In vivo pharmacological evaluation of a novel dual-target inhibitor and methylprednisolone as Alzheimer's disease treatments

Student's name: Bocheng Zhang, MRes of Pharmacy

Student's number: 14331661

Supervisors: Dr. Zheying Zhu; Dr. Lisa White

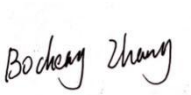
September 2022

Declaration

I understand the nature of plagiarism and that it is a serious academic offence.

I confirm that no materials in this project have been plagiarised and I consent to my work being run through a plagiarism checker.

Student: Bocheng Zhang

Signature: ……  **……**

Date: …30/09/2022……

Abstract

Background: Alzheimer's disease (AD) is the most usual type of dementia, which often occurs in the late stage of life. As the population increases, the ageing problem has become more serious. Some researchers predicted that the number of AD patients would triple worldwide. Even though AD is a tough challenging disease, there are still no effective drugs or therapies that could modify and overcome it.

Objective: This report illustrates the evaluation of a new inhibitor compound **19n** (AChE + metal) and methylprednisolone (MP) on An acute Aβ toxic mouse model, to prove whether the tested compounds have anti-AD efficacy in vivo.

Design and methods: An acute Aβ toxic mouse model was established and the short-term evaluation Y maze and long-term evaluation Morris water maze were applied to test whether the cognitive impairment was improved by **19n** and MP. After testing, the brain samples were collected for measuring the levels of cytokines and inflammatory factors.

Results: The results of behavioural evaluation demonstrated that after both **19n** and MP were treated, the time consumption of finding the hidden platform was decreased and the proportion of time spent in the quadrant containing the platform was elevated. Besides, no toxicity was observed in both two compounds at the described dosage.

Conclusion: Both **19n** and MP could alleviate Aβ-induced cognitive decline.

Acknowledgement

This study was proved by the University of Nottingham and China Pharmaceutical University. First I want to appreciate my supervisors Dr. Zheying Zhu and Dr. Lisa White. Thank you for the help in my whole period of study and the chance to experience academic life at China Pharmaceutical University. I appreciate your guidance and support in my study.

Next, I would like to thank Xinuo Li and Yuan Sun, who taught me a lot about my experiment skills and critical thinking.

Finally, thanks to the company of my girlfriend Yahui Liu and my parents, who always sustain my decisions and support for my research.

Contents:

List of figures

List of abbreviation

αCTF	α-C-terminal fragment
βCTF	β-C-terminal fragment
Aβ	β-amyloid
Acetyl-CoA	Acetyl-coenzyme A
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	AChE inhibitors
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease family
Al	Aluminium
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ASC	Adaptor apoptosis-associated speck-like protein containing a CARD
BACE1	β-APP cleaving enzyme 1
CaMPK II	Ca/calmodulin-dependent protein kinase II
CARD	C-terminal caspase recruitment domain
Cd	Cadmium
CDK	Cyclin-dependent kinases
cGAS	Cyclic GMP-AMP (cGMP-AMP) synthase
ChAT	Choline acetyltransferase
CHT1	Choline transporter
CNS	Central nervous system
CQ	Clioquino
CRISPR/ Cas 9	Clustered Regularly Interspaced Short Palindromic Repeat/ CRISPR associated protein 9
Cu	Copper
DG	Dentate gyrus
Don	Donepezil
EOAD	Early-onset AD
ER	Endoplasmic reticulum
FDG-PET	Fluorodeoxyglucose PET
Fe	Iron
GSK3β	Glycogen synthase kinase 3β
H_2O_2	Nonionic type hydrogen peroxide
HFIP	Hexafluoroisopropanol

HO^-	Hydroxyl radical
IL	Interleukin
IRF3	Interferon regulatory factor 3
ISGs	Interferon stimulating genes
LOAD	Late-onset AD
MARK	Microtubule affinity regulating kinase
MP	Methylprednisolone
MTBD	Microtubule-binding domain
MTDLs	Multi-target-directed ligands
mtDNA	Mitochondrial DNA
MWM	Morris water maze
NBM	Nucleus basalis of Meynert
NF-κB	Nuclear factor-κB
NFT	Neurofibrillary tangles
NLRP3	NOD-like receptor thermal protein domain associated protein 3
NMDA	N-methyl-D-aspartate
NO)	Nitric oxide
O_2^-	Superoxide radical anion
$ONOO^-$	Peroxynitrite
PFA	Paraformaldehyde
PKA	Cyclic-AMPdependent kinase
PPs	Protein phosphates
PQBP1	Polyglutamine binding protein 1
PRD	Proline riched domain
PRRs	Pattern recognition receptors
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PUFAs	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sAPPα	Soluble APP alpha
sAPPβ	Soluble APP beta
STING	Stimulator of interferon genes
TBK1	Tank binding kinase 1
ThT	Thioflavin T
TNF	Tumour necrosis factor
TREM2	Triggering of the receptor expressed on myeloid cell 2
VAChT	Vesicular acetylcholine transporter
Zn	Zinc

1. Introduction

1.1 Alzheimer's disease

Neurodegenerative disease is a group of diseases, such as cerebral infarction, Huntington's disease, Parkinson's disease and amyotrophic lateral sclerosis *etc*. Alzheimer's disease also belongs to this category and it also not only belongs to but also the representative species of dementia. It was first discovered by Alöis Alzheimer when he was treating a woman named Auguste Deter aged 51 in 1907. Due to the strange symptoms cannot understand questions or forgetting things immediately, he paid more attention to this patient. After she died, he did some research on her brain and find amyloid plaques and tangles formed by neuronal fibrils. In 1911, this disease was named by his name and the result of his autopsy had become hallmarks of AD. Based on the descriptions given by Alzheimer's and clinical observations, AD patients always suffer memory deficiency, also sometimes they could not recognize the people or substances which are familiar to them, this situation is named cognitive impairment or cognitive deficiency. As the disease processing, such episodes occur frequently in the late time of AD, patients may lose most of their body function which finally leads to death [1]. AD could be divided into two types, one is late-onset AD (LOAD) which is the most usual type that happens in people who are approximately aged over 65, and the age of morbidity of the other type is around 30 years old, which is called early-onset AD (EOAD), it also named the familial form of AD (FAD) due to it may inherit to filial generation. Genome wide association studies by using single

nucleotide polymorphisms to deal with potential biases, and several genes had been found that were related to LOAD, such as GAB2 and LRAT [2]. EOAD has been reported that may happen between 30 to 50 years old, which is regulated by the mutation in some genes: one is the amyloid precursor protein (APP) that is a kind of transmembrane protein after combination work hold by several enzymes, the final product is β-amyloid (Aβ), the other is presenilin family (PSEN) which could combine with nicastrin, PEN2 and APH-1 to form γ secretase, one vital enzyme in cleaving APP, and it contains two types, presenilin 1 (PSEN1) and presenilin 2 (PSEN2) which have similar construction and functions. As the population raise, the ageing problem would be vital and serious. The number of AD patients has been estimated by scientists in Europe will become double and triple all over the world in 2050 [3]. Despite the issue deteriorating and scientists focused on solving this problem, there is no effective therapy or drug which could alleviate AD.

Nowadays, AD is still diagnosed by two pathogenic features based on Alöis Alzheimer's research: the gathering of Aβ to form amyloid plaques out of cells and neurofibrillary tangles (NFT) to destroy the normal structure in cells [4,5]. Both two hypotheses influence the direction of research and development of new drugs, especially the Aβ hypothesis [6]. Besides, several other mechanisms support the pathology of AD to make it a complicated neurodegenerative disease.

