish of them are shown to be protective and restorative. However, clinical testing of the growth factors is constrained by the poor penetration through blood-brain barrier (BBB) due to its peptidyl nature. Thus small molecules with neurogenesis or neuronal differentiation-promoting property might have therapeutic usage in AD and other related neurodegenerative diseases.

T-006, the novel compound under investigation in this thesis, is

synthesized by conjugation of J147, which showed broad neuroprotective effects *in vitro* and *in vivo* (Cates et al. 2011; Chen et al. 2011; Prior et al. 2013), and tetramethylpyrazine (TMP), the main active ingredient extracted from traditional Chinese medicinal herb Chuanxiong (*Ligusticum wallichii Franchat*). The structural modification is aimed to augment the bioactivity and discover new multi-functional drug candidate for the treatment of neurodegenerative diseases.

In the following literature review section, AD is introduced, as the representative of neurodegenerative diseases, in terms of epidemiology, etiology, pathogenesis with current available clinical treatments and diagnostic methods. The relationship between AD and oxidative stress, NMDA receptor over-activation, neuronal apoptosis and neuronal differentiation is equivalently presented as background knowledge for this study. The features of another two neurodegenerative disorders (PD and ALS) are also briefed to offer an all-round picture of neurodegenerative disorders as well as further highlight the vital role of oxidative stress, apoptosis and neurogenesis. The research result of T-006 are subsequently presented and discussed in detail.

1.1 AD

German neurologist and psychiatrist Alois Alzheimer first recorded a rapid progressing memory loss of his 52-year old patient, thereafter, extensively described as a dementia syndrome at the beginning of the 20th century(1987). After more than a century, AD has become the most prevalent neurodegenerative disease and major cause of dementia as well as a great healthcare challenge in the 21st century. AD prevalence shows an age-dependent manner, affecting 7 - 10% of the population over age 65 and about 40% of individuals over age 80 (Thies et al. 2011).In the USA, someone develops AD every 67 seconds(Alzheimer's_association 2015) and between 2003 to 2013, deaths attributed to AD increased 71 percent(Alzheimer's_association 2015; Hebert et al. 2001).China, starting with a high base of AD patients (6 million) will experience a rapid increase growth (314 - 336%) and reach 26.1 million on 2040 due to the aging of the population(Hampel et al. 2011). Besides posing devastating damage to patients themselves, AD is also becoming a financially critical problem for the society. About 13% of population aged over 65, according to recent estimation, has AD; and the percentage tops at 43% for population aged over 85. In addition, AD is one of the most costly diseases in the world. The long-term healthcare is needed besides the intense attention on AD patients. The total cost on AD patients aggregated to 226 billion USD in 2015 despite more

than 15 million unpaid caregivers, mostly family members of the patients(Alzheimer's_association 2015). A foreseeable worse situation is inevitable in the future if not more actively dealt with. The irreversible aging of population will lead to increasing AD cases in the future. It is shown in the recent projection that total cost of AD in 2050 is estimated to be 1.1 trillion USD.

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 in hippocampal and cortical region (Michaud et al. 2012). Early clinical manifestation of AD is often difficulty in remembering new information such as recently happened event or conversations. Later symptom includes impaired communication, confusion, poor judgement and ultimately difficulty speaking, swallowing and walking (Albert et al. 2011). In AD brain, progressive neuronal loss, extracellular amyloid aggregation along with its end-product senile plaques and intracellular neurofibrillary tangles are widely accepted as the pathological hallmarks of AD. Of note, the changes of brain on cellular level may begin 20 or more years as early before symptom appear (Jack et al. 2009; Villemagne et al. 2013).

1.1.1 Risk factors

1.1.1.1 Age

Age is still the leading risk factor of AD. In late-onset AD, which takes up the vast majority of AD cases, the risk correlate with the increase of age after 65 and rise up to 40% in the population over 80. Nevertheless, AD is not a normal part of aging and age along is insufficient to cause the disease.

1.1.1.2 Genetics

Based on upon age of onset and familial aggregation, AD is divided into two types: early-onset familial AD and late-onset sporadic AD. Unlike the much more prevalent sporadic AD, familial AD only takes up 1% of the total AD cases and shows autosomal-dominant inheritance features with the onset before 60-65 years of age. Mutations in three genes: *APP, PS1* and *PS2* are shown to cause this subtype of AD and all this three genes found to be involved in the production or processing of amyloid-β peptide, the principle component of senile plaques(Goate et al. 1991; Levy-Lahad et al. 1995; Sherrington et al. 1995). The *APP* gene, encodes amyloid precursor protein itself and *PS1, PS2* encodes the proteins that form catalytic center of the γ-secretase complex, which plays vital role in Aβ over-production. Of note, these genetic mutations in familiar AD have been used to create animal model of AD which is widely used on the bench of AD research.

Despite the early success in discovering genetic linkage in the familiar

early-onset AD, the sporadic late-onset AD, which is the vast majority of AD case, displays heterogeneous and complex nature in terms of genetic underpinning. During the last 30 years, extensive studies have been conducted to uncover the elusive AD genes, identifying more than 500 genes that are thought to be potential risk factors. However, the only strongly confirm risk factor is apolipoprotein E (*APOE*) genotype, with the ε4 allele being risky and the ε2 allele being protective (Corder et al. 1993; Strittmatter et al. 1993). *APOE*4 homozygotes has a life-time risk of AD of 50% and heterozygotes 20-30%, compare with the overall risk of 11% for men and 14% for women irrespective of *APOE* genotype. In recent years, genome wide association study, driven by high-throughput genotyping technology has greatly propelled genetic research in AD and suggested gene variations affecting more molecular pathways other than Aβ are implicated in the etiology of AD(Fig.1.1)(Jones et al. 2010).

1.1.1.3 Family history

Late-onset AD is also called sporadic AD, which means family history is not necessary for the occurrence of the disease. However, individuals are more likely to develop AD when there is one AD patient as a first degree relative and when there is more than one patient, the risk is even higher (Green et al. 2002; Lautenschlager et al. 1996).

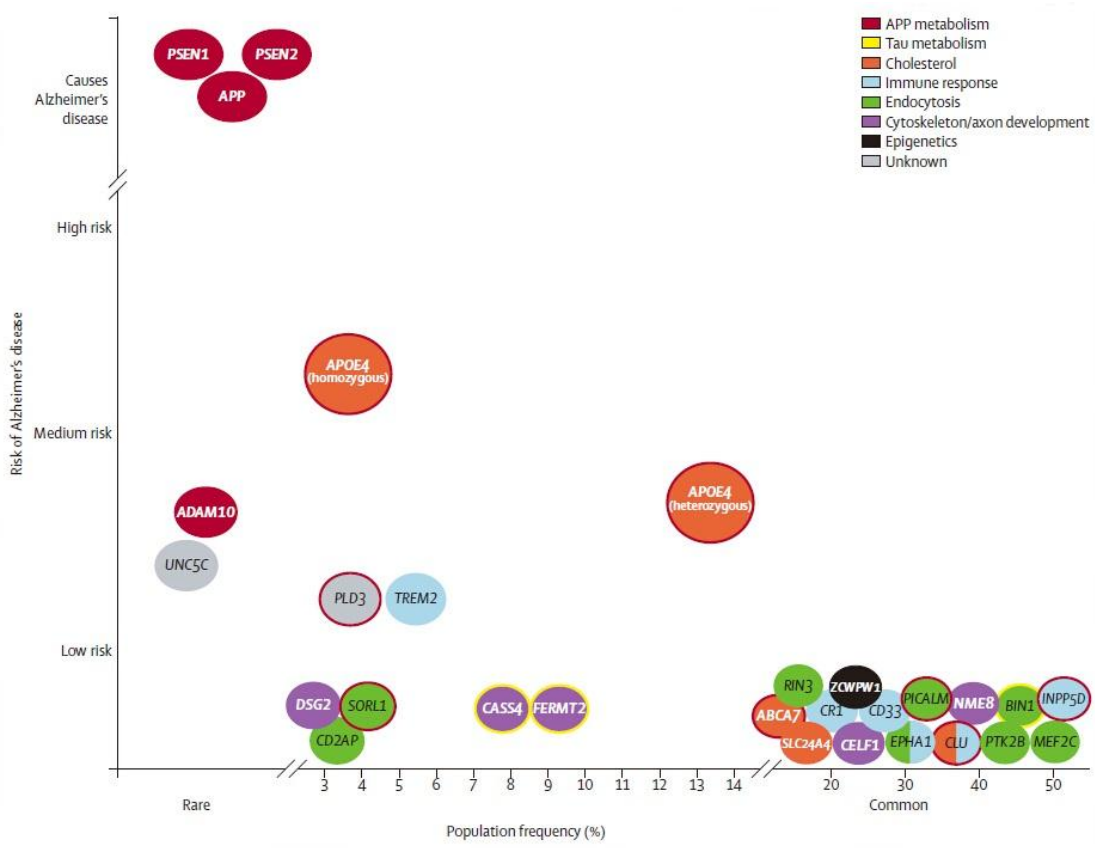


Fig.1.1 Schematic overview of genes linked to AD The colors in the key show the pathways in which these genes are implicated. The central role of APP metabolism is demonstrated in this figure. The figure was adapted from Karch et al (Karch et al. 2015).

1.1.1.4 Education

Years of normal education is another indicator for the risk of AD. People with fewer years of education, compared with those with more years of education, are found to be more prone to develop AD (Sando et al. 2008; Stern 2012). The hypothesis of "cognitive reserve" was proposed to address this situation, stating that more years of education enables brain to build more connections between neurons that provide alternate route for the neuron-to-neuron linkage and compensate to the early neuronal change in AD(Roe et al. 2007; Stern 2006). Some scientists also put up other explanations for this phenomenon including lower socioeconomic statue-caused downgrade of healthcare and lack of mental stimulation in the context of occupation.

1.1.1.5 Cardiovascular disease risk factors

Cardiovascular disease is not only the current top killer among all diseases, but also, suggested by growing evidences, closely linked with AD occurrence(Ronnemaa et al. 2011). Brain is an organ that nourished by the most sufficient blood flows in the body and a healthy heart guaranteed enough blood to meet the oxygen and energy need of nervous system. AD and cardiovascular disease also share some risk factors including smoking (Anstey et al. 2007), midlife obesity(Loef et al. 2013), diabetes(Gudala et al. 2013) and midlife high cholesterol(Solomon et al. 2009), which further prove the relationship of the two diseases.

1.1.2 Hypothesis for the pathogenesis of AD

1.1.2.1 Cholinergic dysfunction and AChE toxicity

Dysfunction of cholinergic system is among the earliest pathological findings of AD (Bowen et al. 1983; Davies et al. 1976)(Fig.1.2). Cholinergic neuron loss together with reduced acetylcholine (ACh), choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) are found to be correlated with the dementia severity and thus became the corner stones of the cholinergic hypothesis, the first hypothesis of AD pathogenesis(Auld et al. 2002). Initial proposal of the “cholinergic hypothesis” lead the direction of pharmarcotherapeutic development strategies with the outcome of cholinesterase inhibitor (AChEIs), which was the only class of FDA approved drugs for AD treatment until 2003. However, clinical observations and recent studies revealed only modest cognitive recovery along with unfavorable side effects of the AChEI therapy, which implies the cholinergic system impairment a consequential and un-central, rather than causative and central role in the pathogenesis of AD(Del Villar et al. 2004; Mega et al. 1999).

AChE is previously regarded as an enzyme with the only function of ACh hydrolyzing. However, recent studies indicate that AChE is far more capable than ACh termination. Intracellular AChE is found to mediate neuronal

apoptosis induced by different stimuli (Zhang et al. 2002). AChE activity is also observed to be increased around the Aβ plaques, despite the overall depressed expression of it(Talesa 2001). Moreover, AChE interacts with Aβ and increases the aggregation of abnormal Aβ protein. Therefore, AChE inhibitor could ameliorate the development of AD by both restoring cholinergic system and inhibiting Aβ aggregation.

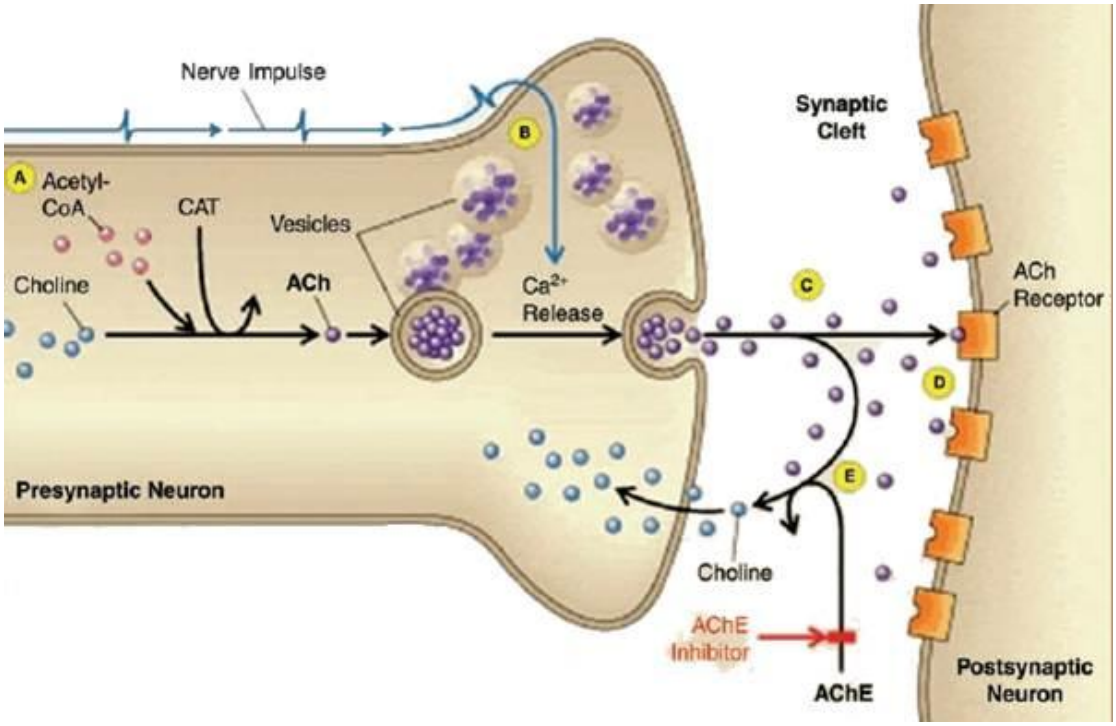


Fig.1.2 Schematic overview of the Cholinergic hypothesis in AD.

Acetyl-CoA and choline is synthesized to ACh by ChAT. B. Synthesized ACh is sealed into the vesicles. C. When action potential reaches the terminal, extracellular Ca^{2+} influx triggers the release of ACh. D. After release, ACh may bond to and activate Ach Receptors. E. ACh is efficiently split by AChE into choline and acetate, which reuptake by presynaptic neuron. The figure is adapted from Craig et al.(Craig et al. 2011)

1.1.2.2 Aβ cascade hypothesis

Aβ is a ～4 KD peptide derived by endoproteolysis from amyloid precursor protein (APP). APP is sequentially cleaved by β- and γ-secretase rather than α-secretase to form Aβ. Senile plaques, one of the major pathological feature of AD, is composed of Aβ fibrils(Masters et al. 1985). Researcher found that mutations in genes encoding APP and γ-secretase result in the over-production of Aβ in familiar early-onset AD patients (Cai et al. 1993; Citron et al. 1992). The genetic linkage of APP mutation and AD occurrence strongly supported the amyloid hypothesis, stating that Aβ peptide plays central role in the pathogenesis of AD and is the proximal cause of multiple AD pathological hallmarks. It is bolstered by the lines of evidences that Aβ fibrils are toxic in cultured neurons(Pike et al. 1991) and addition of Aβ augmented tau pathology in mice models (Gotz et al. 2001).

Although, AD is characterized by the extracellular deposition of senile plaques composed of Aβ fibrils, current researches indicates that the extent of amyloid plaque pathology is poorly correlated with clinical severity of dementia and in AD patients and animal models, the occurrence of senile plaques is not accompanied by NFT all the time, which indicate that deposit of Aβ fibrils is not causative events in AD(Golde 2003). Increasing lines of evidences suggest that soluble Aβ oligomers are the pathogenesis factor to induce synaptic loss and progressive cognitive decline, that leads to an

modification of the Aβ hypothesis(Tu et al. 2014). Aβ oligomers, also termed as Aβ-derived diffusible ligands (ADDLs) are found to be more readily assembled by Aβ1-42 peptides. In human AD postmortem brains, Aβ oligomer level is found to be closely paralleled with synaptic reduction and cognitive impairments in the same area of brain, which is further confirmed in transgenic mouse models that Aβ oligomer over-production induces synaptic loss(Selkoe 2002).