1.1.1 Mechanisms of AD

1.1.1.1 cholinergic hypothesis

The cholinergic dysfunction could be the revolutionary proposition of the whole AD research period, which on the grounds of the cerebral cortex losing presynaptic cholinergic markers [7], nucleus basalis of Meynert (NBM) located in the basal forebrain could regulate the cortical cholinergic nerves distribution in AD [8], and illustration that cholinergic antagonist could deteriorate memory deficiency but agonists worked as the opposite effect [9, 10]. Acetylcholine (ACh) is a kind of chemical substance which was discovered by its message transfer function between nerves in 1936, subsequently, these substances had been defined as neurotransmitters. ACh was the first discovered and synthesized neurotransmitter used by some neurons mainly released ACh for information transfer, which is named cholinergic neurons, and it is a vital part of the cholinergic system [11]. This system has been considered that is related to learning and some researchers believed that ACh was associated with memory [12, 13, 14]. The appearance of ACh needs a synthesis procedure, by fusing choline and acetyl-coenzyme A (acetyl-CoA). These two participants are abundant in the cytoplasm and they are important to several physiological activities. The course of composing ACh mainly happens in the cytoplasm of neurons, especially those which primarily via ACh to process information transfer, both two substrates are catalyzed by the enzyme choline acetyltransferase (ChAT) [15]. After the procedure, ACh could be packaged in a 40nm diameter sphere named synaptic vesicles and sent into the interspace between the presynaptic membrane and postsynaptic membrane. By targeting nicotinic receptors or muscarinic receptors situated on the postsynaptic nerve terminal to process information transfer [16]. However, the enzyme acetylcholinesterase (AChE) which participates in numerous physiological responses plays the role of transferring ACh into choline and acetate to inactive its function in the synaptic

cleft, thus to minish information exchange between cells [17]. Based on the strong relationship between ACh and hippocampus-dependent memory, the amount of ACh plays a crucial role [18, 19]. Therefore, increasing the ACh by inhibiting AChE had become a method to treat AD [20].

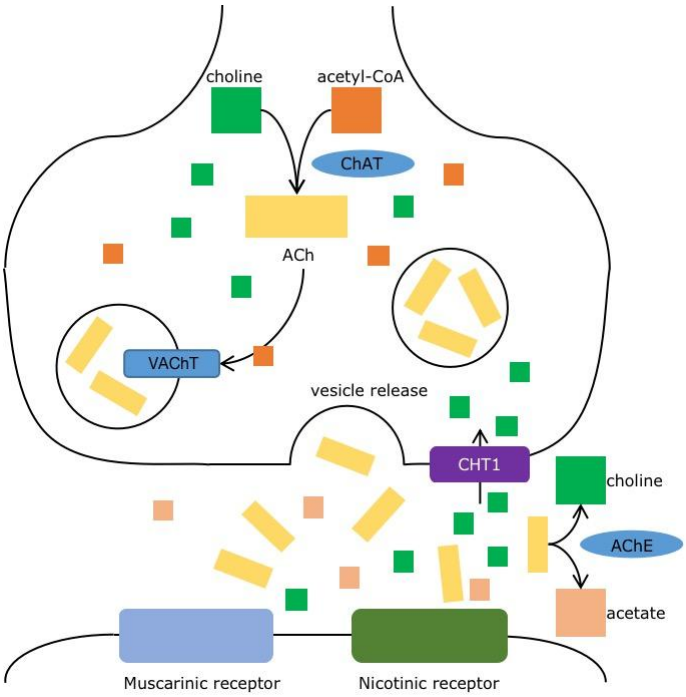


Figure 1: Physiology of acetylcholine synthesis, release and inactivation. Acetylcholine (ACh) synthesis happens in the final part of the presynaptic nerve, the enzyme choline acetyltransferase (ChAT) promotes choline to interact with acetyl-coenzyme A (acetyl-CoA). Once the synthesis is finished, the vesicular acetylcholine transporter (VAChT) transports ACh from the cytosol into synaptic vesicles. When presynaptic neuron depolarization, the vesicles containing ACh would be released into the synaptic cleft where they could target nicotinic receptors and muscarinic receptors on the postsynaptic nerve terminal. The excessive neurotransmitters are catalyzed by the enzyme acetylcholinesterase (AChE) to form choline and acetate, which are returned by a reuptake mechanism via high-affinity choline transporter (CHT1) [11].

1.1.1.2 metal hypothesis

Dysregulation in the homeostasis of metals also could impact AD progress [21]. In AD brains, aluminium (Al) was enriched, especially in NFT and senile plaques [22, 23]. For the biometals, the high-affinity region held by Aβ could combine with copper (Cu) and zinc (Zn), and APP could also interact with them via the N-terminal metal-binding domain [24]. Cu and Zn could modify Aβ and shift it towards aggregation rapidly [25]. Chelation composed of Cu or iron (Fe) with Aβ could restrain oxidative phosphorylation which induces ROS production [26]. For the heavy metals, cadmium (Cd) could induce cholinergic neuronal cell death and mercury (Hg) has a high affinity with tubulin further associated with the suppression of tubulin polymerized to microtubulin, which further leads to NFT [27]. To conclude, based on these clues, it is obvious that metals are contributed to the progression of AD from disparate directions. It hints to us metal might be a suitable and potential therapeutic target as well.

1.1.1.3 Neuroinflammation hypothesis

Besides Aβ plaques and NFT, activation of microglial also plays a crucial part in AD. Inflammation is the protective mechanism of the body, but excessive inflammatory responses would induce tissue damage [28]. The microglial is the basic immune cell resisting inflammatory activities induced by several encephalopathies or infected by bacteria or viruses in the central nervous system (CNS), which regulates cell death or waste products that produce elimination during inflammation [29]. In the normal state, the microglial plays a vital role in protecting CNS, such as tissue maintenance or confronting the injury response and it is the first cell which responds to cerebral injuries [30, 31]. Also, the excessive Aβ could be recognized by

pattern recognition receptors (PRRs) which are always located in the out layers of cells, especially the immune cells. In CNS, microglial cells are the PRRs carriers, they catch the combination signal and activate phagocytosis to mediate Aβ degradation [32]. There is a crucial gene expressed in the microglial named TREM2 that plays the role of maintaining homeostasis of the brain and regulating the autophagy of cell debris as well as Aβ in neurons [33]. One research illustrated that mutations in TREM2 would increase the risk of AD which also proved the dependency between microglial and AD [34]. However, activation of microglial is not always a good thing. After Aβ is recognized by PRRs, the nuclear factor-κB (NF-κB) pathway is evoked. This signal pathway could be evoked normally after external stimulation which induces the release of pro-cytokines, such as tumour necrosis factor (TNF) which always induces cell necrosis or partial inflammation and some members of the interleukin (IL) family, a group of peptide regulate the immune responses, such as interleukin-1β (IL-1β) which belongs to monokines and chemokine IL 8, and the assemblage of NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome [32, 35]. For one thing, excessively produced pro-cytokines could induce and enhance further glial activation and upregulation of the expression of BACE1, which may induce a great amount of Aβ production, resulting in neuron damage or death [32]. For the other thing, NLRP3 was found and combined with adaptor apoptosis-associated speck-like protein containing a CARD (C-terminal caspase recruitment domain) (ASC) and activated protease caspase 1, which combine and form a hexamer that works as the core of Aβ aggregation [32, 36]. Based on the mechanism and the importance of inflammation in AD, evaluating the levels of some cytokines could also be a diagnostic criterion [37].

1.1.1.4 Aβ hypothesis

In the last two decades, the Aβ cascade hypothesis was the most important milestone of the whole period of research on AD. To illuminate how the Aβ appears, the APP must be mentioned. It is proteolyzed in a modal procedure which is initially cleaved by β secretase, such as BACE1, and followed is γ secretase. After the modification by these two proteases, the pathogenic Aβ peptides are produced. In this process, soluble APP β (sAPPβ) is the product and is released after the first cleavage, the β-C-terminal fragment (βCTF) suffers the second cleavage and forms Aβ [38, 39]. Then Aβ would be released out of the cell, and it would be degraded. Nevertheless, the ability to clear Aβ in aged individuals or pathological conditions would be declined, due to the Aβ accumulated in the interspace of neuronal cells and the appearance of amyloid plaques also named senile plaques, which may lead to neurotoxicity, neurodegenerative and neuronal cell necrosis or death. It has been reported that the appearance of plaques may promote the accumulation of Tau protein, which induces neuron impairment [40, 41]. However, APP firstly could be hydrolyzed by α secretase, which produces sAPPα and αCTF, after cleaving by γ secretase. α secretase belongs to a disintegrin and metalloprotease family (ADAM), which contains ADAM17, ADAM10 and ADAM9 [42]. One research illustrated that ADAM10 deficiency could induce APP prone to produce Aβ so that it could be a probable aim for treating AD [43].

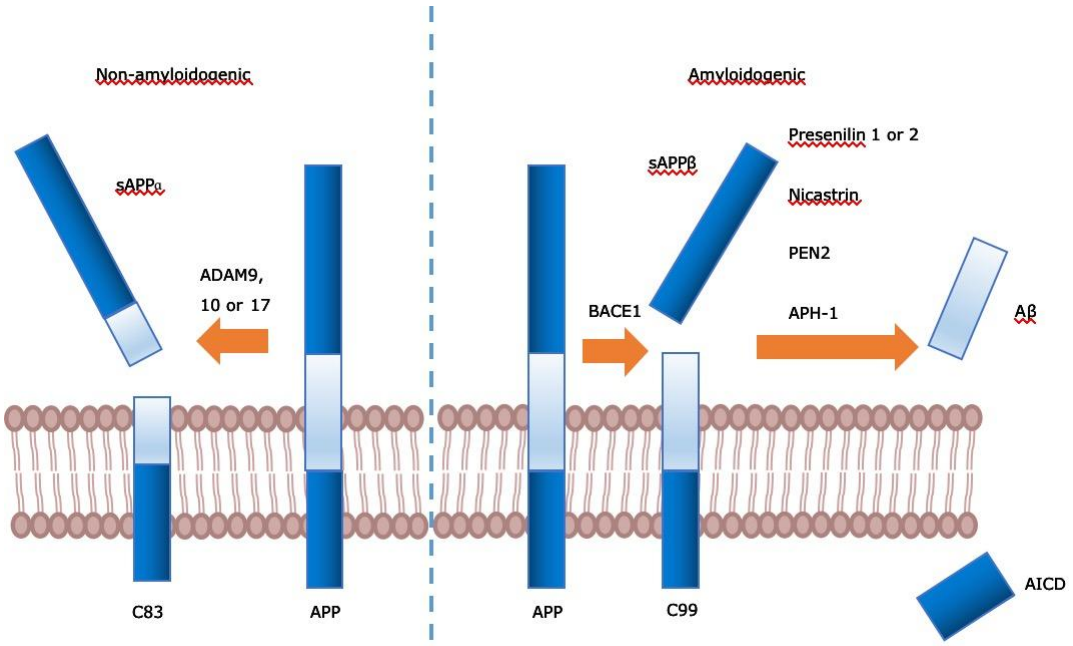


Figure 2: Non-amyloidogenic and amyloidogenic proteolytic processes of APP. The first pathway cannot produce pathological Aβ which is processed by a disintegrin and metalloprotease (ADAM) family, such as ADAM17, ADAM10 and ADAM9. After the cleavage, APP would produce the membrane-tethered domain C83 and release soluble APP alpha (sAPPα). For the pathological pathway, APP is initially hydrolyzed by β-APP cleaving enzyme 1 (BACE1) to produce sAPPβ and C99. After sAPPβ release, the intracellular domain C99 would be sequentially cleaved by γ secretase which is composed of several parts: presenilin 1 or 2 (PSEN1 or PSEN2), nicastrin, PEN2 and APH-1, which may form Aβ and APP intracellular domain (AICD). Finally, Aβ would be released out of the cell and form Aβ plaques [44, 45].

1.1.1.5 Hyperphosphorylation Tau protein hypothesis

Tau protein is always known as a microtubule-associated protein. Microtubule is an important factor in composing basic cell structure, thus tau protein plays a key character in maintaining cytoskeleton stabilization, especially in neurons [46]. Alternative splicing is a process that changes sequences of exons after transcription, which may produce several different transcripts, thus different proteins would be translated based on these transcripts. The MAPT gene could translate human tau protein, which could transcript six transcripts through this process, and encode six different tau proteins finally [47].

The six isoforms differ from zero, one or two at the beginning of the sequence which is called N-terminal insert (0N, 1N and 2N) and three or four repetitive sequences (3R or 4R) between the proline riched domain and C-terminal domain, named microtubule-binding domain. The amounts of isoforms which contain 3R and 4R are approximately the same in a normal brain, but in tauopathies, their distribution cannot be equal [48]. For the structural role, tau is mainly localized in axons of neurons, which could promote axonal microtubule stability [49,50]. Also, tau could regulate mitochondrial dynamics and functions as well as mitochondrial transport [51]. These functions of tau protein would be altered as a phosphorylation event occurs. Besides the appearance of Aβ plaques, AD patients may suffer tau protein hyperphosphorylation which results in the formation of NFT, and then attenuate the ability of binding to tubulin, leading to its detaching from microtubules, which may cause cell disability or cell death [52]. Besides, some reports have illustrated that the appearance of NFT could induce synapse loss in transgenic mice [53, 54]. Tau phosphorylation is regulated by such kinases which could be classified into two kinds: proline-directed kinases and non-proline-directed kinases. For the proline-directed kinases, the glycogen synthase kinase 3β (GSK3β) must be mentioned. It localizes to neurons and associates with the microtubules. It has been considered that the expression of GSK3β elevating, which may shift tau towards phosphorylation [55]. Lucas et al. proved that increasing the expression of GSK3β in the brain could elevate the level of tau phosphorylation in numerous regions by using immunoblot analyses in transgenic mice [56]. Besides, cyclin-dependent kinases (CDK) including CDK2 and CDK5 are both associated with tau phosphorylation [57]. For the non-proline-directed kinases, including microtubule affinity regulating kinase (MARK), cyclic-AMP dependent

kinase (PKA) as well as Ca/calmodulin-dependent protein kinase II (CaMPK II) [58]. However, there are some phosphates which may dephosphorylate phosphorylated tau protein. Normally, there are four phosphoserine/phosphothreonine protein phosphates (PPs) distributed in the brain, PP1, PP2A, PP2B and PP2C [58, 59]. One research illustrated that CDK5 could phosphate PP1 at T320, which may suppress the activity of PP1 [60]. The other research demonstrated tau phosphorylation and total tau was regulated by GSK3*β* through PP2A [61]. Based on the findings, an imbalance of kinase and phosphate might be the fundamental reason for tauopathy, thus keeping homeostasis of these two factors might be a novel target for alleviating AD.

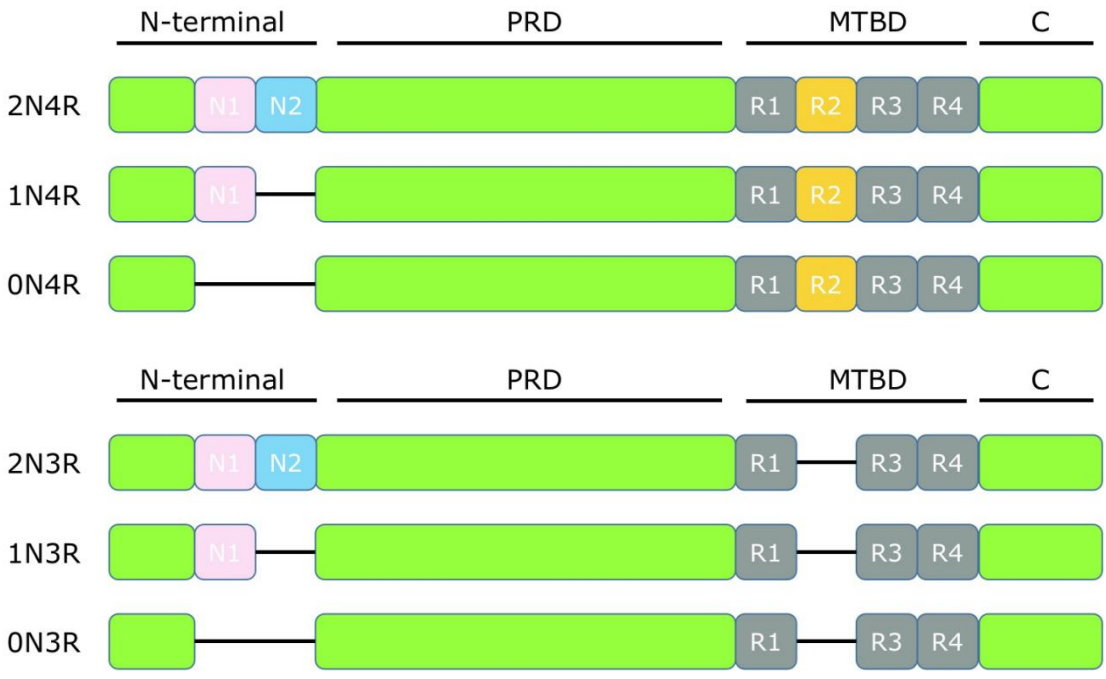


Figure 3: Differences between six isoforms of tau protein. The six isoforms are 2N4R, 1N4R, 0N4R, 2N3R, 1N3R and 0N3R. In N-terminal, different amino acid sequences encoded by exon 2 and exon 3 could form three types: both of these two exons are excluded, including one exon to form 1N (exon 2) or included both exons. The central part of tau is the proline

riched domain (PRD). The alternative splicing happens in exon 10 which is situated in the microtubule-binding domain (MTBD) causing the formation of 3R or 4R. The C-terminal of all six isoforms is the same [48].