1.1.2.3 Tau phosphorylation

Tau protein is one of the three microtubule-associated proteins, maintaining neuronal microtubule network. Tau protein pathology, featured by the intracellularly accumulated neurofibrillary tangles, is a hallmark of AD (Fig.1.3). To date, *in vitro* and *in vivo* studies show that dysfunctions in tau protein are attributed to the abnormal hyperphosphorylation of tau, which results the "imbalance of between the activities of tau kinases and tau phosphatases"(Raskin et al. 2015). Among these enzyme, glycogen synthase kinase-3 (GSK-3) and cyclin-dependent protein kinase-5 (cdk5) is found to be associated with all stages of neurofibrillary pathology in AD (Pereira et al. 2005; Perez et al. 2003).

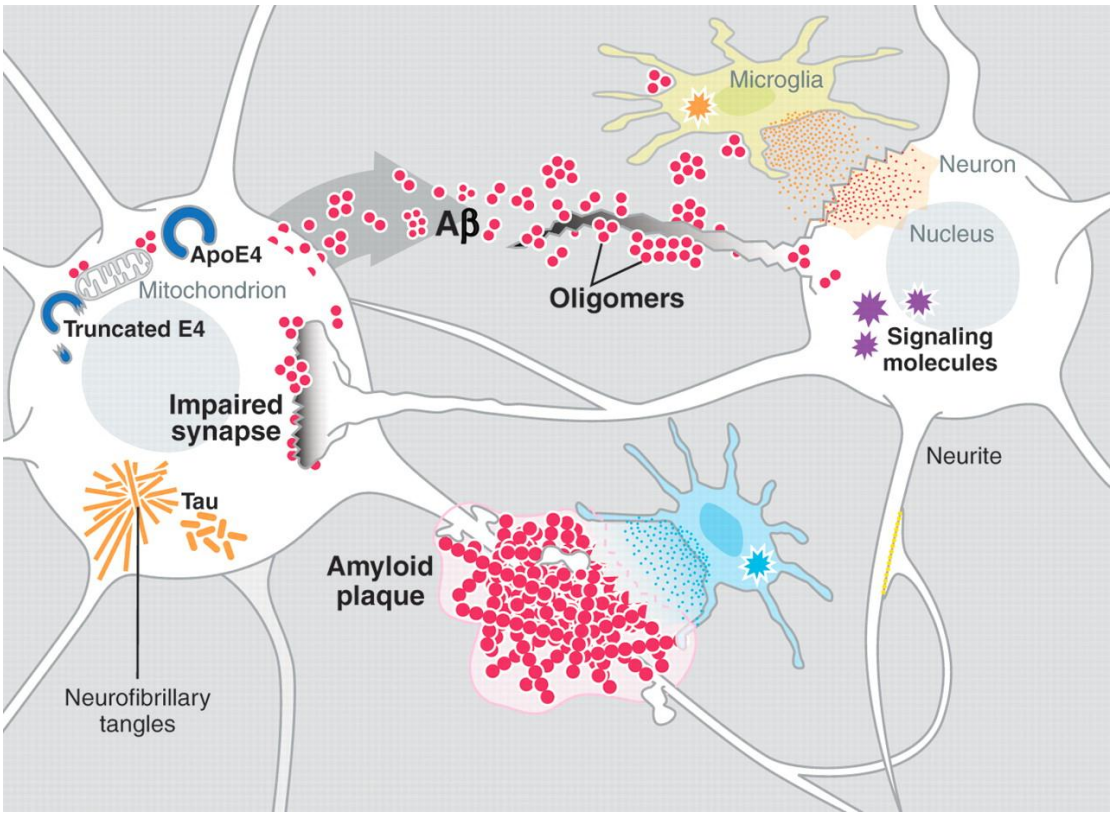


Fig.1.3 Aβ and tau protein presumed to participate in AD pathogenesis

In neurons and other brain cells, Aβ peptides are produced and may aggregate into a variety of assemblies including Aβ oligomers, fibrils or amyloid plaques. Tau promotes Aβ-induced neuronal injury and also exerts independent adverse effects. Microglias, depending on the signaling cascades and functions engaged, could be beneficial or harmful. The figure is adapted from Roberson et al. (Roberson et al. 2006)

1.1.2.4 Glutamate excitotoxicity

Glutamate is an amino acid that serves as one of the main excitatory neurotransmitters and metabolite in the CNS and involves in all aspects of cognition and forming of memory. The N-Methyl-D-aspartate receptor (NMDAR) which locates in the post-synaptic membrane is activated by glutamate and thus triggers the influx of Na^+ and Ca^{2+} (Olsen and Sonnewald 2014). However, under pathological condition, 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). Under pathological stimuli, excessive release of glutamate triggers over activation of NMDAR, resulting in an augmented intracellular Ca^{2+} influx. The elevated Ca^{2+} level mediates the glutamate-induced neural excitotoxicity. Also, the overproduction of NO, mitochondria malfunction, ER stress and eventually neuronal apoptosis are all attribute to the disrupted Ca^{2+} homeostasis and the ensued cascade of signaling pathways(Hu et al. 2013)(Kritis et al. 2015)(Fig.1.4). Post mortem examination shows that glutamatergic pyramidal neurons, as well glutamate receptors undergo substantial loss in the cerebral cortex and hippocampus in AD(Morrison et al. 1997). Moreover, a dysfunction of remaining neurons, caused by the decreased reuptake of glutamate by glial cells, is also discovered in several brain regions (Seki et al. 2008).

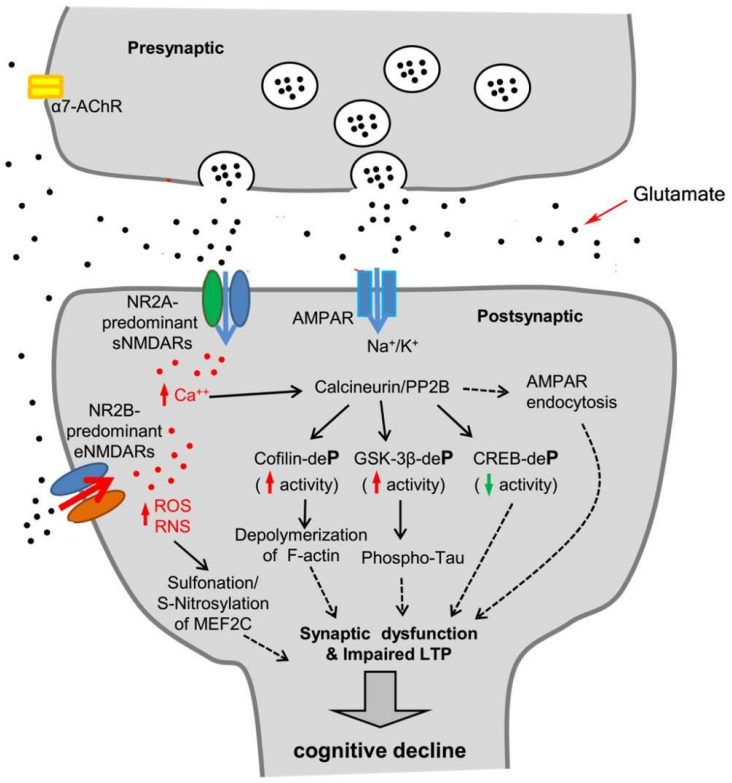


Fig.1.4 receptors and pathways involved in the glutamate-induced excitotoxicity.

Over flow of glutamate caused excitotoxic neuronal cell death. The excitotoxic neuronal death is mediated in part by overactivation of NMDA-type glutamate receptors, followed by the overproduction of NO, mitochondria malfunction, ER stress and eventually neuronal apoptosis, which are all attribute to the disrupted Ca^{2+} homeostasis and the ensued cascade of signaling pathways. The figure is adapted from Wang et al(Wang et al. 2010).

1.1.2.5 Oxidative stress and metal ion homeostasis

Consuming 20% of the body oxygen, brain has a high level of oxygen demand.

Oxidative stress is suggested to play a critical role in neuronal damage and

the resultant progression of AD. It occurs early in the AD brain, even before the significant plaque pathology onset (Perry et al. 1998). A variety of oxidative stress markers are found in the AD brain, especially in Aβ plaques and NFTs (Butterfield et al. 2003). Increased levels of byproducts of lipid peroxidation are found in urine and patients of AD. And antioxidants treatment may delay the progression of AD (Sano et al. 1997).

One source of oxidative stress in AD is proposed to be Aβ. Aβ induces oxidative stress from its methionine residue 35, and cause neuronal apoptosis by consequence generation of ROS in hippocampal neurons and cortical synaptosomal membranes (Tamagno et al. 2003). Further, Aβ-induced oxidative stress can dys-regulate the functions of synaptic and promotes Aβ deposition, up-regulates genes related to apoptosis and mitochondrial metabolism in transgenic APP mice (Reddy et al. 2004). Moreover, oxidative stress can also induce the production of Aβ; aggravate amyloidogenesis and phosphorylation of tau protein, which induce a vicious cycle of pathogenesis of AD(Radi et al. 2014)(Fig.1.5).

Impaired homeostasis of metal ion is also an important feature of enhanced oxidative stress in the AD brain. A wealth of studies show the increased level of redox-active metals such as iron and copper, which both capable of incite various oxidative stresses including free radical formation, lipid peroxidation and DNA oxidation(Jomova et al. 2010). Furthermore,

copper, which bind to Aβ with a high affinity, forms hyper-metalized Aβ and resistant to cellular clearance (Hung et al. 2010).

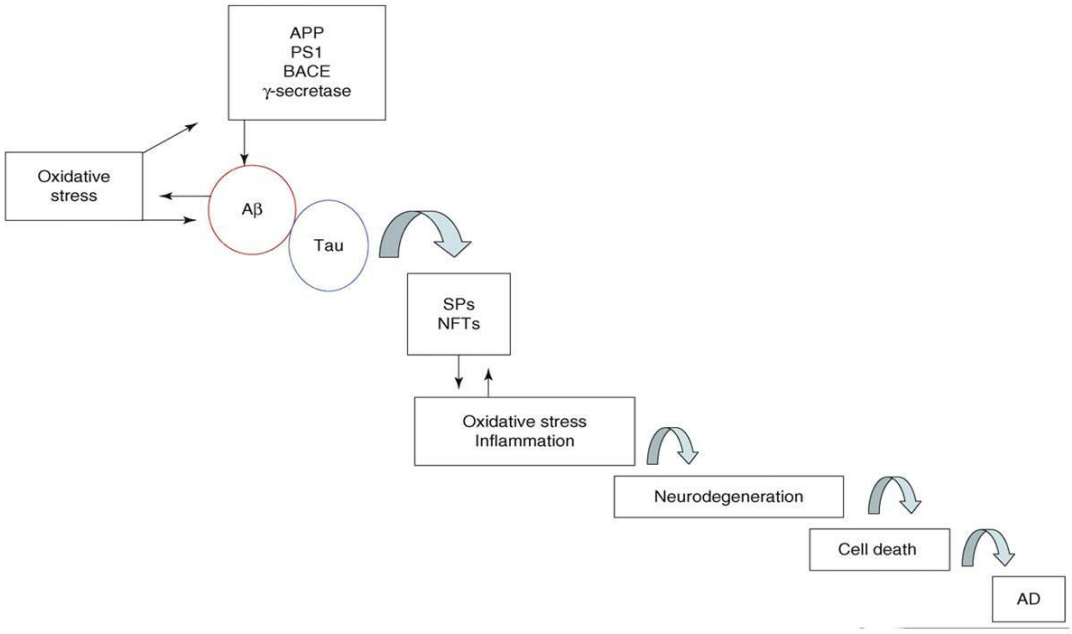


Fig.1.5. Oxidative stress plays a vital role in the progression of AD. APP levels are increased by oxidative stress and thus Aβ production is promoted. Aβ itself, with oxidant ability, creates a vicious cycle on APP levels and oxidative stress, which leads to neuron degeneration.

1.1.2.6 Neurotrophic factor deficiency

Neurotrophic factors are a family of small versatile proteins that play vital roles in neuronal survival, differentiation, growth and even apoptosis under physiological condition(Schindowski et al. 2008). Among these neurotrophins, NGF and BDNF are found to be closely related with cholinergic system dysfunction and pathogenesis of AD. As the founding member of the family, NGF was identified in 1950's and extensively studied thereafter. It is well established that NGF could promote survival in basal forebrain cholinergic neurons and increase activity of ChAT enzyme(Allen et al. 2013). The transgenic mouse model of NGF deficiency demonstrates cholinergic neuronal loss and memory decrease as well as other AD-like pathology such as senile plaques and NFT(Capsoni et al. 2000). ProNGF, the precursor form of NGF is also discovered. Unlike mature NGF, which bind TrkA, TrkB or TrkC receptors, proNGF bind with $p75^{NTR}$ and induces apoptosis. In AD models, the ratio of proNGF/NGF increases, which is correlated with the progression of neuronal death.

1.1.3 Treatments of AD

Although the exact mechanism of AD remains elusive, the huge and increasing number of AD patients represents enormous need for anti-AD drug, which seems to be good opportunity for drug companies and institutions.

However, past a few decades witnessed AD became a graveyard for costly drug development and clinical tests. It is shown that during 2002 and 2015, 244 compounds were tested in 413 clinical trials and only 1 drug is approved by FDA: a failure rate of 99.6%. Meanwhile the failure rate of anti-cancer drug is 81%(Rafii et al. 2015). Current pharmacological approaches, including clinically approved and treatment developing in the pipeline, can be broadly grouped into 3 parts: symptomatic treatment, disease modifying treatment and preventive treatment.

1.1.3.1 AChE inhibitors

Due to the longest history of cholinergic hypothesis for pathogenesis of AD, AChE inhibitors have been the most widely investigated. The fact that 4 out of 5 FDA approved drugs including Cognex® (tacrine), Aricept® (donepezil), Razadyne® (galantamine) and Exelon® (rivastigmine) are AChE inhibitors reflects the success of them, albeit tacrine is now rarely used due to its hepatotoxicity.(Michaelis 2003). Although clinical use of AChE inhibitors in mild to moderate AD patients shows modest but reproducible effects in cognitive and memory functions, the improvement is proved to be impermanent, unable to halt the progression of AD or prolong the survival period of patients. Besides the above mentioned FDA approved drugs, huperzine A, an active *Lycopodium* alkaloid isolated from Chinese herb

Huperzia serrata (Thunb) Trev (Qian Ceng Ta) was also proved to be a "potent, selective and well-tolerated inhibitor of AChE" (Zhang 2012). Huperzine A has been widely used in china but unable to be commercialized worldwide due to the patent issues.

1.1.3.2 NMDA receptor antagonist

As mentioned above, impairment of glutamatergic neurons is involved in the progression of AD. Development of NMDA receptor antagonist is another route for the discovery of anti-AD drug. Due to the wide-spread nature of NMDA receptor and glutamatergic neurons in the neocortex and hippocampus(Fonnum 1984), many agents failed in clinical tests as a result of serious side-effects until memantine, an uncompetitive, moderated affinity antagonist of NMDA receptor with a fast on-off rate was discovered (Lipton et al. 2004). As the latest FDA approved drug, memantine is also the only drug for the treatment of moderate to severe stage of AD (Hellweg et al. 2012). Given the linkage of excitotoxicity and protein aberrant in AD, memantine is also found to inhibit Aβ-induced cytotoxicity *in vitro* and memory deficits *in vivo*(Lipton 2006).

1.1.3.3 Therapies targeting Aβ and tau

Amyloid hypothesis is the most prevailing theory of AD pathogenesis by now. Given the pivot role of Aβ in AD, the whole amyloid cascade, from

Aβ production to aggregation until Aβ clearance, has been a constant focus for drug developers.

The first strategy is the blocking of Aβ generation. As previously mentioned, sequential cleavage of APP by β- and γ-secretase leads to the production of Aβ. Hence, a number of compounds was created aiming for the inhibition of β-secretase (BACE-1) and γ-secretase while vast majority of which failed in pre- or clinical tests due to the substrate complicacy of both enzymes. Among the survivors, a small-molecular inhibitor of BACE1 name MK-8931 has entered clinical phase III, showing significant CSF Aβ level decrease(Forman et al. 2012; Stone et al. 2013).

The idea of passive anti-amyloid immunotherapy comes from the discovery in which immunization of Aβ shows decreased amyloidogenesis and improved cognitive function in mouse models of AD. Thereafter, a number of monoclonal antibodies targeting various domains of Aβ have been developed and show anti-aggregation and Aβ clearance activity. The representatives are solanezumab(Doody et al. 2014), crenezumab(Cummings et al. 2014) and gantenerumab(Panza et al. 2014).

Besides above, active anti-amyloid immunotherapy, in another word vaccines, has also been developed and entered clinical trials as well as antibodies targeting tau protein(Rafii and Aisen 2015).