1.1.1.6 ApoE

Aβ plaques and NFTs are two important symbols of AD, which could be linked by apolipoprotein E (ApoE) [62]. ApoE contains three isoforms which differ from each other at the 112 and 158 amino acids: ApoE4 is Arg112 and Arg158, ApoE3 is Cys112 and Arg158 and ApoE2 is Cys112 and Cys158 [63]. It had reported that a single ε4 allele could rose the risk of AD by about 3 folds, the increase of AD risks promoted by two copies of the ε4 allele is 12 times higher than those individuals who possess two copies of the ε3 allele, however, AD risk could be declined by ε4 allele at about 0.6 fold [64, 65]. Due to the relationship between ApoE and AD, scientists have focused on this gene. One research demonstrated that by using the thioflavin-T assay which could be marked the Aβ fibrils proved the existence of ApoE could promote Aβ fibrillogenesis and increase the amount of Aβ fibrils [66]. Sanan et al. also proved that ApoE3 combined with Aβ is slower than ApoE4, thus accelerating the fibril generation [67]. These mechanisms may induce the formation of Aβ plaques. Besides, the dying or impaired neurons or synapses are provided feasible conditions for tau protein accumulation with the help of ApoE [63].

1.1.1.7 Mitochondrial dysfunction and oxidative stress

Primarily, the impaired glucose metabolism was detected by using fluorodeoxyglucose PET (FDG-PET) which illustrated in living patients

the consumption of glucose is lower normal individuals, which hinted glycolysis reduction, as well as synaptic dysfunction, might occur in AD brains [68, 69]. This finding illustrated altered metabolic pathways in AD patients, which are associated with mitochondrial dysfunction [70, 71]. Mitochondrial dysfunction is always associated with oxidative stress [72]. Oxidative stress can be considered as the balance between pro-oxidants and antioxidants was disturbed which is associated with the redox reaction [73]. Also, the amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are increased when oxidative stress occurs. There are several species of ROS and RNS, such as the ionic type superoxide radical anion (O_2^-), hydroxyl radical (HO^-) and peroxynitrite ($ONOO^-$) and nonionic type hydrogen peroxide (H_2O_2) and nitric oxide (NO) [74]. The brain is a high-energy consumption organ, so the possibility of suffering oxidative stress is higher than in other organs [75]. The neuron is the basic functional unit of the brain, the metabolic rate of neurons is higher than the other cells so it is weak in confronting oxidative stress [76]. The CNS, especially the neuron, is rich in polyunsaturated fatty acids (PUFAs) which could be oxidized by reactive oxygen species (ROS), resulting in lipid peroxidation [77, 78]. Besides, hydrazide reactive protein carbonyl is a symbol of oxidation impairment of protein, proved by some research that protein carbonyls were discovered in some zones of the brain, such as the hippocampus, parietal lobe and superior middle temporal gyri, in the individuals who suffered AD [79, 80]. In addition, DNA oxidative damage also emerges in AD brains which could be described as DNA double strands breaking, DNA and protein crosslinking or base modification happening in the hippocampus and cerebral cortex [81, 82]. Damage to RNA is similar to DNA and it is prone to happen in cytoplasmic RNA rather than nuclear RNA in AD [83]. However, as oxidative stress

develops, the concentrations of antioxidants such as bilirubin, uric acid, albumin etc, are found as declining trends in AD patients [84]. Summarily, AD-associated mitochondrial dysfunction and oxidative stress could be described as the imbalance between oxidants and antioxidants which may form a vicious circle. Keeping homeostasis between these two factors might remit AD.

1.1.1.8 The cellular phase hypothesis

Besides above-mentioned hypothesis, it was also argued that cellular context also contributed to the process of AD. To begin with, initial vascular damage, such as hypoxia and hypoperfusion, which associated with the damage of blood brain barrier, owing to the abnormal clearance and metabolism of Aβ and Tau which may lead to vascular issues. Following, the decline of synaptic plasticity which could lead to brain dysfunction and neuronal connection disruption was found in AD mouse models. Last bu not least, the appearance of Aβ also influenced the way of astrocytes working, due to inflammatory biomarkers release abnormity [85].

1.2 *In vivo* model

In the preclinical research of diseases such as the mechanisms or the biomarkers, an appropriate experimental model plays a crucial role, especially *in vivo* model. Those models could help researchers comprehend the diseases in more detail and in general. AD is a multiple mechanisms disease and there are no effective therapies to overcome it, thus animal models in developing new drugs are vital. There are several types of AD mouse models: acute AD mouse models and transgenic mouse models [86]. The erection of the acute AD

mouse model is by injecting Aβ into a healthy mouse brain, and the success of this model had been proved by such research [87]. Even though this method is convenient and time consumption is lower, the Aβ seeding is hard to be observed [88, 89]. Initially, the transgenic mouse model carried a human APP single mutation, which induced APP overexpression [90]. Subsequently, several mutations in FAD-related genes carried in one mouse could lead to more serious pathological features and the appearance of cognitive impairment is earlier, such as APP/PS1. 5×FAD is one of the APP/PS1 mouse models, which carried $APP^{K670N\,/\,M671L}$; APP^{V717I}; APP^{1716v}; $PS1^{M146L}$; $PS1^{L286V}$ [91]. The merits of this model are it could steadily express excessive Aβ at 1.5 months old and cognitive impairment appears at 4-6 months old [92].

1.3 Current therapies

Currently, though AD is still a tough question and scientists spent a great amount of time and money to push forward the research, the outcome is not optimistic. There are mainly five kinds of licensed drugs which could be divided into two categories, one is AChE inhibitors (AChEI) and the other is N-methyl-D-aspartate (NMDA) receptor antagonist. The function of AChEI is by inhibiting the function of AChE to enhance the number of ACh at synapses, and further promote information exchange between neurons, this group contains donepezil, rivastigmine, tacrine and galantamine [93, 94]. Following, superfluous glutamate facilitates calcium ions influx which may cause a great amount of neuronal cell death, so the NMDA receptor antagonist was designed to block the ion channel to reduce the current flow, this category includes only memantine [95]. Besides, there is a combination therapy composed of donepezil and memantine

was also proved for those severe AD patients who were continuous taking donepezil as the stable therapy [96]. In addition, scientists also proved some other compounds to modify AD. Verubecestat (MK-8931), a kind of BACE 1 inhibitor, was terminated after the placebo-controlled phase Ⅲ trial, which illustrated a lack of effect despite it demonstrated that reducing the level of $A\beta_{1\text{-}40}$ as well as $A\beta_{1\text{-}42}$ in the cerebral spinal fluid of patients who suffered AD from mild stage to moderate stage and healthy individuals in phase Ⅰ trial, and based on the analysis of the trial it was believed that verubecestat for the patients with high accumulation of $A\beta$ cannot have clinical benefit [97, 98]. TRx0237 is a tau aggregation inhibitor, and also failed to show the treatment benefit in the phase Ⅲ trial [84]. Although thus compounds or therapies could attenuate the symptoms of AD to some extent, they still manifest a crucial need to develop more effective and secure AD drugs.

1. 3. 1 **19n**

It is obvious that AD is a disease associated with several factors and supported by a great number of hypotheses, the multi-target-directed ligands (MTDLs) therapy might be available for AD treatment. Herein, a compound named **19n** was composed of parts of the donepezil and clioquinol was developed. Donepezil is the most widely used AChEI worldwide used to treat AD. Clioquinol is a metal chelator, that precisely targets Cu and Zn, which was proved by Silvio and Umberto that it could down-regulate $A\beta$ level in plasma [99]. By fusing them, **19n** mainly plays the function of AChEI and chelate metal ions to play the role of multi-targeting against AD.