1.1.3.4 Antioxidant

Brain tissue is found to be more vulnerable to free radicals and in the pathogenesis hypothesis of AD, oxidative stress stands in an important place as it can be the mediator of a few other pathogenic factors and induces progressive neuronal death. Therefore, the possible benefit of antioxidants in treatment of AD has been studied. Vitamin C (ascorbic acid) and vitamin E, the representatives of aqueous phase antioxidant and lipophilic antioxidant, has been tested in several *in vitro*, *in vivo* and clinical studies(Butterfield et al. 2002; Kontush et al. 2001; KONTUSH et al. 2004). Plant extracts with anti-oxidative stress ability has also been used widely and the usage is differed from culture to culture. In China, people studies herbal medicine with intelligence- or memory-enhancing functions for thousands of year and discovers numerous plants, such as *ginkgo biloba*, with antioxidant and neuroprotective properties. Egb761 is an extract of *ginkgo biloba* and has been approved for the treatment of dementia in Germany. In clinical tests, Egb761was proved to increase cognitive function in AD patients (Le Bars et al. 1997). Variety of nutraceuticals with anti-oxidative activity i.e. curcumin, apigenin, epigallocatechin gallate (EGCG) and resveratrol also arouse interest of researcher in their potential for AD prevention and treatment (Kim et al. 2015).

1.1.3.5 Neurotrophic factors

Neurotrophic factors represent for a family of proteins which is essential in neuronal survival, differentiation, growth and even apoptosis. The linkage of neuronal disorders and the deficiency of neurotrophic factors in brain indicate a high therapeutic potential. As the representative of neurotrophins, NGF is transported to basal forebrain cholinergic neurons through axon and replenish of NGF by injection ameliorates the neuronal loss and impaired learning and memory in rats AD models(Kromer 1987; Williams et al. 1986). However, clinical testing of the growth factors is constrained by the poor penetration through BBB due to its peptidyl nature. In recent years, advantage in NGF administration has been pursued. NGF gene therapy, in which fibroblasts genetically modified to express mature NGF are implanted into the forebrain of AD patients, has been tried and lead to significant cholinergic neuron growth and present a new possibility in therapeutic use of neurotrophins(Tuszynski et al. 2005).

1.1.4 Neuronal apoptosis

1.1.4.1 Apoptosis in AD

In AD, apoptosis is the leading type of death in functional neurons (Schindowski et al. 2003). In the AD brains, apoptosis markers such as increased DNA fragments, activated caspases and altered expressions of members of the Bcl-2 family have been found in the process of neuronal death

(Radi, Formichi et al. 2014). However, by activating pro-survival signals, neurons may still be rescued in the middle of apoptosis. Thus the prevention or delaying of neuronal apoptosis has become a focus regarding the search of AD therapy (Huang et al. 2012).

1.1.4.2 Oxidative stress-induced apoptosis

Oxidative stress plays a vital role in the apoptosis of neurons and the signaling transductions underlying oxidative stress are complex. In general, the PI3-K/Akt pathways may inhibit neuronal apoptosis and activation of the c-Jun N-terminal kinase (JNK) and the p38 pathways may promote cell death. The role of extracellular signal-regulated kinase (ERK) is more complicated that it may either promote or inhibit apoptosis depending on the different cell types and stimulators (Radi, Formichi et al. 2014; Yao et al. 1995).

Also known as protein kinase B (PKB), Akt is a down-stream serine/thronine kinase of PI3-K. Under exposure of oxidative stress, Akt is activated through growth factor/growth factor receptor systems. The anti-apoptosis effect of growth factors such as vascular growth factor (VEGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) is found to be mediated by activating Akt through stimulation of individual factors (Klotz et al. 2000; Wang et al. 2000). Activated Akt diminish the uprise of pro-apoptotic proteins such as Bad, caspase-9 and GSK3β and in

turn further inhibits apoptosis (Grimes et al. 2001; Tulasne et al. 2008).

The JNK and p38 of MAPK pathways are activated by a wide range of oxidative stresses. P53 may be used as the mutual mediator of pro-apoptotic signaling of JNK and p38 pathways. Under oxidative stress, both pathways have been shown to increase the phosphorylation of p53 and regulating p53 expression level (Bulavin et al. 1999; Fuchs et al. 1998).

As one of the vital compartment of MAPK family, the ERK cascade (Raf, MEK and ERK) plays critical roles in growth, stress and differentiation, either phosphorylating target proteins in the cytosol or regulating transcription factors in nucleus (Kolch 2000). Mainstream of the researchers suggest the ERK pathway to be a survival factor upon oxidant injury because 1) oxidative stress reduces the activation of ERK and 2) enhancers of ERK activation promotes survival in cells under oxidative stress (Guyton et al. 1996). However, certain stimulants or stresses may also cause the activation of the ERK pathway, leading to cell apoptosis in certain cell types (Brand et al. 2001; Mebratu et al. 2009).

1.1.4.3 Glutamate-induced apoptosis

Glutamate is recognized as the primary neurotransmitter in the CNS. In the process of learning and memory, NMDA receptor, as one of the three

ionotropic glutamatergic receptors, plays fundamental role by means of long-term potentiation (LTP)(Wang and Qin 2010). However, over-activation of NMDA receptor by accumulated glutamate leads to excessive influx calcium ion, increasing consequent oxidative stress and activating pro-apoptotic signaling pathways(Fig.1.6). In the AD brains, the excessive release of glutamate is also found to be accompanied with the disruption of intracellular energy homeostasis. The prolonged over-activation of the NMDA receptor leads to the loss of neurons and failure of synaptic plasticity.

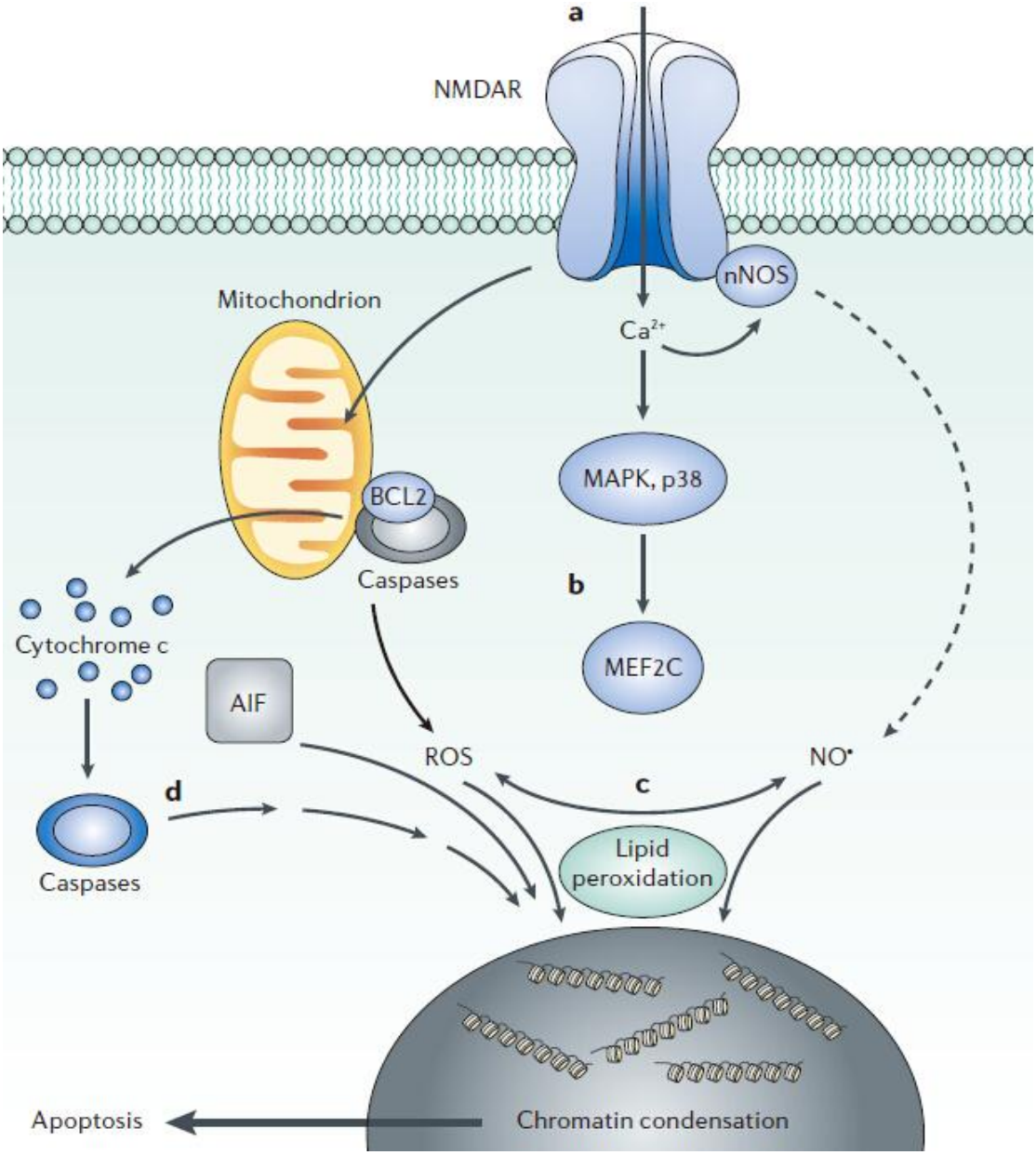


Fig.1.6 Scheme of the pro-apoptotic pathways triggered by excessive NMDAR activity.

The pathway includes (a)NMDAR overactivation; (b) p38-MEF2C pathway was activated; (c) free radicals induces toxic effects such as lipid peroxidation; and (d) down-stream apoptosis related pathways were initiated; Cyt c, cytochrome c; nNOS: neuronal nitric oxide synthase. The figure is adapted from Lipton et al (Lipton 2006).

1.1.5 Neurogenesis and neuronal differentiation

1.1.5.1 Neurogenesis and learning and memory

NSCs is a kind of self-renewing and multipotential stem cell which could further differentiate into neurons and glia in adult human brain (Rusznak et al. 2016; Sharpless et al. 2007). Neurogenesis has been proved in two areas of brain by recent researches: the subventricular zone (SVZ), where NSCs could differentiate into neurons and migrate to the olfactory bulb, and the subgranular layer (SGL), where NSCs could differentiate into granule neurons (Lazarov et al. 2010). In contrast, other brain regions have a limited capacity to maintain neurogenesis. Newly formed neurons in the adult brain play an important role in learning and memory. Neurogenesis in SVZ could regulate synaptic plasticity in the olfactory bulb (Jin 2016). Long-term olfactory memory could be impaired by focal irradiation and the hence diminished neurogenesis in SVZ (Lazarini et al. 2009).

1.1.5.2 Neurogenesis and impairments of neuronal differentiation in AD

Impairment of neurogenesis is observed in the AD brain. The neurogenesis areas of SGL and SVZ are influenced by senile plaques and NFTs during the progress of AD. Moreover, the APP and PS1 mutant mice have shown impaired neurogenesis that begins prior to the formation of Aβ plaques and declination of memory (Demars et al. 2010). Furthermore, impairment of neurogenesis in AD could also account for the functional loss

of neuronal progenitor neurons in differentiating into neurons. As progressive decline in memory and learning is considered as the primary characteristic of AD, it is hypothesized that impaired neurogenesis might exacerbate the impairment of memory and learning in AD by affecting hippocampal and olfactory neural circuits (Lazarov and Marr 2010). Therefore, drugs capable of promoting neuronal differentiation and neurogenesis might have therapeutic potential in the treatment of AD.

1.2 Other neurodegenerative disease

Besides AD, the feature of progressive neuronal loss in a certain area of central nervous system is also shared by other neurodegenerative disorders like PD, HD and ALS. In the following section, two neurodegenerative diseases which are also commonly diagnosed: PD and HD are briefly introduced and may offer a deeper insight into the complexity of these diseases.

1.2.1 PD

Parkinson's disease (PD) is the second prevalent neurodegenerative disorders following Alzheimer's disease among the elderly (de Lau et al. 2006; Hirtz et al. 2007). PD is characterized clinically by cognitive dysfunctions and motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements (Lang et al. 1998; Lang et al. 1998).

Neuropathological hallmarks of PD includes 1.the presence of Lewy bodies composed mostly of alpha-synuclein and ubiquitin and 2.the loss of substantia nigra pars compacta (SNpc) dopaminergic neurons (Dauer et al. 2003). The PD related genetic alternations includes autosomal dominant point mutations or gene duplications or triplications in the α-synuclein gene, and point mutations in the ubiquitin-C-terminal hydrolase-L1 (UCH-L1) and the leucine-rich repeat kinase (LRRK2) genes, as well as autosomal recessively inherited mutations in Parkin, PINK1, DJ-1 and the ATPase13A2 genes. Several of the genetic mutations related to mitochondrial dysfunction are also directly associated with early-onset forms of PD(Singleton et al. 2013). Nevertheless, the pathogenesis of late-onset sporadic PD is still elusive. Mounting evidences suggest that oxidative damages induced by ROS and RNS is intimately linked with the progression of dopaminergic neuron loss(Chung et al. 2010; Hanrott et al. 2006; Lin et al. 2006). Increased oxidative lesions exerted on proteins such as α-synuclein will leads to the misfolding and degradation of proteins which finally forms lewy body(Xu et al. 2002). In addition, a steep decline of neurogenesis and neuronal differentiation are also found in PD(Simuni et al. 2008).

1.2.2 ALS

In year 2014, the “Ice bucket challenge” swept internet and greatly aroused people’s awareness of ALS. ALS is a motor neuron disease that is

characterized by a loss of motor neurons in spinal cord and cerebral cortex. Compared with other neurodegenerative diseases, ALS is equivalently fatal but in a more rapid way: most of the ALS patients die within 3 years after diagnosis. In the autosomal dominate form of ALS, antioxidant superoxide dismutase 1(SOD1) gene mutation has been identified to be responsive pathogenesis factor while in sporadic form, the pathogenesis factor is still yet to be clarified. In the past few years, by virtue of powerful sequencing technology, more than 20 genes has been putatively associated with the pathogenesis of ALS(Marangi et al. 2015). But widely different genetic causes of ALS appear to ultimately converge into common pathogenic pathways like mitochondria dysfunction and ER stress, which both lead to imbalance of redox and calcium level(Manfredi et al. 2016). Moreover, NMDA receptor over activation and reduction of differentiation of NSCs are also observed in ALS brains (Liu et al. 2006).

1.3 T-006 is a novel anti-Alzheimer's agent derived from tetramethylpyrazine and J147

There is currently no approved causal treatment for AD and 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 with the hope of discovering a disease-modifying drug.

1.3.1 Introduction of Tetramethylpyrazine

Tetramethylpyrazine (TMP), is an alkaloid extracted from *Ligusticum wallichii* Franchat, which has been used for a long history on heart, kidney, and brain diseases in traditional Chinese medicine(Xu et al. 2003). Previous experimental studies demonstrated the diverse pharmacological activity of TMP such as anti-inflammation(Chang et al. 2007; Liao et al. 2004), calcium antagonism(Pang et al. 1996),and free radical scavenging(Shih et al. 2002; Zhang et al. 2003). In addition, the protective effects of TMP in central nervous system injuries challenged by multiple exogenous stimuli has been reported in the *in vitro* and *in vivo* models. It protects neuronal cells against traumatic, ischemic brain or spinal cord injury (Fan et al. 2006; Gao et al. 2008). Moreover, TMP promotes proliferation and differentiation of neural stem cells (NSCs) from rat brain (Tian et al. 2010) and protect rat retinal cell degeneration (Yang et al. 2005; Yang et al. 2008). Preclinical assessment of the ADMET of TMP demonstrated that TMP can permeate the blood brain barrier in multiple animal models (Lou et al. 1986; Qi et al. 2002; Tsai et al. 2001). Toxicity test revealed a low toxicity in animals with an oral LD_{50} of 1910mg/kg in rats and 1436mg/kg in mice. TMP tablet, TMP-HCl injection are both prescription available for a treatment of cardiovascular disease in

China (Tan 2009).was shown to have multiple functions, including prevention of oxidative stress-induced neuronal death, blockade of calcium channel, and enhancement of mitochondrial biosynthesis(Li et al. 2010; Liu et al. 2010; Tan 2009). All above suggested TMP is a compound extensively studied and utilized with promising effects in multiple neurological disease models.

1.3.2 Introduction of J147

J147 is originally a derivative of Curcumin. Curcumin, as one of the most important active compounds in turmeric, has also been extensively studied for treating neurodegenerative disorders. Although mounting evidences have demonstrated the impressive neuroprotective effects of curcumin including in transgenic AD mouse models (Lim et al. 2001), the clinical use of curcumin is constrained mainly by its poor absorption. The structural modification of curcumin never stops. Using curcumin and cyclohexyl-bisphenol A (CBA), scientists from Salk institute created a series of hybrid molecules. CBA is a compound that “has neurotrophic activity which curcumin lacks” (Liu et al. 2008). They picked out a best agent named CNB-001. A large number of derivative of CNB-001 were then developed and among these derivatives, an orally active compound named J147 was shown to possess broad neuroprotective effects, including inhibiting oxidative stress, reducing trophic factor withdrawal-induced neurotoxicity, promoting hippocampus long-term potentiation and preventing the reduction of energy metabolism(Cates, Cates-

Gatto et al. 2011; Chen, Prior et al. 2011; Prior, Dargusch et al. 2013).