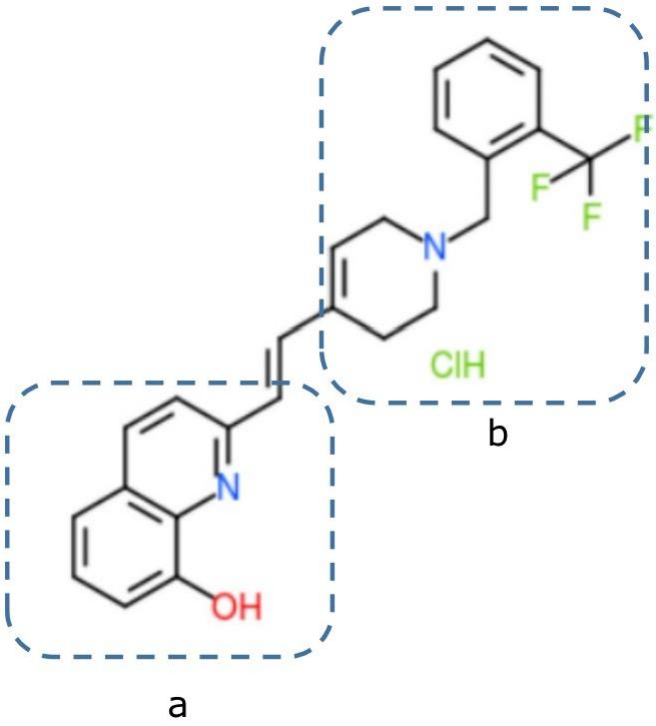


Figure 4: Structure of compound **19n**. It is fused by donepezil and clioquinol. Part a play the role of metal ions chelating activity and part b work as AChEI.

1. 3. 2 Methylprednisolone

Steroid is a group of compounds for treating inflammation and autoimmune diseases [100]. Because of the strong relationship between neuroinflammation and AD, it was suggested that it could be a good direction to alleviate AD [101]. One research proved that a low dosage of prednisolone attenuated the cognitive deficiency compared to the patients who were given a placebo and the level of inflammation marker was decreased [102]. Besides, as the inhibiting of glucocorticoids, the amount of activated microglial cells and the concentration of cytokines were elevated [103]. Methylprednisolone (MP) is a kind of glucocorticoid which belongs to one category of steroids, which is used for anti-inflammation [104]. Based on the previous studies, we explored whether it could ameliorate AD from attenuate neuroinflammation.

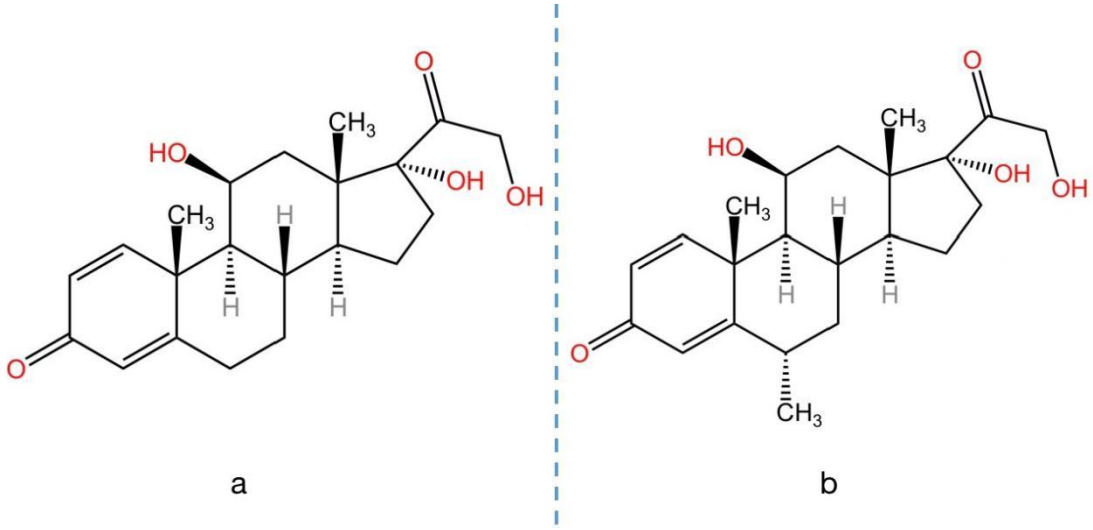


Figure 5: Structures of prednisolone and methylprednisolone. Methylprednisolone (b) is the ramification of prednisolone (a), which has better lipophilicity and can cross the blood brain barrier.

1. 4 Objective

19n is a dual targets compound which could inhibit AChE and chelate with metal ions. MP is a kind of glucocorticoid which could alleviate inflammation. The objective of this present study was from establishing an acute AD mouse model to test these two compounds by using animal ethology to evaluate whether they could improve the symptoms of AD.

2. Methods

2.1 ThT assay

1mg A$\beta_{1\text{-}42}$ (MCE, HY-P1363) was dissolved in 1080 ml 1 % ammonium hydroxide to achieve an Aβ stock solution (200 μM), which was separated into small samples and stored at -80 ℃. The groups were designed as followed: Aβ alone, Aβ + Cu^{2+}, Aβ + Donepezil, Aβ + Clioquinol, Aβ + **19n**, Aβ + Cu^{2+} + Donepezil, Aβ + Cu^{2+} + Clioquinol, Aβ + Cu^{2+} + **19n** (50 μM), Aβ + Cu^{2+} + **19n** (100 μM), Aβ + Cu^{2+} + **19n** (150 μM).

For the copper-induced Aβ aggregation experiment, the HEPES solution (pH = 7.5) was prepared by mixing 20 mM HEPES (H3375-250g, Sigma) and 150 mM NaCl. The HEPES solution was used to dilute the Aβ stock solution to make it achieve 75 μM. Cu^{2+} was acquired from $CuCl_2 \cdot H_2O$, which was dissolved in deionized water to achieve a 50 mM final concentration $CuCl_2$ solution, and then mixed with the same HEPES solution which was used before to 75 μM. Donepezil and clioquinol were dissolved by DMSO to get 150 μM, and **19n** was prepared in three concentrations, 150 μM, 300 μM as well as 450 μM. For the Aβ alone group, mixed the 10 μl Aβ (75 μM) and 20 μl HEPES buffer to achieve a 25 μM of Aβ solution. For the Aβ plus Cu^{2+} group, mixed the 10 μl Aβ (75 μM), 10 μl $CuCl_2$ solution (75 μM) and 10 μl HEPES buffer to achieve a 25 μM of Aβ solution and Cu^{2+} solution. For Aβ plus tested compounds, mixed the 10 μl Aβ (75 μM), 10 μl tested compounds (150 μM) and 10 μl HEPES buffer to achieve a 25 μM final concentration of Aβ and 50μM final concentration of tested compounds. For the donepezil and clioquinol treatment groups, 10 μl Aβ (75 μM), 10 μl $CuCl_2$ solution (75 μM) and 10 μl tested

compounds to achieve 25 μM final concentration of Aβ and copper and 50μM final concentration of tested compounds. For the **19n** treatment group, was divided into three concentrations and followed the methods given above to achieve 50 μM, 100 μM and 150 μM final concentrations of **19n**. All the groups were incubated at 37 ℃ for 24 hours. Then, we took a 20 μl sample from each group and diluted it with 180μl 50mM glycine-NaOH (pH = 8.0) which contained 20μM thioflavin T (ThT) (T3516-5g, Sigma) at room temperature and incubated for 5 minutes. Finally, testing the fluorescence intensity at 450 nm (excitation wavelength) and 485 nm (emission wavelength).

2.2 TEM assay

To detect the structure of copper-induced Aβ aggregation, we use a transmission electron microscope (JEM-1400) (TEM) for imaging. Samples preparation is similar to the copper-induced Aβ aggregation experiment, the HEPES solution (pH = 7.5) containing 20 mM HEPES (H3375-250g, Sigma) and 150 mM NaCl was used to dilute A$\beta_{1\text{-}42}$ (MCE, HY-P1363) stock solution to achieve 75 μM. The sample preparation was the same ThT assay, and the groups are designed as followed: fresh A$\beta_{1\text{-}42}$, A$\beta_{1\text{-}42}$ alone, A$\beta_{1\text{-}42}$ + Cu^{2+}, A$\beta_{1\text{-}42}$ + Cu^{2+} + Donepezil, A$\beta_{1\text{-}42}$ + Cu^{2+} + Clioquinol, A$\beta_{1\text{-}42}$ + Cu^{2+} + **19n**.

We placed a 10 μl sample on a carbon-coated copper grid for 10 minutes. Before imaging, all the samples were need staining. We stained each sample with 10 μl phosphotungstic acid for 5 minutes, drained off the excess solution and followed by secondary staining as the same procedure. Put all samples under the infrared lamp to dry off for 7 minutes. After draining the solution, all the specimens are ready

for imaging by TEM. The samples we used in this experiment were completely dissolved in this buffer.

2.3 Evaluation of AChE-induced Aβ aggregation

Hexafluoroisopropanol (HFIP)-treated E22G β-amyloid (SP-Ab-11_0.1, JPT) was managed by DMSO to achieve a final 200 μM stock solution. Then 13500 g 10 minutes centrifuging was followed, and subsequently transferred the supernatant into a new vial which was ready for the subsequent experiment. To judge the consequence of using AChEI regulated Aβ aggregation rate, 2 μl compounds at appropriate concentration were added into vials, respectively, and 2 μl Aβ stock solution (200 μM), followed by 20 μl AChE enzyme (#C3389-500UN, Sigma-Aldrich ltd) (2U/ml) which dissolved in 1 × PBS (pH = 8.0). After 24 μl of the mixed solution was prepared, another 76 μl of 1 × PBS (pH = 8.0) was added to the mixture to reach 100 μl in each vial. All the samples were incubated for 24 hours at indoor temperature. Then, 100 μl of 5 μM ThT (T3516-5g, Sigma) was added to all samples and incubated at room temperature for 1 hour. Tecan spark microplate reader was used to detect the fluorescence intensity (emission at 490nm, excitation at 450nm). Results were analyzed by the formula: (Fb – Fi) / (Fb -Fo) x 100% [105]. Fb: blank control only mixed with ThT; Fi: samples containing Aβ, AChE, AChEI and ThT; Fo: samples containing Aβ, AChE and ThT.

2.4 Experimental animal and group allocation

The experimental animal we chose is C57BL/6. All the mice were male and aged about 6 weeks and were acquired from Changzhou cavens

experimental animal corporation. We put 5 mice in one cage and housed them at room temperature (24-26 ℃) and humidity (30 %-40 %). The room is 12 hours light and 12 hours dark cycle. Mice were fed with pure water and chow *ad libitum* changing every two days. The mice for testing compound **19n** were grouped as followed: control group (n = 8), control + A$\beta_{1\text{-}42}$ (n = 8), A$\beta_{1\text{-}42}$ + Donepezil (n = 8), A$\beta_{1\text{-}42}$ + **19n** (n = 8); for MP: control group (n = 10), control + A$\beta_{1\text{-}42}$ (n = 10), A$\beta_{1\text{-}42}$ + MP (n = 10) . The number of mice in each group is to make calculation meaningful and easy to calculate statistical significance. Don, **19n** and MP were dissolved in 40 % PEG400 and 60 % NaCl solution and both of the final concentration was 0.5 mg/ml. The dosages of donepezil, **19n** and MP were 5 mg/kg, 15 mg/kg and 25 mg/kg, severally, given by intragastric administration once a day and last for 14 days. To evaluate the toxicity of compounds, the mice's body weights were measured and recorded during this period. On the eighth day, the Y maze was used to test short-term spatial learning. Morris water maze started on the ninth day and last for six days. On the fourteenth day, the mice were sacrificed. All animal experiments were approved by the Animal Ethics Committee of China Pharmaceutical University (SYXK-(苏)2021-0011) and mouse care followed the guidelines of the National Institutes of Health Guide and Care of Laboratory Animals.

2.5 Acute AD mouse model

We purchased 5 mg human A$\beta_{1\text{-}42}$ (P9001-1 mg, Beyotime) from Beyotime, dissolved by 5ml hexafluoroisopropanol (HFIP) to achieve 5 mg/ml Aβ stock solution then separated it into 5 shares and volatilized HFIP in two days. After that, 500 μl PBS was added into each tube to acquire 2 mg/ml Aβ stock solution and incubated in a

37 ℃ incubator for 7 days, then stored at -80 ℃. 20 g-25 g mice were chosen for erecting model. We anaesthetized mice with 4% chloral hydrate at 0.1 ml/10 g dosage. Fixed the mice on stereotaxic apparatus, sheared the skin on the skull and used hydrogen peroxide to wrap the meninx. The zone we selected to inject is the lateral ventricle. We found 0.22 mm underneath the coronal suture, 1mm on the right of the sagittal suture and 2.5 mm in depth. Then, 5 μl 2 mg/ml Aβ was used for each mouse at 1 μl/min injection speed by micro syringe and then maintained for 5 minutes. Finally, sutured the wound and sterilized it with iodine.

2.6 Spontaneous alternation Y maze

Y maze is the common evaluation for detecting spatial memory and recognition in a short time, which doesn't include any training [106]. This test relies on the rodents such as mice are prone to probe new environments. The Y maze is 25 cm deep and consists of three rectangular arms (30 cm) separated by 120 degrees, which are defined as A, B, C, and the triangle formed in the centre of the Y maze was defined as an isolation area, which means it did not belong to any arm. This task aimed to record the sequences of the arms that mice entered and spontaneous alterations. One spontaneous alternative behaviour is defined as the combination of three different arms, such as ABC, ACB, BAC etc. We considered once two hind legs of mice leave the isolation area the arm would be recorded. We hold the tail of the mouse and slowly put it down into the Y maze until its forepaws reach the surface of the Y maze and 10 minutes recording was followed. After testing, put the mouse back in its cage and sprayed the Y maze with 75 % ethyl alcohol, which for sterilization and

covered the odour. The proportion of the alteration is (spontaneous alternation * 100 %) / the entire number of entered arms minus 2.

2.7 Morris water maze

It lasted for six days, acquisition days lasted five days and the sixth day was testing. The aim of the morris water maze (MWM) is to train mice to seek the under-surface platform after five days of practice [107] and recording the track, distance, velocity, escape latency (how long before finding and reaching the platform) and the ratio of time spent in the aimed area of each group of mice in 90 seconds. The experiment was conducted in a pool, filled with water and added moderate titanium dioxide to become opacified. The temperature of the water in the poor was maintained at 22 - 24 ℃. The pool contained isometrical four quadrants named Q1, Q2, Q3 and Q4, a black circle platform was placed in the third quadrant (Q3) 1.5 cm under the water surface. All the mice were released at the edge position of the first quadrant, about 2 cm over the water surface and each time only placed one mouse at the same position. As the mouse was released, the record started until the mouse reached the platform and stayed on it for five seconds or if the mouse didn’t finish the task in 90 seconds. On acquisition days, if mice cannot find the platform, place mice on the platform for 10 seconds after 90 seconds of recording, but on training days this procedure is not needed. After each training, wiped dry each mouse and put it back into the cage.

2.8 Tissue preparation

On the fourteenth day, mice are ready for
sacrifice. Paraformaldehyde (PFA) was dissolved in PBS to achieve a

4 % PFA solution, stored at 4 ℃ and keep out of the light. We anaesthetized mice with 4 % chloral hydrate and fixed anterior limbs and posterior limbs on cystosepiment. Find the right auricle and cut it, and then the dark blood would flow out. Inject the needle 2 mm deep from the left ventricle and infuse 50 ml PBS (pH = 7.4) at a 10ml/min speed for 5 minutes. The samples for immunofluorescence need to be fixed. After PBS perfusion, 50 ml 4 % PFA is perfused from the left ventricle for 5 minutes. After heart perfusion, the brains for immunofluorescence were fixed in 4 % PFA kept out of light at 4 ℃. The others were collected by 6-wells plates and stored at -80 ℃.

2.9 ELISA

A portion of the cortex was collected for ELISA, and proper 1 × PBS (pH = 7.4) and PMSF (PBS: PMSF = 100: 1) were added to dilute samples (weight: volume = 1: 9). Then, the mixtures were ground on ice and repeated frozen thawing the homogenate for several times. Following, centrifuged the homogenate at 4 ℃, 5000 × g for 10 minutes. Finally, collecting the supernatant for detection. Quantitative determinations of TNF-α and IL-1β by using corresponding kits based on the protocol supplied by the corresponding kit (TNF-α: E-MSEL-M0002, Elabscience; IL-1β, E-MSEL-M0003, Elabscience).

2.10 Immunofluorescence

4 % PFA fixed the brain samples for 24 hours from they took out and after changed to 30 % sucrose solution which was used to move the water for 48 hours until they sank into the bottom of the centrifuge

tubes which were used to preserve the brains, sliced into 30μm thickness on freezing microtome at -20 ℃. The slices were washed 3 times in PBS for 5 minutes each. To improve penetration of antibody, incubating in 0.3 % Triton X-100 (ST795, Beyotime) for 20 minutes, then blocked by 10 % donkey serum for 1 hour. The slices were incubated with the primary antibody, Anti Iba1 Rabbit for Immunocytochemistry, (1:600, 019-19741, Fujifilm), overnight at 4 ℃. Followed was 5 minutes rinse by PBS for 3 times, then incubated with secondary antibody (anti-rabbit; 1: 800) at indoor temperature for 1 hour. Rinsed 5 minutes of the slices three times followed. Counterstained the sections with DAPI (1: 1000) for 20 minutes, finally rinsed by PBS three times, 5 minutes each. Mounted sections on the slides and dropped antifade mounting medium before coverslipped. Images were captured and edited by Cytation5, which was with the same settings.