1.3.3 The design of T-006

In this study, we utilize a widely used and relatively simple strategy in drug development: combining or modifying the compounds already possessing some desired activity. Also in many cases, unexpected activities may be found in the novel molecule that are distinct from the original compounds. Herein, our compound T-006 was created by replacing the methoxyphenyl group of J147 by TMP, with the hope of possessing the pleiotrophic pharmacological activities of both compounds with stronger potency(Fig.1.7).

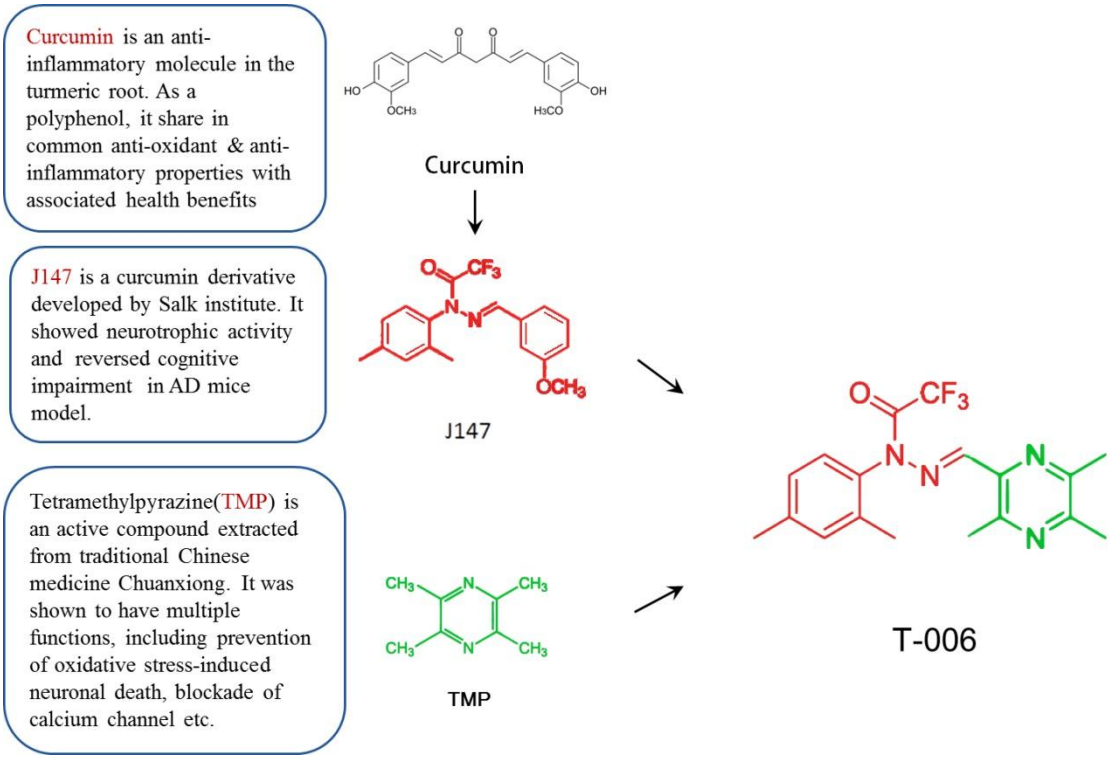


Fig.1.7 T-006 is a conjugation of curcumin derivative J147 and TMP.

1.4 The aim of the study

Neuronal damage in AD and other related neurodegenerative disease is initiated by various factors but may converged into some common pathways such as oxidative stress induced neuronal apoptosis and impairment of neurogenesis(Lazarov and Marr 2010; Pohanka 2013). Moreover, clinical trials during past 10 years exhibit little progress in development of compounds targeting single pathogenic factor. Thus, multi-functional agent with the properties targeting these vital pathogenic mechanisms could have therapeutic significance. T-006 is chemically designed to interfere with

multiple pathways related with AD. Therefore, in my research, I would like to achieve following goals:

1. To elucidate the neuroprotection and mechanisms by which T-006 protects against glutamate-induced neuronal apoptosis
2. To investigate the anti-oxidative activity of T-006 under H_2O_2 challenge.
3. To validate the anti-AD activity of T-006 in *in vivo* model of AD

Chapter 2
Materials and Methods

2.1 Chemicals and reagents

T-006 was synthesized as previously described(Chen et al. 2015). All cell culture media were purchased from Gibco (Carlshand, CA, USA) unless otherwise stated. LY294002, PD98059, U0126, SB415286, SB203580 and SP600125 were obtained from Calbiochem (San Diego, CA, USA). Glutamate, Fura-2/AM, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorescein diacetate (FDA), propidium iodide (PI), Hoechst 33342, tacrine, memantine, atropine, dibutyryl cAMP (dbcAMP), mecamylamine, MLA, K252a and tubocurarine were obtained from Sigma Chemicals (St Louis, MO, USA). Cytotoxicity detection kit (LDH) was obtained from Roche (Indianapolis, IN, USA). Hydroxyphenyl fluorescein(HPF), 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA),3-amino,4-aminomethyl-2′,7′-difluorescein diacetate (DAF-FM), dihydrorhodamine 123 (DHR123) and JC-1 were from Molecular Probes (Invitrogen, Carlsbad, CA, USA).Recombinant NGF was purchased from R&D Systems (Minneapolis, MN, USA).

2.2 Primary cell cultures

8-day-old Sprague–Dawley rats were used extract CGNs (The Central Animal Facilities of Hong Kong Polytechnic University) as described in

previous publication (Subramaniam et al. 2005). Briefly, after harvesting, CGNs were seeded at a density of 2.7×10^5 cells/cm2 and maintained in basal modified Eagle's medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, and penicillin (100 U/ml)/streptomycin (100 μg/ml). Cytosine arabinoside (10 μM) was added to the culture medium 24 h after plating to inhibit the growth of non-neuronal cells. Under this protocol, 95–99% of the cultured cells were CGNs. 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℃ for 15 mins. The isolated neurons were resuspended in neurobasal medium containing 10% FBS, 0.25% glutamine and penicillin (100 U/ml)/streptomycin (100 μg/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 μg/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 PC12 cell cultures

The mouse pheochromocytoma PC12 cells (American Type Culture Collection, Manassas, VA, USA), were grown in high glucose DMEM medium with 12.5% (v/v) horse serum, 2.5% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. Cells were seeded in 96 well plates (100 μL/well) at a concentration of 1×10^5 cells/mL. Cultures were maintained at a 37 ^oC humidified atmosphere containing 5% CO_2.Experiments were carried out 24 h after the the seeding of cells.

2.4 Cell viability assessment

Cell viability assessment was performed by the MTT assay. The assay was done under the specifications of the manufacturer. Briefly, cells were seeded in 96-well plates. 10μl MTT reagent (5 mg/ml) was added to 100 μl of medium in each well and then incubated for 4 h in a humidified incubator at 37℃. After the incubation, medium was removed and replaced by 100 μl DMSO. After plate-shaking for 10mins, the absorbance of the samples was

measured at a wavelength of 570 with 655 nm as a reference wavelength.

2.5 Neurite outgrowth induction and assessment in PC12 Cells

20 ng/mL nerve growth factor was used to induce PC12 cells differentiation for 48h in the culture medium of DMEM containing 1% FBS and 1% HS.

The quantification of neurite-bearing cells was carried out as previously described (Hu et al. 2015). Neurite-bearing PC12 cells were observed and photographed after treatment using a phase-contrast microscope with a 10× objective lens and a digital camera. If at least one neurite grew longer than the diameter of the cell body, the cell was deemed as positive for neurite outgrowth. For each sample, approximately 300 cells in 6 randomly chosen visual fields were counted.

2.6 Measurement of mitochondrial membrane potential

Primary cortical neurons were used to detect the mitochondrial membrane potential and dye JC-1 was employed as the molecular probe. After seeding, Primary cortical neurons were incubated in neurobasal medium supplemented with 10% FBS and 4mM glutamine in 12-well plates at a density of 3-4×10^5 cells/well for ten days. neurons were then incubated with

T-006 or TMP at the indicated concentrations 2 h prior to the treatment of 100 μM *t*-BHP for 24 h. After washing with JC-1 buffer, 2 μM JC-1 dye was incubated with neurons forfor 10 mins. Fluorescence intensity was determined at 488 nm excitation and 529 nm/590 nm dual emissions by a microplate reader.

2.7 Measurement of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS)

Pretreatment of T-006 or TMP at indicated concentrations on PC12 cells for 2 h was followed by 100 μM *t*-BHP stimulation for 24 h. Intracellular ROS (such as hydrogen peroxide and hydroxyl radical) were detected using H_2DCF-DA (10 μM), HFP (5 μM). Intracellular RNS (such as nitric oxide and peroxynitrite) were detected by DAF-FM (10 μM) and DHR123 (5 μM), respectively. The fluorescence intensity was determined using a multi-detection microplate reader. The fluorescence values of the treated group were normalized to the fluorescence of the control cells.

2.8 FDA and PI staining assay

To further investigate the condition of the neurons after various treatment, CGNs were stained with two specific fluorophores, FDA and PI. Living

granule neurons were stained with fluorescein formed from FDA and emits green fluorescence while PI intercalates with DNA of dead neurons and emits red fluorescence (Li et al. 2005). Briefly, after incubation with FDA (10 μg/ml) and PI (5 μg/ml) for 10 min, the neurons were washed with PBS, then examined and were photographed using a fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA).

2.9 Hoechst staining assay

As previously described (Zhang, Yang et al. 2002), chromatin condensation, a hall mark of apoptosis was detected Hoechst 33342 staining. CGNs grown in a 6-well plate were washed with ice-cold phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS. Cells were then stained in Hoechst 33342 (5 μg/ml) for 5 min at 4℃. Nuclei were visualized using a fluorescence microscope at indicated magnification.

2.10 Calcium uptake determination.

Quantitative measurements of intracellular calcium concentration were performed in CGNs grown on PLL-coated 96-well plates at 8 Div. Fura-2 AM was used as the probe. Experimental procedures followed the manufacturer's

manual. Briefly, at the end of pretreatment of varies drugs, cells were incubated with complete medium containing 5μM Fura-2/AM at 37℃ for 30 min. The cells were then washed with PBS and incubated at 37℃ for another 10 min prior to glutamate addition and measurement. Intracellular Ca2+ concentration by a fluorescence spectrometer (F-4500 HITACHI Japan) at an excitation wavelength of 340-380nm and emission wavelength at 510nm. The concentrations of Ca2+ were expressed as percentage of non-treated group at 0 min time point.

2.11 Immunocytochemical Staining

Cortical neurons were used for immunocytochemical staining. After incubation and treatments, the neurons were fixed in 4% paraformaldehyde containing 10% sucrose for 10 mins. Then the neurons were blocked in blocking buffer (0.5% bovine serum albumin, 0.1% Triton X-100, and 5% goat serum) for 1 h under room temperature. Neurons were then blocked by 5% BSA followed by incubation overnight with anti-gap-43 antibody in blocking buffer (Santa Cruz) under 4°C. Neurons were then washed with PBST 3 times and incubated in HRP-conjugated anti-mouse secondary antibody for 1 h. The neurites were observed and photos were taken with a fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA).

2.12 Western blot assay

CGNs were used in the western blot assay. Western blot analysis was performed as described previously (Hampel, Prvulovic et al. 2011). In brief, CGNs were harvested using cell lysis buffer. After 15 min of lysis on ice, the samples were centrifuged at 14 000 g at 4℃ for 10 min. We then assess the protein concentration with BCA assay and the final concentration was determined by linear-regression with BSA as standards. SDS sample buffer were then added and samples boiled for 5 min. The protein (30 μg) was resolved in a 10% SDS–polyacrylamide gel. After SDS-PAGE, the gel was taken out and placed on a gel holder cassette with a piece of PVDF membrane which soaked in 100% methanol in prior. After transferring, the PVDF membranes was blocked with %5 fat-free milk. Then the membrane were incubated with specific primary antibody overnight at 4°C. After washing with PBST for 3 times, a secondary HRP-linked antibody were then used for another 1h. Blots were finally developed using an ECL plus kit (Amersham Bioscience, Aylesbury, UK) and exposed to Kodak autoradiographic films.

2.13 HuAPPswe/PS1ΔE9 transgenic mice

The APP/PS1 transgenic mice carrying two transgenes. Before the experiments, the two muted genes: mouse/human chimeric APP/Swe and human PS1ΔE9 were characterized by PCR.

The mice of 4-5 months old were used in the experiments and each group contains 4 mice. In T-006 and J147 treatment group, transgenic mice were orally given 2 mg/kg T-006 and J147 respectively for 14 consecutive days. And the vehicle groups: the control transgenic mice group and wild type group were given an equal volume of saline containing 3% DMSO.

An electric Y-Maze (Sanxin Laboratory Instrument Company, Zhangjiagang, China) was used to investigate spatial memory of each group. Minor modifications were made according to the procedure described by Ye et al (Ye et al. 2010). Briefly, one hour after drug administration, the Y-Maze test was implemented. As shown in figure 3.3.1A , the Y-Maze test was done on days 1, 2 and then every other day. The index of average correct ratio and average training time were chosen.

2.14 Data analysis and statistics

In section 3.1, results are expressed as mean ± SEM from at least three independent experiments and in section 3.2 and 3.3, results are expressed as

mean ± SD from at least three independent experiments. Analysis of variance followed by Dunnett's test, Duncan's multiple range tests or t test were used for statistical comparisons. Levels of $p< 0.05$ were considered to be of statistical significance.

Chapter 3
Results

3.1 T-006 elicits robust neuroprotection against glutamate-induced excitotoxicity in primary cerebellar granule neurons

3.1.1 T-006 blocks neuronal loss induced by glutamate in NGF-differentiated PC12 cells

For a preliminary investigation into the potential application of T-006 in the treatment of AD, the PC12 cell model, differentiated by NGF, was employed. The differentiated PC12 cells were challenged by glutamate and the effect of T-006 pretreatment was examined. As shown in Fig.3.1.1, T-006 efficiently blocked the glutamate-induced cell viability decrease and morphological changes including damaged cell body and broken neurite network.

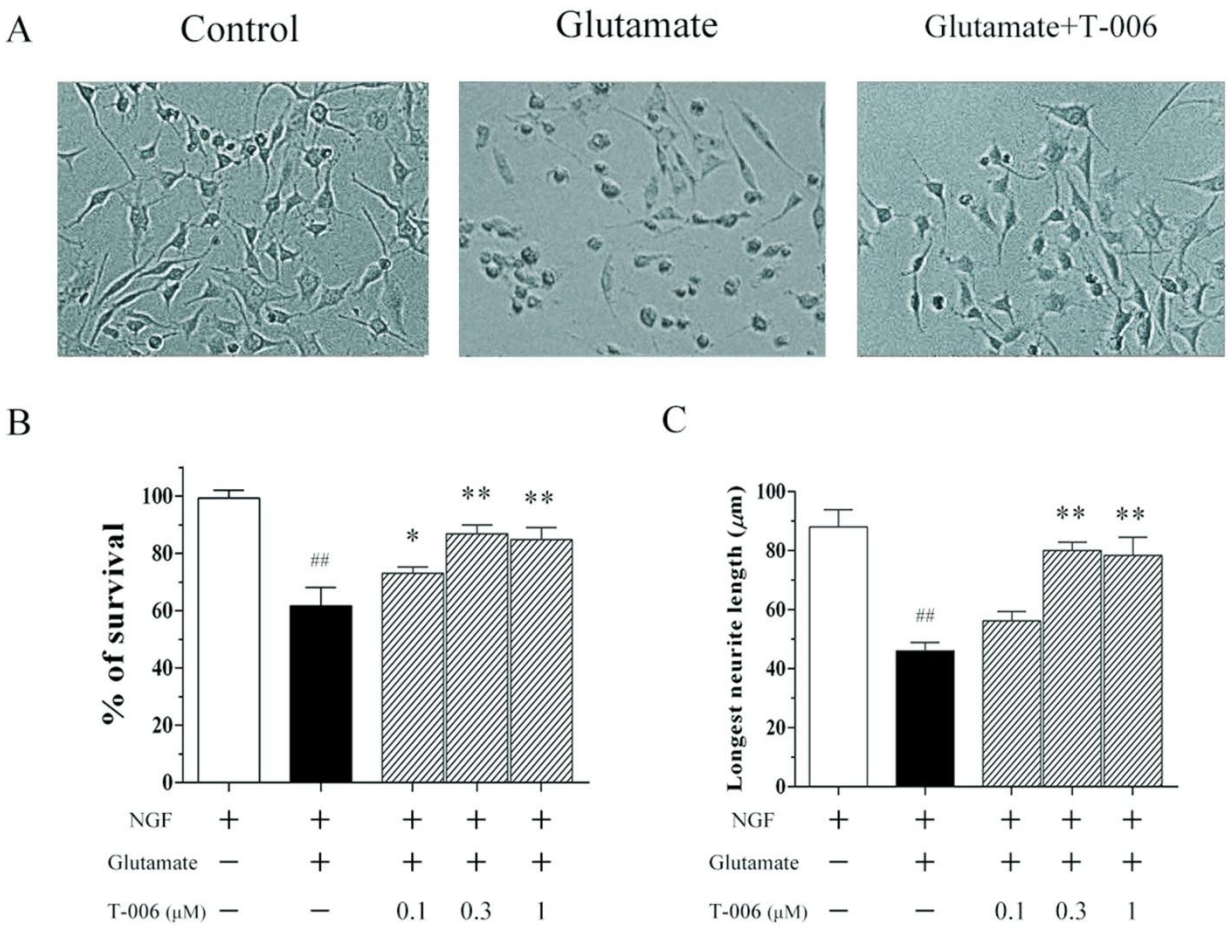


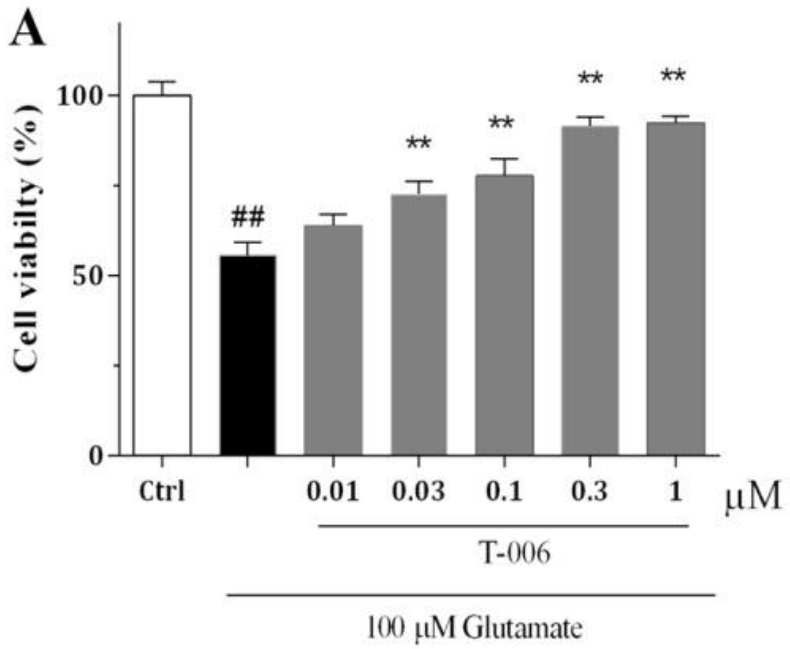
Fig.3.1.1. T-006 protects differentiated PC12 against glutamate-induced toxicity.

(A) T-006 protected PC12 glutamate-induced impairment on neurites. Differentiated PC12 cells were pretreated with T-006 for 2h and then incubated with 20 mM glutamate for 24 hours. Photos were taken by a phase-contrast microscope. (B) The decreased cell viability induced by glutamate on differentiated PC12 cells were prevented by treatment of T-006. Differentiated PC12 cells were pretreated with T-006 for 2h and then exposed to 20mM glutamate for 24 hours and then subject to MTT assay(n=6). (C) The shortening of neurite length induced by glutamate was prevented by T-006. Differentiated PC12 cells were pretreated with T-006 for 2h and then incubated with 20mM glutamate for 24 hours. The neurite length was

measured by ImageJ software. (n=6) ##, $p<0.01$, compared to NGF-treated only group; *, $p<0.05$ and **, $p<0.01$, compared to the glutamate group.

3.1.2 T-006 prevents glutamate-induced cell death more potently than memantine

According to previous researches in our group, 100 μM glutamate which yielded about 50 % neuronal loss of CGNs was used in this current study (Hu, Cui et al. 2013). T-006, ranging from 0.01 to 1 μM was used for the investigation of its neuroprotection against glutamate-induced excitotoxicity. As shown in Fig.3.1.2A, T-006 concentration-dependently prevented glutamate-induced toxicity and the maximum protection reached 90.5±1.3%. To compare the neuroprotective effects between T-006, memantine and TMP, CGNs were pretreated with T-006, memantine or TMP, and then glutamate was added for 24 h. The EC_{50} of these 3 compounds were also calculated as in the table in Fig.3.1.2B, the EC_{50} values of T-006 is 59.4 nM and that of Memantine is 4.58 μM, indicating that the neuroprotection of T-006 against glutamate-triggered excitotoxicity is stronger than memantine. The parental molecule TMP did not show significant protection (Fig.3.1.2B).



B

Potency of drugs

Drug	EC_{50}
T-006	59.4nM
Memantine	4.58μM
TMP	not detected

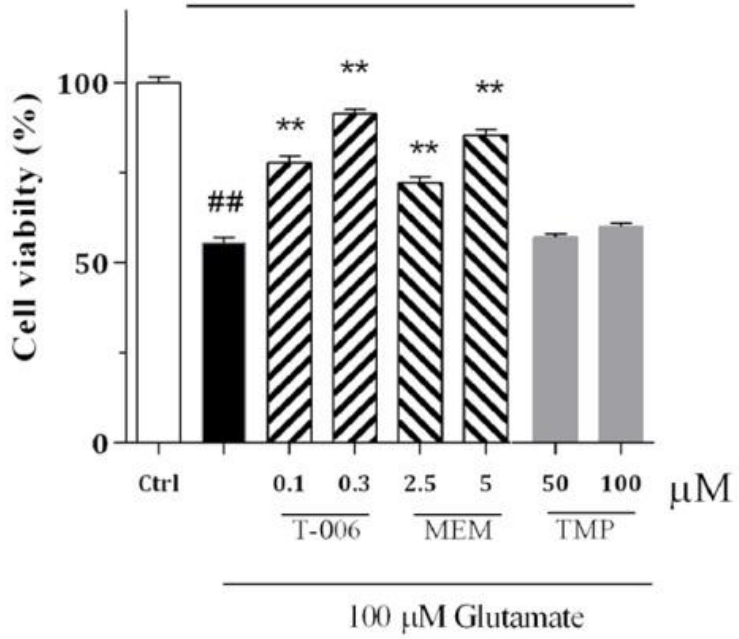


Fig.3.1.2 T-006 prevents glutamate-induced excitotoxicity more potently than memantine and TMP.

(A) T-006 antagonized glutamate-induced neuronal death. At 8 DIV. CGNs were pre-treated with T-006 (0.01 μM to 1μM) for 2 h, and then incubated with 100 μM glutamate for additional 24 h. MTT assay was used to detect cell viability(n=6). (B) T-006 protected CGNs under glutamate more potently than memantine (MEM) and TMP. At 8 DIV, CGNs were pre-treated with T-006 (0.01 μM,0.3 μM), MEM(2.5μM,5μM) or TMP(50μM,100μM) for 2 h, and then incubated with 100 μM glutamate for additional 24 h, and finally subjected to MTT reduction assay(n=6). The EC_{50} of T-006 were calculated by nonlinear regression using GraphPad Prism software, 7 dosages of T-006 were used. ##, $p<0.01$, compared to control and **, $p<0.01$, compared to the glutamate group.

3.1.3 T-006 substantially blocks glutamate-induced apoptosis in primary cultured CGNs

To further demonstrate the neuroprotection of T-006 is through an anti-apoptotic manner, Hoechst staining and western blotting were performed. After counting apoptotic bodies, a 56.2±5.6% apoptosis rate after the 24h treatment of glutamate was shown. As exhibited in the images of phase contrast microscopy and FDA/PI double staining (Fig.3.1.3A), T-006 at 0.3 μM significantly blocked the loss of neurons. And the glutamate-induced morphological changes of CGNs such as cell body shrinkage and neuronal network impairment were also prevented by T-006. T-006 also significantly protected neuronal apoptosis induced by glutamate (Fig.3.1.3B). In Fig.3.1.3C, two apoptosis related proteins Bcl-2 and Bax were examined by western blot. It was found that pretreatment of T-006 reversed the glutamate-induced Bcl-2 down-regulation and Bax up-regulation. The Bcl-2/Bax ratio was increased to 76.2±5.4% of control by pretreatment of T-006.

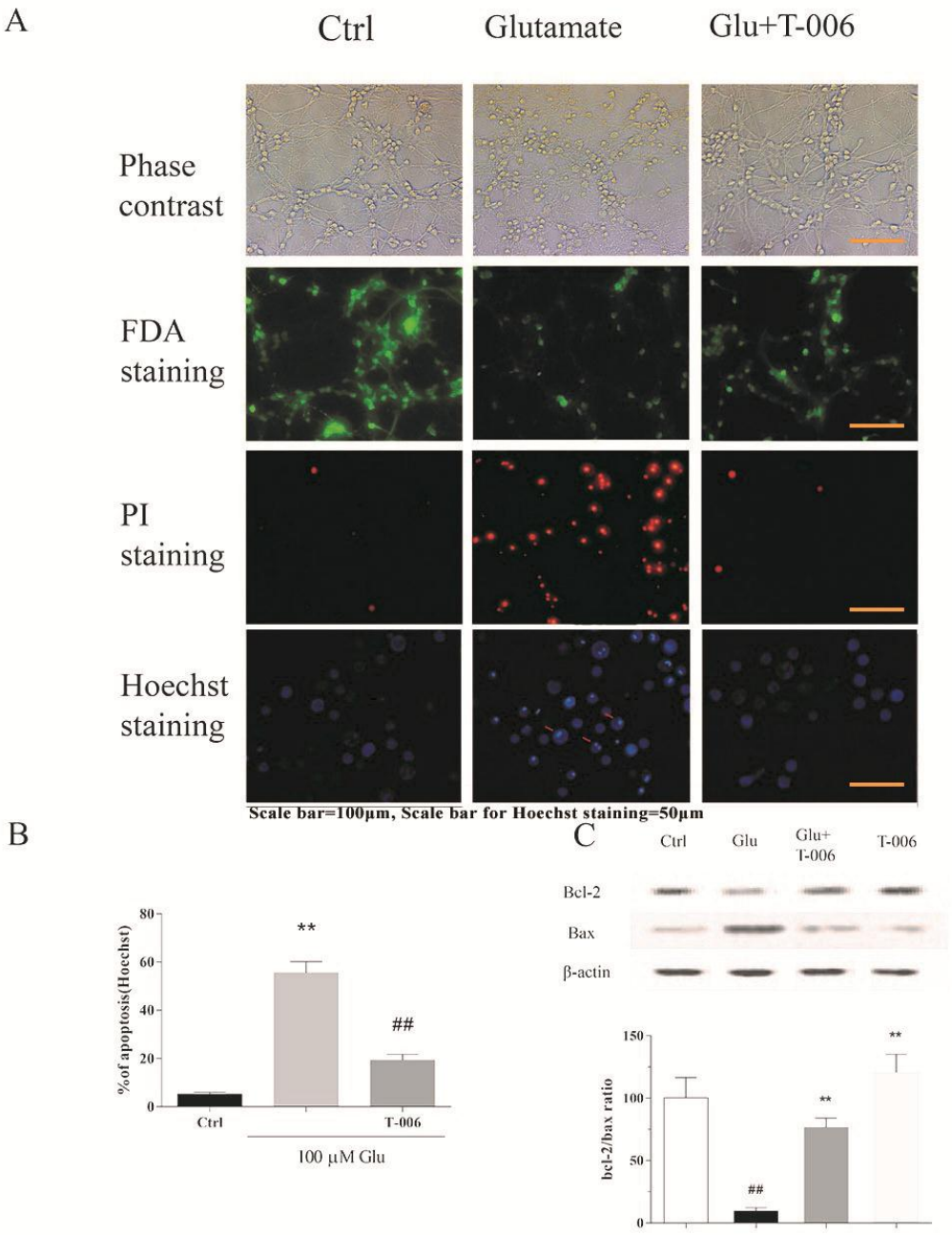


Fig.3.1.3 T-006 attenuates the hallmarks of apoptosis-induced by glutamate in CGNs.

A, At 8 DIV, CGNs were pre-incubated with 0.3μM T-006 for 2h, and then 100 μM glutamate was added. At 24 h after glutamate treatment, CGNs were stained with FDA or PI or Hoechst 33342. Red arrows indicate apoptotic nucleus. B, the counts of apoptotic bodies by Hoechst staining as in A (n=5)

**, p<0.01, compared to control, ##, p<0.01, compared to glutamate group.C, Western blot of the apoptosis related protein Bcl-2 and Bax. ## P<0.01 compared to control, ** P <0.01 compared to the glutamate group.

3.1.4 T-006 prevents calcium overload induced by glutamate in primary cultured CGNs.

Calcium plays a fundamental part in many physiological processes, while excessive influx of Ca^{2+} into neurons may lead to neuronal damage. It is well established that the initial event in glutamate evoked neuronal excitotoxicity in CGNs as well as many neurodegenerative diseases is an extensive entry of Ca^{2+} through the NMDA receptor (Mehta et al. 2013). Thus, the level of intracellular Ca^{2+} concentration reflects the blockage of NMDA receptor. We performed experiments to determine the intracellular accumulation of Ca^{2+} after the glutamate challenge as well as the effect of T-006 pretreatment in preventing Ca^{2+} overload (Fig.3.1.4). As observed from the fluorescence intensity of fura-2/AM probe, addition of 100μM glutamate robustly elevated intracellular Ca^{2+} concentration starting from 2 mins and reached the peak of 372%±19.4% at 15 mins. The pretreatment of NMDA uncompetitive antagonist MK801 nearly fully inhibited the Ca^{2+} elevation. T-006 and memantine both performed partial blockade of Ca^{2+} influx.

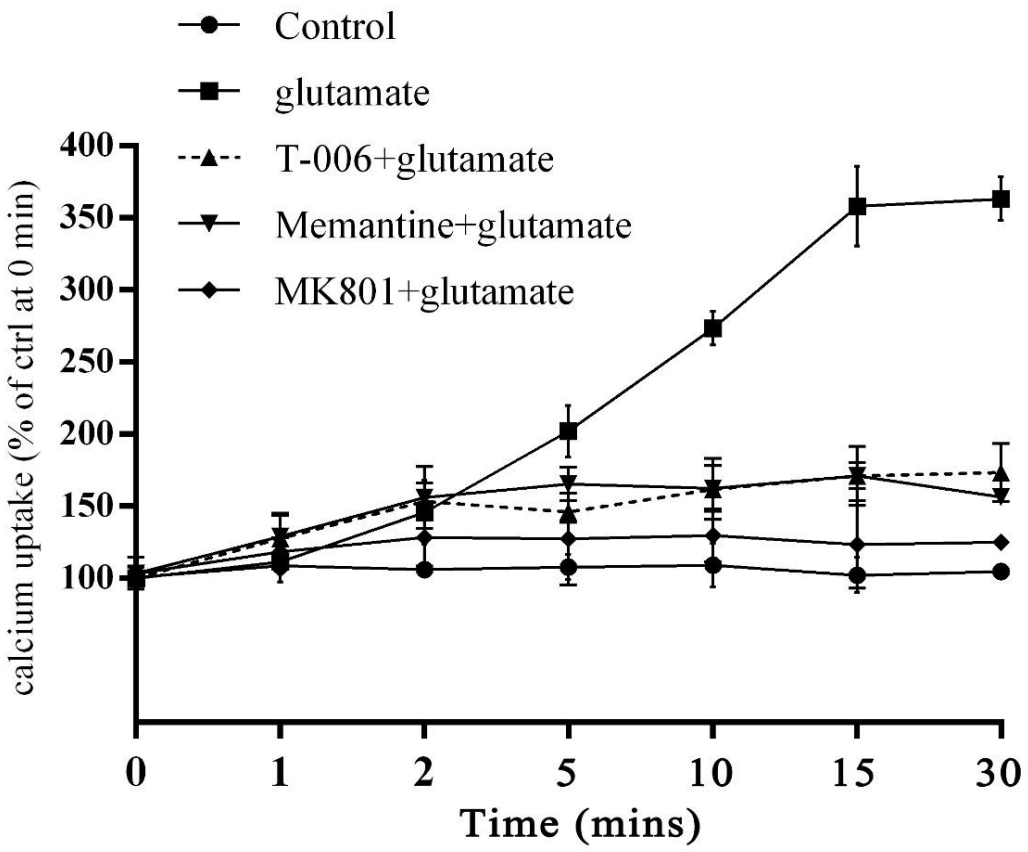


Fig.3.1.4 T-006 prevents calcium overload induced by glutamate in CGNs.