2.11 Antifade mounting medium

0.53 g Na_2CO_3 (solution CO_3^{2-}) and 0.42 g $NaHCO_3$ (solution HCO_3^-) were dissolved in Mill-Q H_2O constant volume of 10 ml, respectively. Mixed 1 ml of solution CO_3^{2-} and 9 ml of solution HCO_3^- and adjusted the pH of the mixture to 8.5. Measured 45 ml glycerol and 5 ml carbonated solution (pH 8.5) and adequately mixed them.

2.12 Statistical analysis

Data were analyzed by Student's *t*-test for single comparison and one-way ANOVA followed by Dunnett's multiple comparisons test. The graphs were performed by GraphPad Prism 8. Graphs are expressed as mean ± SD.

3. Results

3.1 Modulation of Cu^{2+} induced Aβ_{1-42} aggregation by compound **19n**

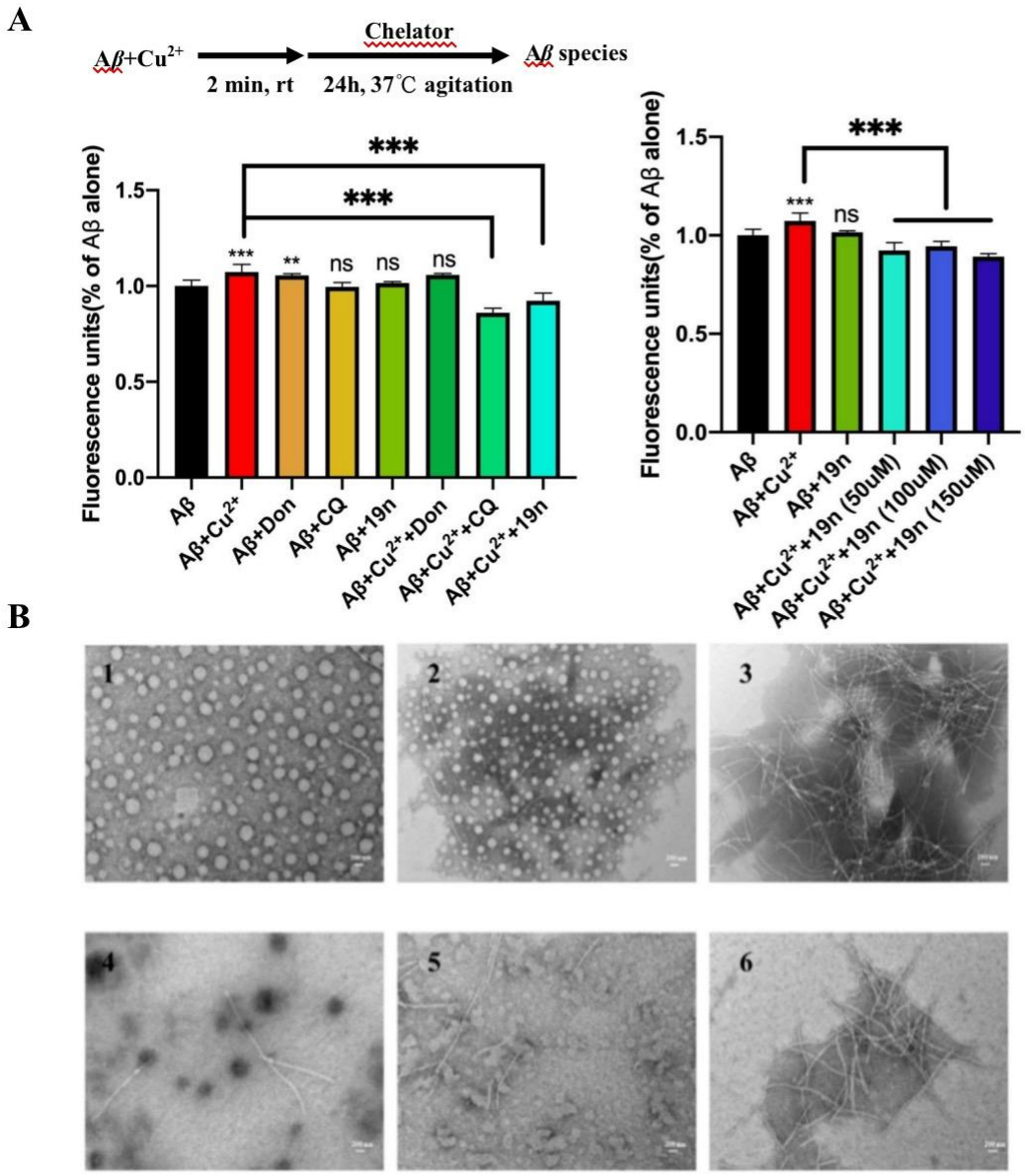


Figure 6: Experiment of testing the function of compounds to suppress Aβ_{1-42} aggregation induced by Cu^{2+}. (A) On the top of the figure is the scheme for the aggregation experiment. On the bottom is using the ThT assay to evaluate the fluorescence intensity, data are expressed as mean ± SD with five independent experiments. Statistical significance was analyzed by Student's *t*-test: ** $p < 0.01$, *** $p < 0.001$, vs. Aβ; *** $p < 0.001$, vs. Aβ + Cu^{2+} (B) TEM images analysis the Cu^{2+} induced Aβ_{1-42} aggregation and the inhibition function of compounds. Continuous numbers: (1) fresh Aβ_{1-42}, (2) Aβ_{1-42} only, (3) Aβ_{1-42} + Cu^{2+}, (4) Aβ_{1-42} + Cu^{2+} + **19n**, (5) Aβ_{1-42} + Cu^{2+} + Clioquino (CQ), (6) Aβ_{1-42} + Cu^{2+} + Donepezil (Don). Experimental conditions: Aβ_{1-42} (25 μM); Aβ_{1-42}: Cu^{2+}: compound=1:1:2; HEPES(20 mM) and NaCl (150 mM); pH 7.5; 37℃.

Metal ions such as Cu^{2+} could facilitate Aβ_{1-42} aggregation to form plaques [108]. **19n** is a chelator which could target Cu^{2+}. Firstly, the ThT assay was based on the ability of ThT could bind with Aβ fibrils to detect the aggregation of Aβ_{1-42} by detecting the fluorescence intensity of Aβ_{1-42} staining by ThT. The changes of Aβ species in morphology were determined by TEM assay (Figure 6). Owing to **19n** being fused by donepezil and clioquinol, both of them were selected as the positive control. Compared to the eight groups, the fluorescence intensity in the Aβ_{1-42} + Cu^{2+} + clioquinol group was the weakest. Based on the allocation of groups, the fluorescence intensity in Aβ_{1-42} + Cu^{2+} group was elevated, and in all three Aβ_{1-42} + compounds, the decreases were not obvious which illustrated the success of Cu^{2+} induced aggregation. It was obvious that Cu^{2+} promote 7.3 ± 1.5% of the aggregation by comparing with Aβ. The volume graph illustrated that compound **19n** suppressed 14.9 ± 1.3% of the aggregation induced by Cu^{2+}, whereas donepezil hardly inhibit the aggregation of Aβ with 1.5 ± 3.3% and clioquinol inhibited 21.2 ± 2.2%. Considering the mechanisms of donepezil and clioquinol, **19n** played the role of chelator not AChEI to inhibit the Aβ aggregates, even though the result was not better than clioquinol. The three compounds plus Aβ groups, by comparing with Aβ alone, demonstrated the experiment was convincible. Following, whether 19n was dose-dependent was tested. I schemed the concentration gradient as 50 μM, 100 μM and 150 μM. From the results, the column revealed that all three concentrations demonstrated a distinct function of restraining the aggregation induced by Cu^{2+}, with 14.9 ± 1.3%, 12.8 ± 1.8% and 18.1 ± 2.9%, respectively (Figure 1 A). It is obvious that the inhibition didn't increase as the concentration elevated, so the dose-dependent was not overt for **19n**.