At 8 DIV, CGNs were pre-incubated 0.3μM T-006, 10μM memantine or 1μM MK801 for 2 h. After incubation with fura-2 AM calcium probes, CGNs were exposed to 100μM glutamate. Fluorescence were detected at indicated time points. (n=3)

3.1.5 T-006 suppresses the activation of MAPK/ERK pathways caused by glutamate

In Fig.3.1.5A, U0126 and PD98059 as MEK inhibitors, exerted modest protection in the glutamate-induced excitotoxicity model in CGNs, which indicate the involvement of the MAPK/ERK pathway (but not JNK or P38, data not shown). To assess the effects of T-006 on the MAPK/ERK pathway, western blot was used to analyze the levels of phosphor-MEK1(S218/S222)/MEK2(S222/S226) and phosphor-ERK1 (T202/Y204)/ERK2(T185/Y187). As shown in Fig.3.1.5B, the levels of phosphor-MEK1/2 and phosphor-ERK1/2 increased and peaked at 2 h after glutamate addition. T-006 at 0.3 μM significantly inhibited the increased phosphorylation of kinases caused by glutamate.

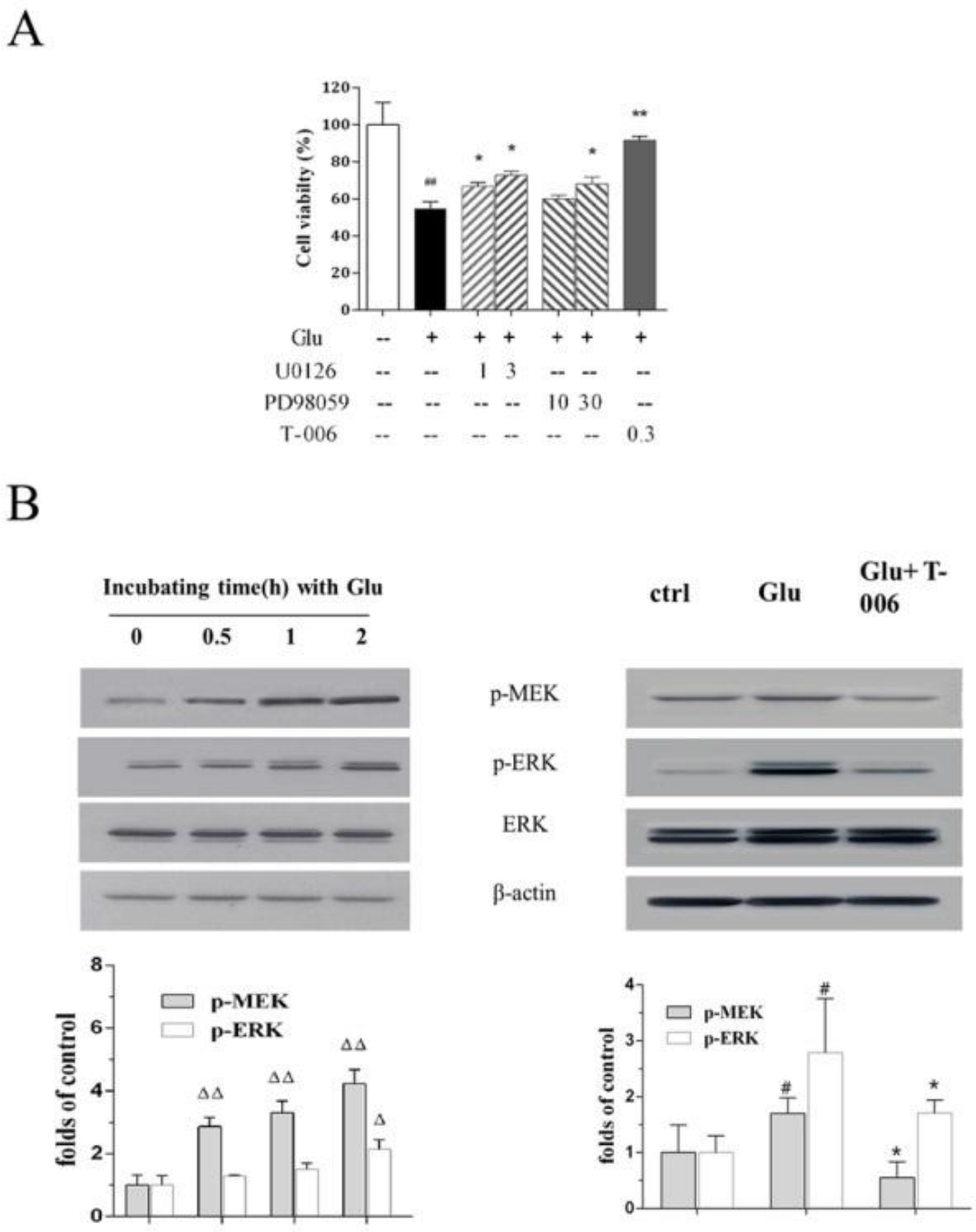


Fig.3.1.5 Inhibition of glutamate-mediated activation of MAPK/ ERK pathway by T-006.

(A) Pharmacological inhibition of MEK by U0126 or PD98059 only

produced partial protection against glutamate-induced neuronal loss compared with T-006. ##p<0.01, compared to control. *p<0.05, **p<0.01, compared to the glutamate-treated group(n=6). (B) Different time points of glutamate (Glu) treatment at 100 μM were used on CGNs. Cells were then collected and proceeded to Western Blot using the anti-bodies against phosphor-MEK, phosphor-ERK, total ERK and β-actin. CGNs were pre-treated with 0.3μM T-006 for 2 h, and then exposed to 100μM glutamate for 2 h, Western Blot was used to detect the specific phosphor-MEK, phosphor-ERK, total ERK and β-actin. $^{\Delta}p<0.05$, compared to group with 0 h of glutamate treatment, $^{\Delta\Delta}p<0.01$, compared to group with 0 h of glutamate treatment. $^{\#}p<0.05$, compared to control, $^{*}p<0.05$, compared to the glutamate-treated group,

3.1.6 T-006 reverses the inhibition of PI3-K/Akt/GSK3β pathway caused by glutamate

The connection of T PI3-K/Akt/GSK3β pathway was associated and the neuroprotection of T-006 against glutamate-induced excitotoxicity was investigated. Firstly, PI3-K inhibitor LY294002 and Akt inhibitor Akt inhibitor IV were used. 50 μM LY294002 or 2 μM Akt inhibitor IV significantly abolished the neuroprotection of T-006 against glutamate challenge (Fig.3.1.6D). To further confirm the effects of T-006 and on the PI3-K/Akt/GSK3β pathway, the levels of p-Ser473-Akt and p-Ser9-GSK3β were also examined by Western blotting. As shown in Fig.3.1.6A and B, glutamate at 100 μM down-regulated the phosphorylation of Akt and GSK3β in a time-dependent manner. After glutamate challenge for 2 h, p-Ser473-Akt and p-Ser9-GSK3β decreased to 48.2 ± 13.1 % and 42.7 ± 8.1 % of the control level respectively, while treatment with T-006, in a concentration-dependent manner, reversed both suppressed kinases without affecting the un-challenged controls (Fig. 3.1.6A&B). Pretreatment of 50 μM LY294002 suppressed the T-006 maintained levels of downstream p-Ser473-Akt and p-Ser9-GSK3β levels (Fig.3.1.6C).

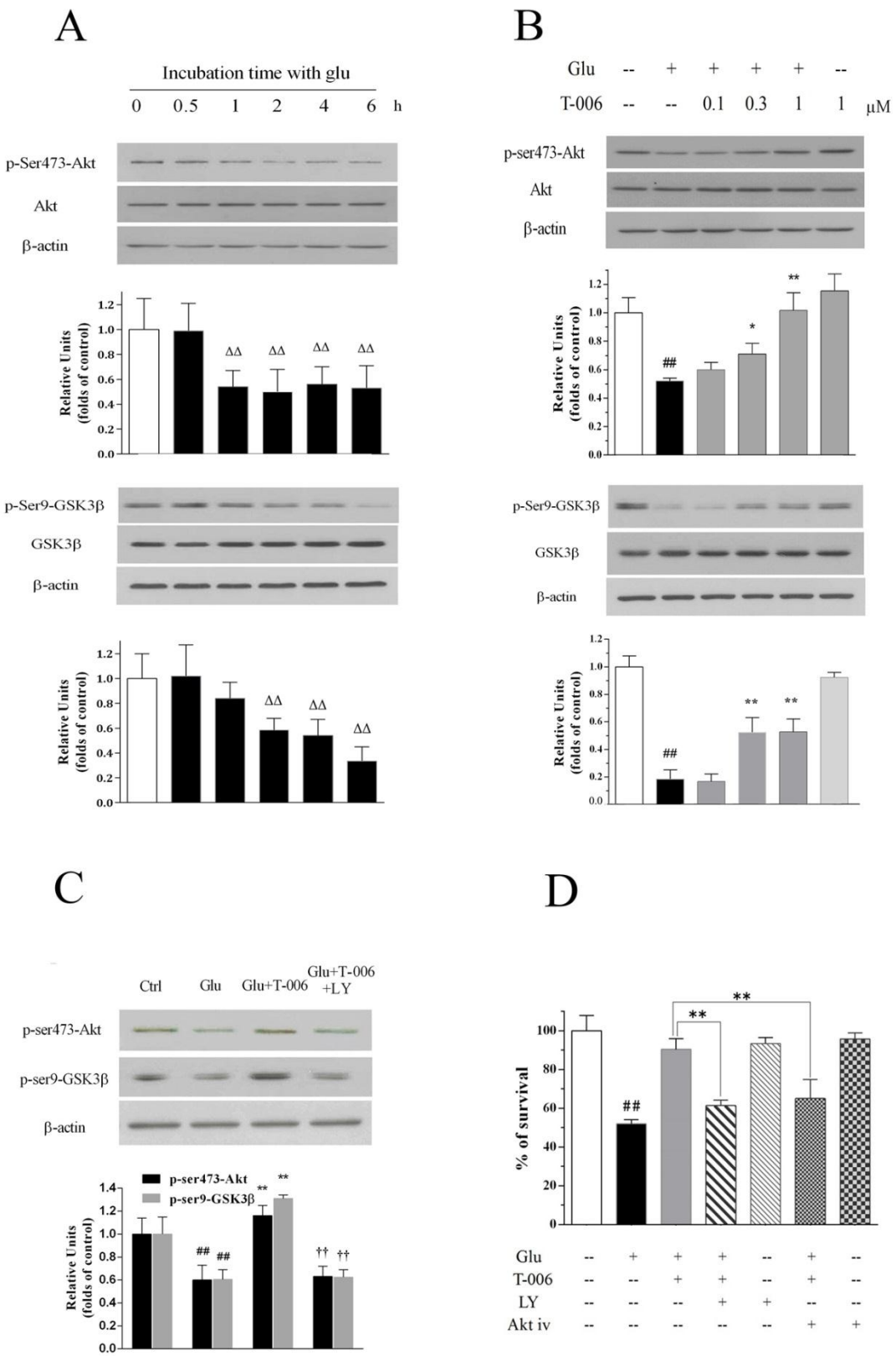

A
Incubation time with glu
0 0.5 1 2 4 6 h
p-Ser473-Akt
Akt
β-actin
Relative Units (folds of control)
ΔΔ
p-Ser9-GSK3β
GSK3β
β-actin
B
Glu -- + + + + --
T-006 -- -- 0.1 0.3 1 1 μM
p-ser473-Akt
Akt
β-actin
##
*
**
p-Ser9-GSK3β
GSK3β
β-actin
C
Ctrl Glu Glu+T-006 Glu+T-006 +LY
p-ser473-Akt
p-ser9-GSK3β
β-actin
p-ser473-Akt
p-ser9-GSK3β
##
**
††
D
% of survival
Glu
T-006
LY
Akt iv

Fig.3.1.6 T-006 attenuates glutamate-induced excitotoxicity by maintaining the activation of PI3-K/Akt/GSK3β pathway in CGNs.

A, 100 μM glutamate significantly suppressed the expression of phospho-Akt and phospho-GSK3β of CGNs in a time-dependent manner. B, T-006 dose-dependently reversed glutamate-induced decrease of phospho-Akt and phospho-GSK3β. C, western blot results showed that PI3-K specific inhibitor partially abrogated the up-regulation of Akt and GSK3β phosphorylation. D, PI3-K specific inhibitor and Akt specific inhibitor abolished the neuroprotection of T-006 through PI3-K/Akt/GSK3β pathway(n=6). $^{\Delta\Delta}p<0.01$, compared to group with 0 h of glutamate treatment. $^{\#}p<0.05$, compared to control, $^{\#\#}p<0.01$, compared to control. $^{*}p<0.05$, compared to the glutamate-treated group, $^{**}p<0.01$, compared to the glutamate-treated group. $^{\dagger\dagger}p<0.01$, compared to T-006 combination with glutamate-treated group.

3.2 T-006 elicits neuroprotection against *t*-BHP-induced oxidative stress

3.2.1 T-006 effectively prevents *t*-BHP-induced neuronal death in PC12 cells and cortical neurons.

Firstly, the dose course and time course of t-BHP–induced cell death were tested. In PC12 cells, cell viability was reduced by about 50% after 24 hours of *t*-BHP treatment. The prior addition of T-006 significantly prevented *t*-BHP-induced PC12 cell viability deduction. When at concentrations higher than 1 μM, T-006 showed nearly complete protection of cell viability. At the same condition, the potency of J147 was slightly weaker than T-006. The effective concentration of J147 started from 1 μM. The efficacy of J147 was similar to that of T-006; at 10-100 μM it almost fully prevented *t*-BHP-induced neuronal death. However, the TMP the parental compound of T-00 only showed neuroprotective effect at high concentration (100 μM) (Fig.3.2.1A).

Primary cortical neurons were also used to validate the neuroprotective effects of T-006 under *t*-BHP-induced challenge. 50 μM *t*-BHP induced about 50% of the cell viability reduction on cortical neurons and T-006 (0.001-10 μM) significantly protected the neurons. The protection was further confirmed by reduced LDH release. TMP (100 μM) also had moderate effect on this model (Fig.3.2.1B and C).

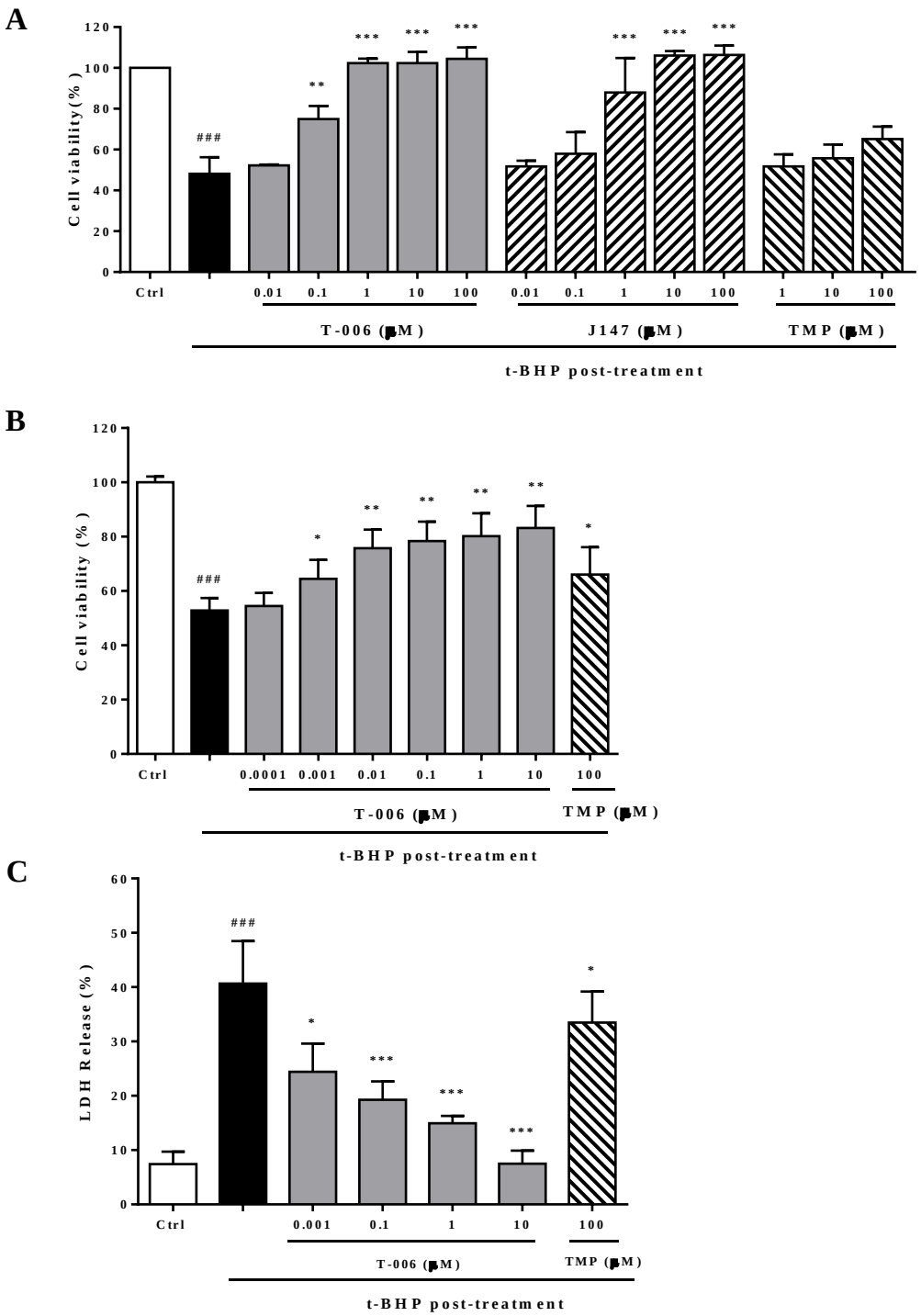


Fig.3.2.1 T-006 effectively prevents *t*-BHP-induced neuronal death.

(A) Cell viability impairment induced by *t*-BHP in PC12 cells were prevented by T-006. Pretreatment of indicated dosages of T-006, J147 and TMP for 2 h was followed by 24h exposure to 100 μM *t*-BHP for 24 h. MTT assay was

used to detect cell viability(n=6). (B) T-006 blocked *t*-BHP-induced neuronal death in cortical neurons. Prior to 24 h exposure of 50 μM *t*-BHP, cortical neurons were treated with T-006 or TMP at various concentrations for 2 h. Cell viability was determined by MTT assay(n=6). (C) T-006 reduced *t*-BHP-induced LDH release in cortical neurons. Prior to 24 h exposure of 100 μM *t*-BHP, cortical neurons were treated with T-006 or TMP at various concentrations for 2 h. LDH release was measured using cell viability detection kit (Roche) (n=6). $^{###}p<0.001$ compared to control group; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to *t*-BHP group.

3.2.2 T-006 potently attenuates *t*-BHP-induced neuronal apoptosis in PC12 cells.

Neuronal apoptosis was characterized after investigation of the neuroprotective effects of T-006 in *t*-BHP-induced cell lesion model. Intracellular ROS and RNS were also evaluated. Stained by Hoechst 33342, number of pyknotic bodies was visualize and T-006 exhibited potent anti-apoptosis activity (Fig.3.2.2A and B). Mitochondria membrane potential ($\Delta\Psi m$) loss is an early indicator for apoptosis. It is shown that *t*-BHP induced loss of $\Delta\Psi m$ and pretreatment T-006 (0.001-10 μM) in PC12 cells significantly restored the $\Delta\Psi m$ alternation(Fig.3.2.2C).

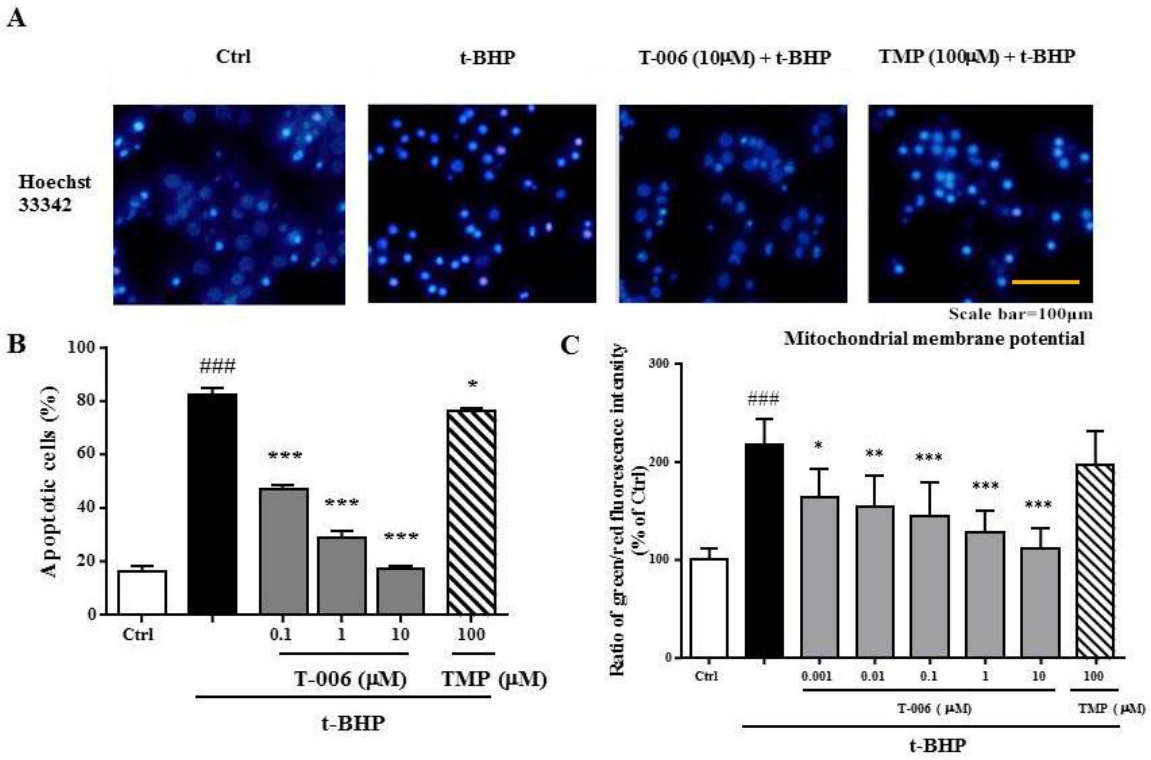


Fig.3.2.2 T-006 attenuates *t*-BHP-induced neuronal apoptosis in PC12 cells.

(A) T-006 prevents *t*-BHP-induced apoptosis in PC12 cells. PC12 cells were pre-incubated with T-006 or TMP at indicated concentrations for 2 h, followed by addition of 100 μM *t*-BHP. 24 h after *t*-BHP challenge, PC12 cells were stained with Hoechst 33342. (B) Statistical analysis of the number of pykonitic nuclei. The number of pyknotic nuclei as the hall mark of apoptosis was counted from representative Hoechst staining photos. For each sample, 6 pics were randomly taken and the results were presented as a percentage of the total number of nuclei counted. (n=6) (C) T-006 prevents *t*-BHP-induced alternation of mitochondrial membrane potential in PC12 cells. After pretreatment of T-006 or TMP for 2 h at the indicated concentrations, PC12 cells were exposed to 100 μM *t*-BHP. The mitochondrial membrane potential was evaluated by staining with the potential sensor JC-1 and analyzed with flow cytometry. Data expressed as percentage of control, $^{###}p<0.001$ compared to control group; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to *t*-BHP group.

3.2.3 T-006 attenuates *t*-BHP-induced increase of ROS and RNS in PC12 cells.

The molecular probes of DCF-DA, HFP, DAF-FM and DHR123 were used for the detection of intracellular production of hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite, respectively. Addition of *t*-BHP was found to substantially elevated the four major markers of ROS and RNS in PC12 cells. The four radicals were suppressed by the pretreatment of T-006 and J147 for 2 h (Fig.3.2.3). However, TMP at 100 μM could only mildly inhibit the overproduction of hydrogen peroxide and nitric oxide, but not hydroxyl radical or peroxynitrite (Fig.3.2.3). In terms of nitric oxide scavenging, T-006 was shown to be more potent than J147 at 0.01 and 0.1 μM. (Fig.3.2.3C).

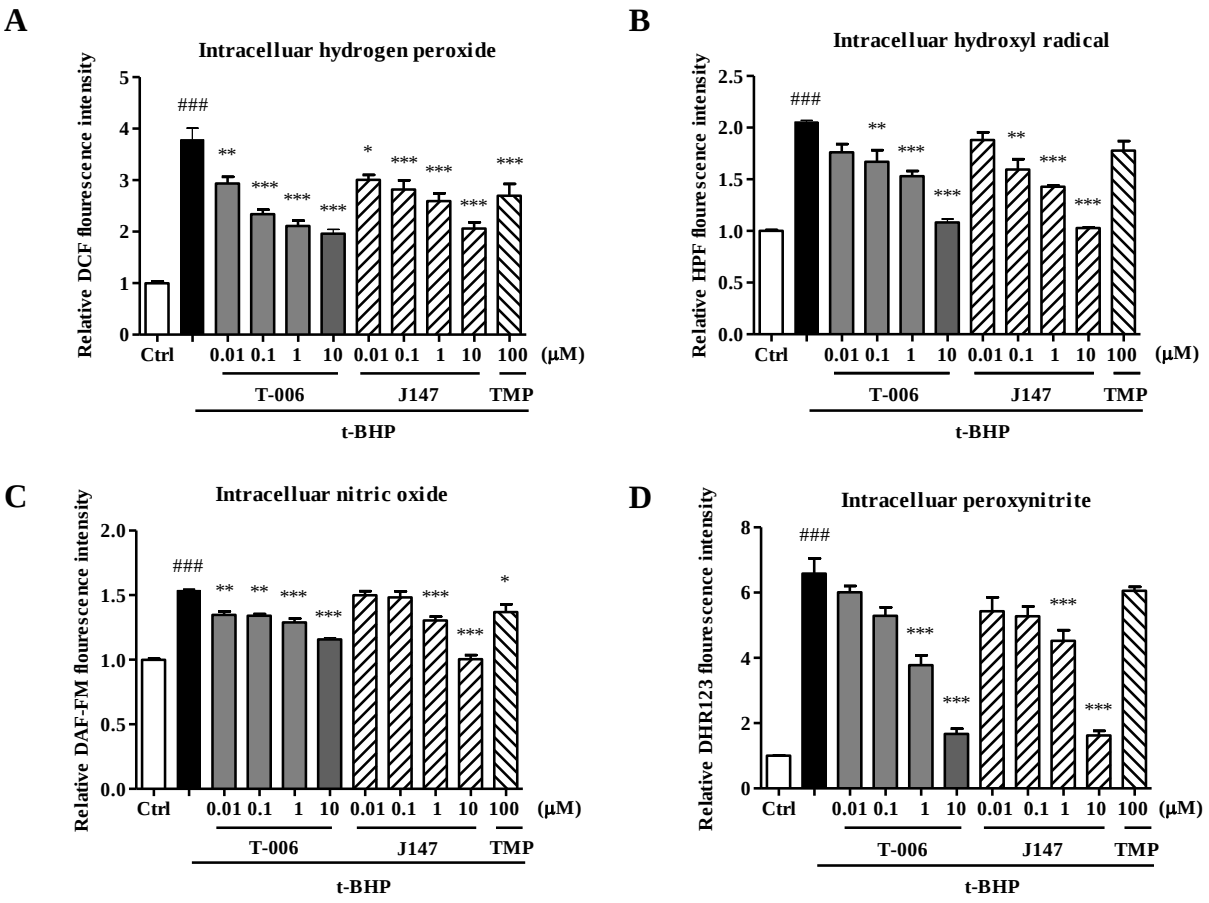


Fig.3.2.3 T-006 attenuates *t*-BHP-induced increase of ROS and RNS in PC12 cells.

Pretreatment of T-006, J147 and TMP at the indicated concentrations for 2 h were conducted on PC12 cells and then exposure of 100 μM *t*-BHP for another 6 h. (A) Intracellular hydrogen peroxide was measured by DCF-DA. (n=6) (B) Intracellular hydroxyl radical was measured by HFP. (n=6) (C) Intracellular nitric oxide was measured by DAF-FM. (n=6) (D) Intracellular peroxynitrite was measured by DHR123. (n=6) $^{###}p<0.001$ compared to control group; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to *t*-BHP group.

3.3 T-006 elicits neuroprotection in *in vivo* model of neurodegeneration

3.3.1 T-006 reduces memory deficits in APP/PS1 transgenic mice

In light of the promising neuroprotective activities shown by T-006 *in vitro*, we further used a transgenic mice model of AD to examine whether T-006 could reduce memory deficits *in vivo*. Previous studies have indicated a clear pathological change and behavioral alternation at 5-6 months in APP/PS1 transgenic mice using in Y maze test (Chen, Prior et al. 2011; Jankowsky et al. 2007). In my study, the transgenic mice aged 5-6 months were daily *p.o.* administrated with 2 mg/kg T-006, 2 mg/kg J147 or vehicle for 2 weeks(Fig.3.3.1A). After the first administration, memories of the mice were assayed in the Y-maze every two days. The study showed memory deficits in transgenic AD mice exhibited by significantly reduced correct ratio and increased training time in the Y-maze test compared with wild type mice (Fig.3.3.1B and C). With the administration of T-006 or J147, transgenic mice displayed significantly increase in average correct ratio and decrease in average training time at as early as day 1 post-treatment (Fig.3.3.1D and E). Moreover, T-006 and J147 significantly decreased the impairment of memory during the 14 days of intervention, suggesting that these drugs could reduce memory deficits in APP/PS1 transgenic mice (Fig.3.3.1F and G).

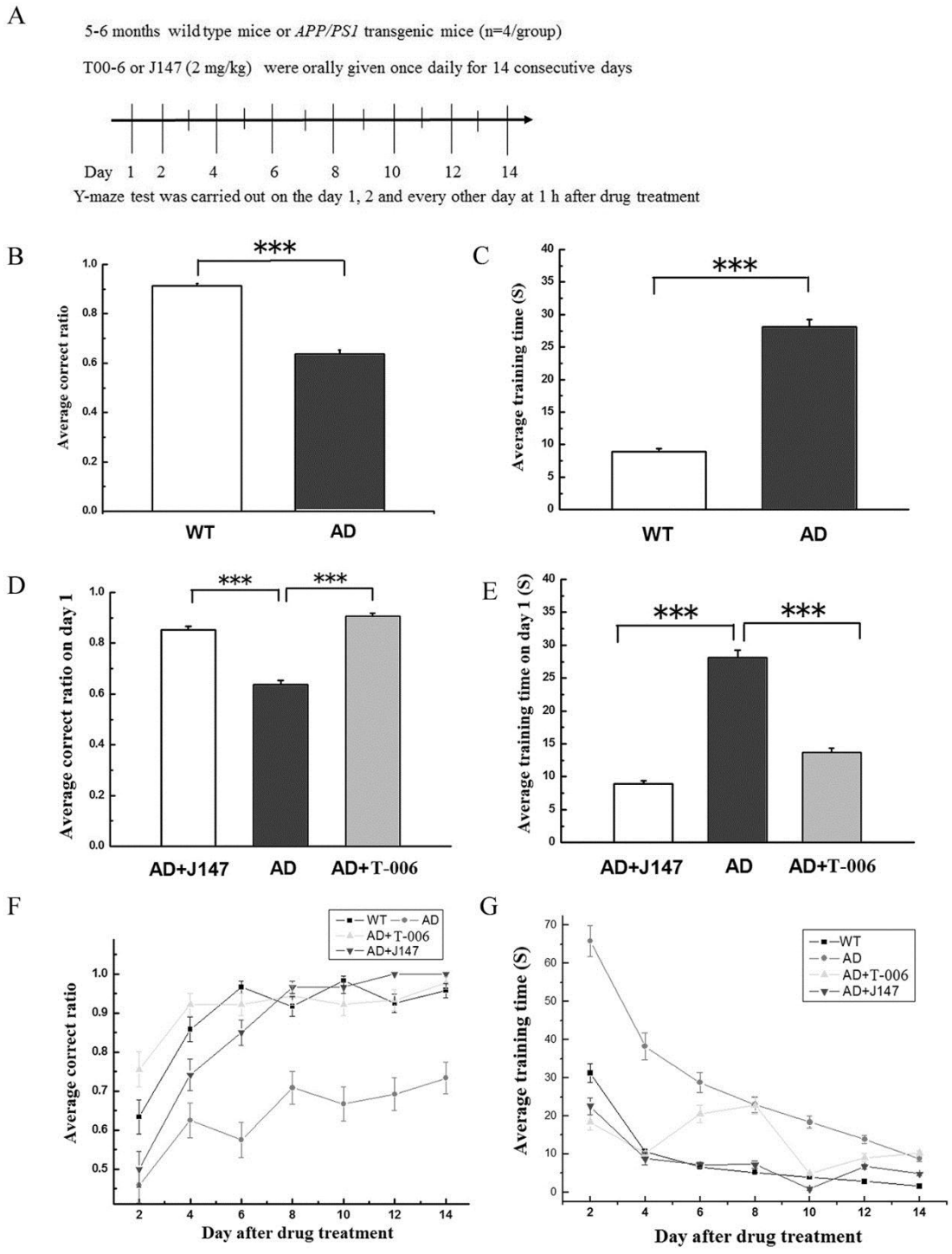


Fig.3.3.1 T-006 reverses cognitive deficits in APP/PS1 transgenic mice.

(A) The diagram of animal experiments. (B, C) Average correct ratio and average training time in APP/PS1 transgenic mice and their wild-type littermates in 14 consecutive days. (n=3) (D, E) Average correct ratio and average training time in mice treated with various agents at day 1 post-

treatment. (n=3) (F, G) Average correct ratio and average training time in mice treated with various agents in 14 consecutive days. (n=3) $^{***}p<0.001$

Chapter 4
Discussion

4.1 T-006 elicits neuroprotection against glutamate excitotoxicity in cerebellar granule neurons via the concurrent regulation of MAPK/ERK and PI3K/Akt/GSK3β pathways

Primary cultures of CGNs from rat have been used for neuroscience researches since 30 years ago. As one of the most prevalent *in vitro* models, CGNs were used to study almost every aspect of developmental, functional and pathological neurobiology (Gallo et al. 1982). The currently known AD pathogenesis factors such as amyloid precursor protein, tau protein, excitotoxicity and NO-mediated toxicity in CGNs were proved to resemble similar signaling transduction features as in hippocampal and cortical neurons (Allen et al. 1999; Canu et al. 1998; Contestabile 2002). Moreover, more than 90% of the neurons in cerebellum are granule neurons, which constitute the largest homogeneous neuronal population in mammalian brain.

Glutamate is one of the most important excitatory neurotransmitters which plays a fundamental role in neural transmission, development, differentiation and plasticity. However, improper management of glutamate level can lead to not only the malfunction of neural signaling properties, but may cause excitotoxicity, leading eventually to neuronal death (Lai et al. 2014; Stanika et al. 2009; Wang and Qin 2010). Excessive glutamate level is followed by over activation of glutamate receptors, resulting in an augmented Ca^{2+} influx, which initiates a cascade of intracellular signaling pathways. It is

claimed that excitotoxic neuronal death is not a uniform event but rather a mixed form of necrosis and apoptosis(Martin et al. 1998). In our study, glutamate-treated CGNs, in line with the opinion, exhibit the characteristics of apoptosis by showing chromatin condensation and apoptosis-related protein alternation and necrosis is also detected PI staining positive. Pretreatment of T-006, as shown in Fig.3.1.1 and 3.1.2, robustly protected CGNs from the glutamate-induced excitotoxicity and the subsequent apoptosis and necrosis.

Differentiated PC12 cells is another prevalent model for the study of neuronal cell death in the aspects of morphological, biochemical, and molecular changes (Mills et al. 1995). For a further insight into the potential application of T-006 in the treatment of AD, we used glutamate to induce excitotoxicity in NGF-differentiated PC12 cells. T-006 was found to provide neuroprotection against glutamate insult, as evidenced by the fact that T-006 significantly reversed the decrease of cell viability and shortening of neurite induced by glutamate. This result, along with the effective protection in the CGN model and previous proposed anti-oxidative activity, may enable T-006 to become a potential anti-AD agent targeting multiple mechanisms.

As described above, the influx of Ca^{2+} through NMDA receptor is known to mediate the glutamate-induced excitotoxicity. We performed experiments to determine the intracellular Ca^{2+} concentration with or without

T-006 pretreatment before glutamate stimulation in CGNs in order to investigate the whether T-006 may interfere with the NMDA receptor-mediated Ca^{2+} overload. In the present study, addition of glutamate robustly increased the intracellular Ca^{2+} concentration and the uncompetitive NMDA receptor antagonist MK801 as a positive control nearly fully inhibited the Ca^{2+} influx. Pretreatment of T-006, similar as memantine, partially blocked the Ca^{2+} overload, which indicates T-006 may be a partial antagonist of NMDA receptor.

Classically, ERK1/2, as one of the best-characterized members of MAPK family, is thought to participate in neurotropic actions like cell growth, differentiation and survival (Fukunaga et al. 1998; Hetman et al. 2004). It was a well-accepted perception that in the MAPK family, JNK/SAPK (stress-activated protein kinase) and p38 MAPK promote cell death, whereas ERK1/2 opposes cell death(Sturla et al. 2005). However, this view is overly simplistic. Increasing evidences demonstrate that both survival and death signals can activate ERK1/2. Over-activation of NMDA receptors and the ensued oxidative stress may cause sustained activation of ERK1/2 which induced the translocation of ERK1/2 to nucleus and promote neuronal death (Kolch 2000; Mebratu and Tesfaigzi 2009). In our study, sustained over-activation of NMDA receptor induced by glutamate motivated the up-regulation of phosphorylation of ERK1/2 and its upstream MEK in a time

dependent manner and mediated the glutamate-induced neuronal death of CGNs. Whereas pretreatment of T-006 exerts its neuroprotection by asignificantly ameliorated the ERK1/2 and MEK up-regulation.

Unlike the controversial role of ERK1/2 plays in the neuronal survival/death, activation of PI3-K/Akt cascade is regarded as a pivotal pro-survival index in neurons(Cantrell 2001). PI3-K, which activated by the recruitment to cell membrane, phosphorylate PIP2 to produce PIP3 (phosphatidylinositol (3,4,5)-triphosphate). Then PDKs (3-phosphoinositide dependent protein kinase)(Knight 2010) were activated. PDK1 phosphorylates Akt at Thr308 and possibly Ser473 residues, resulting in its activation and thereby contributing to the cell survival by attenuation of apoptosis. The activation of GSK3 has been shown to be involved in the neuronal apoptosis (Lee et al. 2008) and Akt is suggested to inhibit the activation of GSK-3 by phosphorylation of ser21 in GSK-3α and Ser9 in GSK-3β. In our study, although T-006 alone did not influence the Akt, it did reverse the glutamate-induced decrease in phosphorylated Akt level. LY294002, as a potent PI3-K inhibitor, blocked the maintenance of p-Akt conducted by T-006. Moreover, a functional involvement of PI3-K/Akt in T-006 protection is verified by the using of LY294002 and Akt inhibitor iv partly abolished the protective effects.

However, it is observed that MEK and ERK inhibitor could only

partially protect glutamate-induced neurotoxicity and Akt or PI3-K inhibitor could only partially abolish the protective effect of T-006. Western blot and pharmacological methods herein indicate that pretreatment of T-006, at the concentration of 0.3μM, concurrently reversed the activation of MAPK/ERK pathway and the inhibition of PI3-K/Akt pathway. It indicates that T-006 play the protection role in this system through both inhibiting MAPK/ERK pathway and restoring PI3-K/Akt pathway. This could also explain the reason T-006 is more potent than its parental compound TMP and the similar agent J147 in the model.

4.2 T-006 attenuates *t*-BHP-induced neuronal apoptosis in PC12 cells

Oxidative stress, including ROS and RNS, is one of most important players in aging and age-related diseases, such as cancer, cardiovascular diseases, chronic inflammation, and neurodegenerative diseases(Halliwell 2006). Oxidative stress is found to be present in the early pathological stage of AD. Under normal physiological condition, oxidative stress and damage are controlled by endogenous antioxidant components and enzymes. However, in the AD brain, significant decrease in levels of antioxidant enzymes are discovered, which make it more vulnerable to oxidative damages (Texel et al. 2011). Therefore, brain-accessible agents which scavenge free radicals or

induce endogenous antioxidant enzymes maintains the potential in treating neurodegenerative diseases like AD and the agents to hit both the targets is believed to be more potent.

We had previously reported that *t*-BHP, a compound which exerts oxidative stress, dose-dependently decrease cell viability in PC12 cells (Cui et al. 2013). Therefore, this model was used to test the anti-oxidative activity of T-006. The results showed that in PC12 cells, T-006 was effective from the concentration of 0.1 μM. When at concentrations higher than 1 μM, T-006 almost fully prevented *t*-BHP-induced cell death, which excels the potency of J147. We also used primary cultured cortical neurons to validate the neuroprotective effects of T-006 against *t*-BHP-induced neuronal death. Cell viability and LDH release were both tested. Pretreatment of T-006 at various concentrations (0.001-10 μM) dose-dependently prevented *t*-BHP-induced neuronal death in cortical neurons. Furthermore, molecular probes of DCF-DA, HFP, DAF-FM and DHR123 were used to detect intracellular ROS and RNS. A substantially elevation of the intracellular levels of hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite, major markers of ROS and RNS were found in PC12 cells. Pretreatment of T-006 significantly prevented the increase of these four radicals.

However, the potent neuroprotective effects of T-006 may not be mainly attributed to its antioxidant activity. First, T-006 produced neuroprotective

effects at very low concentrations, while potent antioxidants even at high concentrations were reported as ineffective in the neuroprotective assays described above (Pohanka 2013). Second, J147, an analog of T-006 that produced almost similar efficacy in all the assays in our study, was reported to produce neuroprotective effects independent of antioxidant property (Chen, Prior et al. 2011). The high potency of T-006, according to our preliminary studies, may attributed to the induction of many pro-survival transcriptional factors, such as erythroid2-like factor 2 (Nrf2) by T-006. Nrf2 controls the expression of many endogenous antioxidant enzymes, which make it one of the most important regulator of redox homeostasis(de Vries et al. 2008). Nrf2 is activated by escaping from the Keap1-mediated proteasomal degradation, allowing Nrf2 translocating to nucleus and bind to the DNA antioxidant response element (ARE)(Calkins et al. 2009). In our study, the potent antioxidative activity of T-006 may be attributed to the Nrf2 activation, which deserves further investigation. Moreover, according to literatures, expression of many neurotrophic factors such as NGF and BDNF were stimulated by treatment of J147(Prior, Dargusch et al. 2013). suggesting that T-006 might also act on these targets. Further experiments are being undertaken in our laboratory to determine the neuroprotective targets of T-006.

4.3 T-006 elicits neuroprotection in transgenic mice model of AD

In vitro study showed that T-006 may be potential multifunctional agent for treatment of AD. We then carried on the study in animal model in terms of learning and memory. T-006 was tested in APP/PS1 transgenic AD mice model.

APPswe/PS1ΔE9 transgenic mouse model, which overexpresses both mutant human amyloid precursor protein (APP) and human mutant presenilin 1 (PS1), is one of the most classic transgenic models of AD(Garcia-Alloza et al. 2006; Jankowsky, Younkin et al. 2007). These mice show pathological hall marks of AD such as elevated Aβ levels, amyloid plaque deposits, cognitive deficit, and neuroinflammation. In our study, 12 transgenic mice and 4 wild type littermates, at 5-6 months age were used. T-006, as well as its origin compound J147 were administrated daily for 14 days. In previous study, J147 has been shown to provide immediate cognition benefits(Prior, Dargusch et al. 2013). T-006, in this Y-maze test, also displayed significant activity in reducing memory deficits in APP/PS1 transgenic mice as potent as J147.

4.4 The multi-target paradigm in drug development

During the past few decades, the single-target paradigm is the mainstream in the industry of drug development. An exquisitely selective ligand which bonds to drug targets was pursued and unwanted side effects

was carefully avoided. By virtue of the developments in molecular biology, scientists are able to discover a key target for a particular disease ensued by designing of a certain molecule to affect the target. With the great success has been achieved, the single-target paradigm will continue being pursued in the future. However, the single target paradigm seems to be incapable in finding effective treatment for complex diseases such as cancer, AD and PD due to their multiple pathogenic mechanisms. Developments of highly selective ligands for the treatments of complex disease have been largely unsuccessful. 99% of the anti-AD drugs showed no improvement in cognitive deficits in AD patients and did not survived phase III clinical trials. New paradigms that address disease etiological complexity has gained increasing acceptance (Geldenhuys et al. 2011). There are two conceivable strategies to hit the multiple targets implicated in the complex diseases. The multicomponent strategy, which has been successfully employed in traditional medicine for thousands of years and in current drug cocktails used for the control of HIV. The other attempt is to employ one compound to hit multiple targets. The latter strategy is more convenient to used but more difficult to fulfill. Multifunctional drug may be considered as the simple version of the network medicine and could be further developed into network medicines, which represent a great promise in fighting against complex diseases. In our study, T-006 is developed by joining J147 and TMP. J147 is a curcumin derivative

and has shown a broad spectrum of neuroprotective effects including oxidative stress scavenging, neurotrophic effects and preventing the reduction of energy metabolism(Chen, Prior et al. 2011; Prior, Dargusch et al. 2013). Tetramethylpyrazine(TMP) is an active compound extracted from traditional Chinese medicine and has been proven to possess free radical scavenging and mitochondrial biosynthesis enhancement effects. Hence, T-006 has promising potential to become a multi-target drug candidate for AD therapy

4.5 Summary and conclusions

. In this study, we investigated the neuroprotective effects and neuronal differentiation promoting effects, as well as the underlying mechanisms, of T-006, a promising anti-Alzheimer's compound derived from Chinese medicinal component tetramethylpyrazine (TMP), *in vitro* and *in vivo*.

(1) The results showed that T-006 prevented glutamate-induced excitotoxicity in primary CGNs (EC_{50}=59.4nM) more potently than memantine, and also superior to those of TMP and the other parental molecule J147. T-006 achieves the anti-excitotoxicity effect via inhibiting MAPK/ERK pathway and activating PI3K/Akt pathways concurrently.

(2) T-006 effectively prevented *t*-BHP-induced neurotoxicity in PC12 cells and primary cultured cortical neurons by significantly scavenging the elevated intracellular ROS and RNS induced by *t*-BHP.

(3) Results of animal studies showed that T-006 reversed learning and memory deficits in APP/PS1 transgenic mice AD model.

Taken together, we have shown that T-006 might be the multifunctional anti-AD leads, which offer not only strong support for the potential therapeutic strategies in the prevention and treatment, but also the molecular insights into the pathological mechanisms of AD.

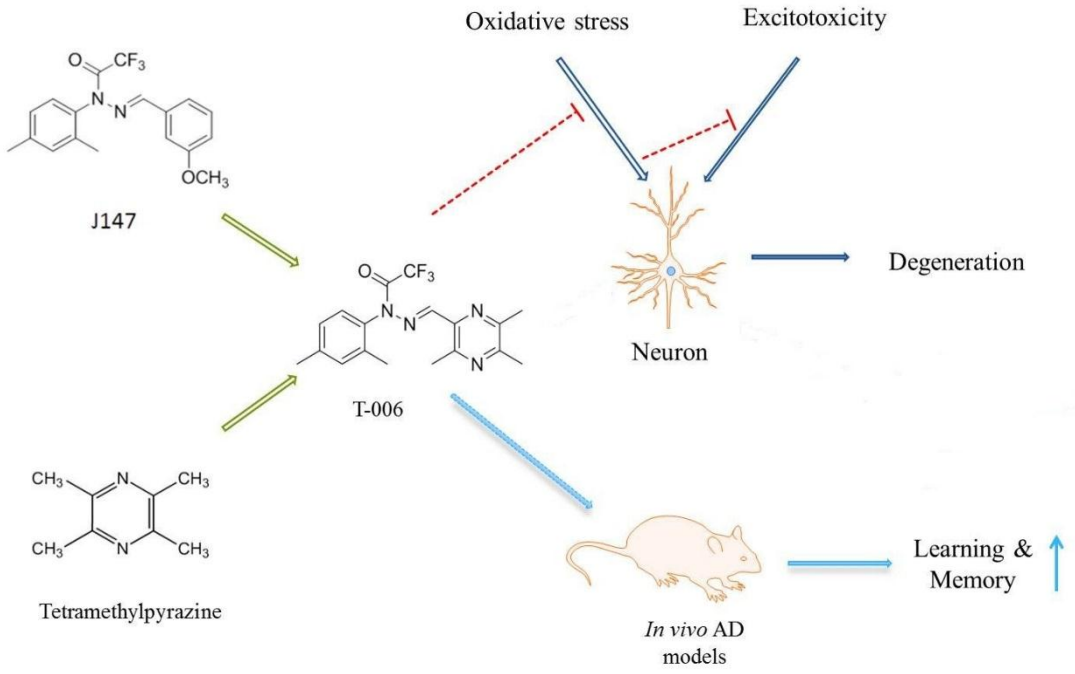


Fig.4.1 The schematic illustration of activities of T-006

4.6 Future studies

1. To further investigate the underlying anti-oxidative mechanisms of T-006

The free radical and oxidative stress theory of aging suggests that oxidative damage is a major player in neuronal degeneration. In our current study T-006 showed potent free radical scavenging activities but the underlying mechanism is hardly touched. Thus, the involvement of T-006 in the oxidative stress-related mechanism including mitochondrial dysfunction and Nrf2/Keap1/HO-1 pathways is investigated thereafter.

2. Interrogating activities of T-006 in other neurodegenerative models *in vitro* *in vivo*

oxidative stress has a well-established pathophysiological feature in AD. Other neurodegenerative diseases e.g. PD is also closely related with oxidative stress and apoptosis, leaving T-006 another realm to probe.

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