Further, distinct morphological changes were observed by the TEM assay. Cu^{2+} treated Aβ illustrated more fibrils were produced in comparison with the samples without treatment. The samples incubated with **19n** or clioquinol demonstrated fewer fibrils, but there are nearly no changes in the number of fibrils after incubating by donepezil (Figure 6 B). Overall, these results made us believe that **19n** could modify Cu^{2+} to induce Aβ aggregation by regulating metal ions.

3.2 **19n** inhibit AChE induced Aβ aggregation

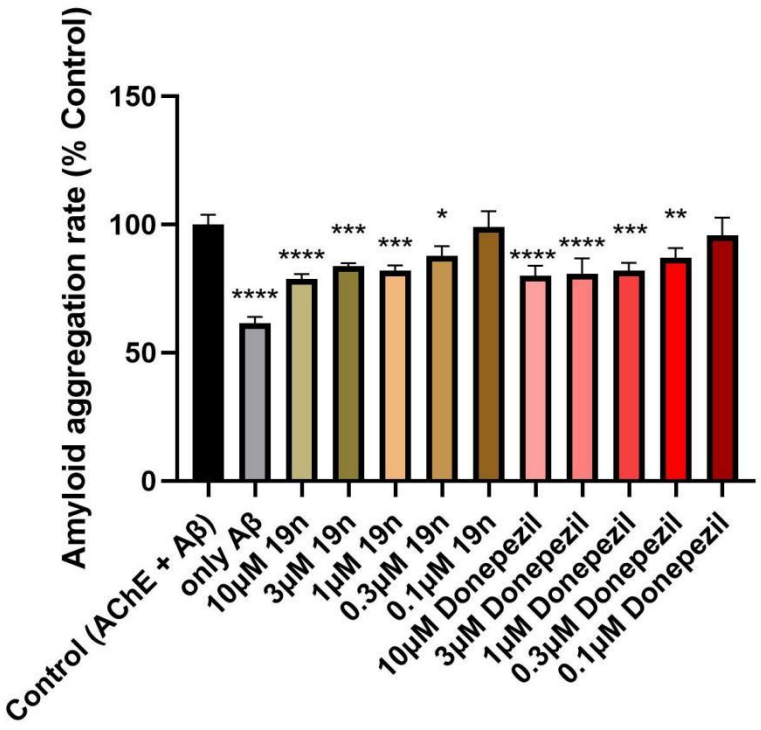


Figure 7: Compound **19n** and donepezil could attenuate AChE and induce $A\beta_{1\text{-}42}$ aggregation from 0.1 μM to 10 μM. Data are expressed as mean ± SD with six independent experiments. Statistical significance was analyzed by one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *** $p < 0.0001$.

Because **19n** could regulate two targets, next we tested the other target of it. The ability to inhibit aggregation was evaluated in vitro. Samples were treated by **19n** and donepezil from 0.1 μM to 10 μM for

24 hours, respectively. From the results, it was clear that 19n could decrease the rate of aggregation in a dose-dependent manner, despite 1 μM being slightly better than 3 μM. Donepezil as the positive control illustrated the same trend, which proved that **19n** had similar inhibiting aggregation potency to donepezil.

3.3 **19n** could improve A$\beta_{1\text{-}42}$-induced cognitive deficit

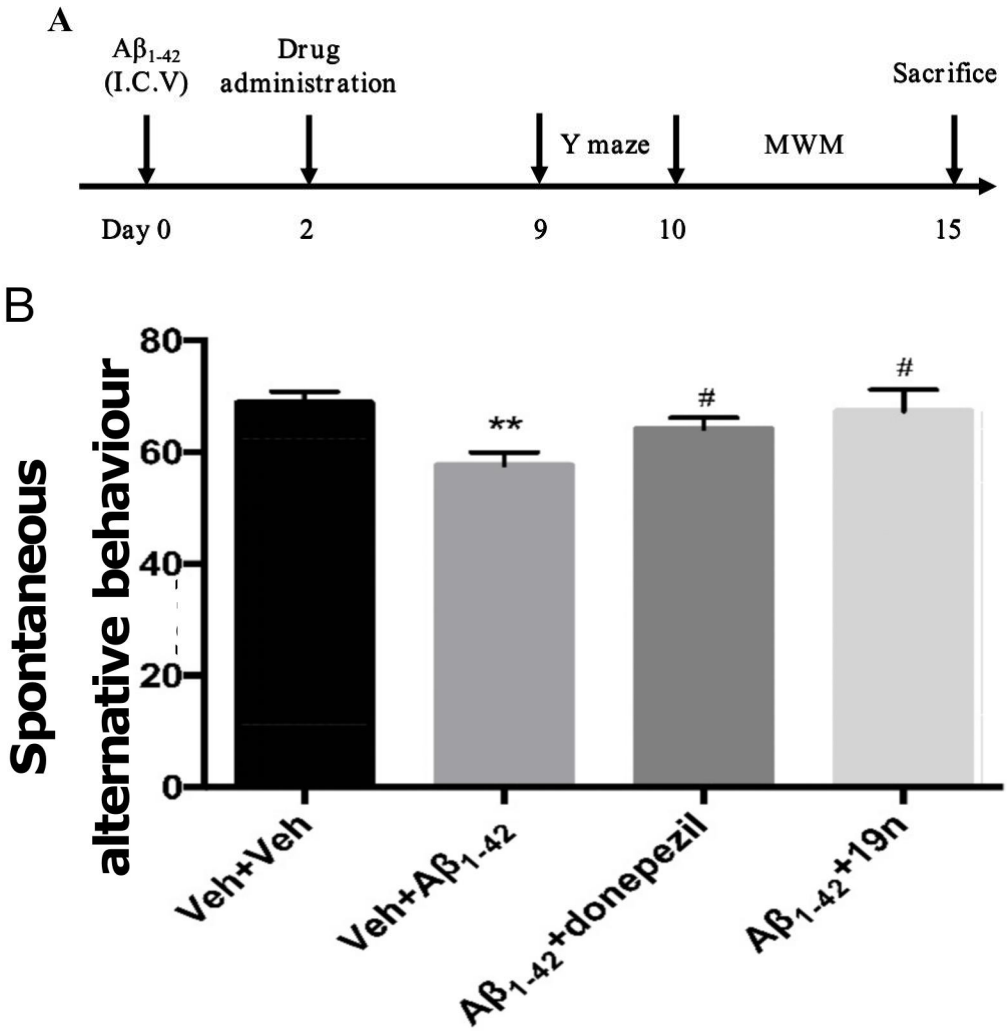
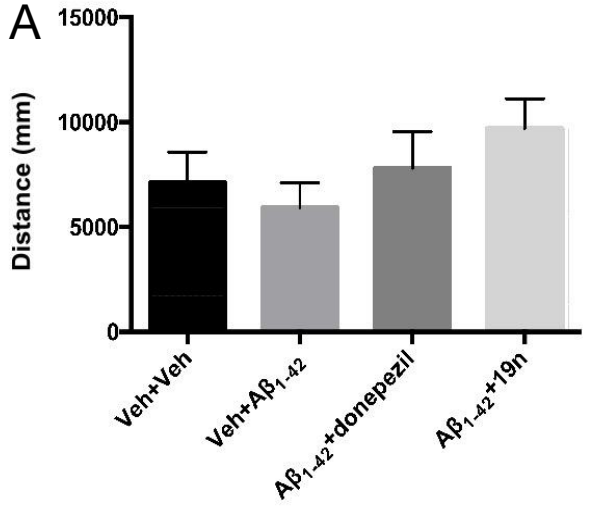
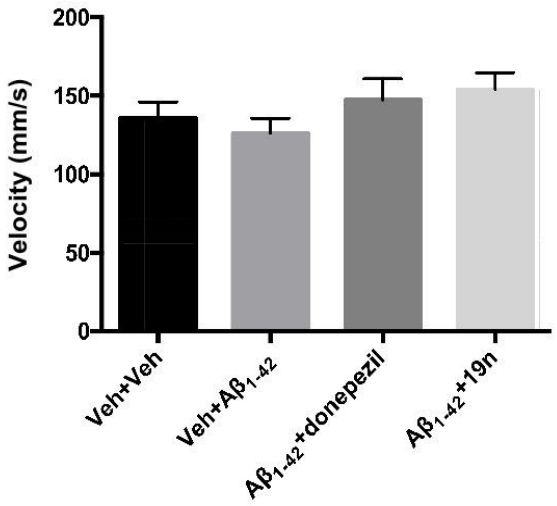


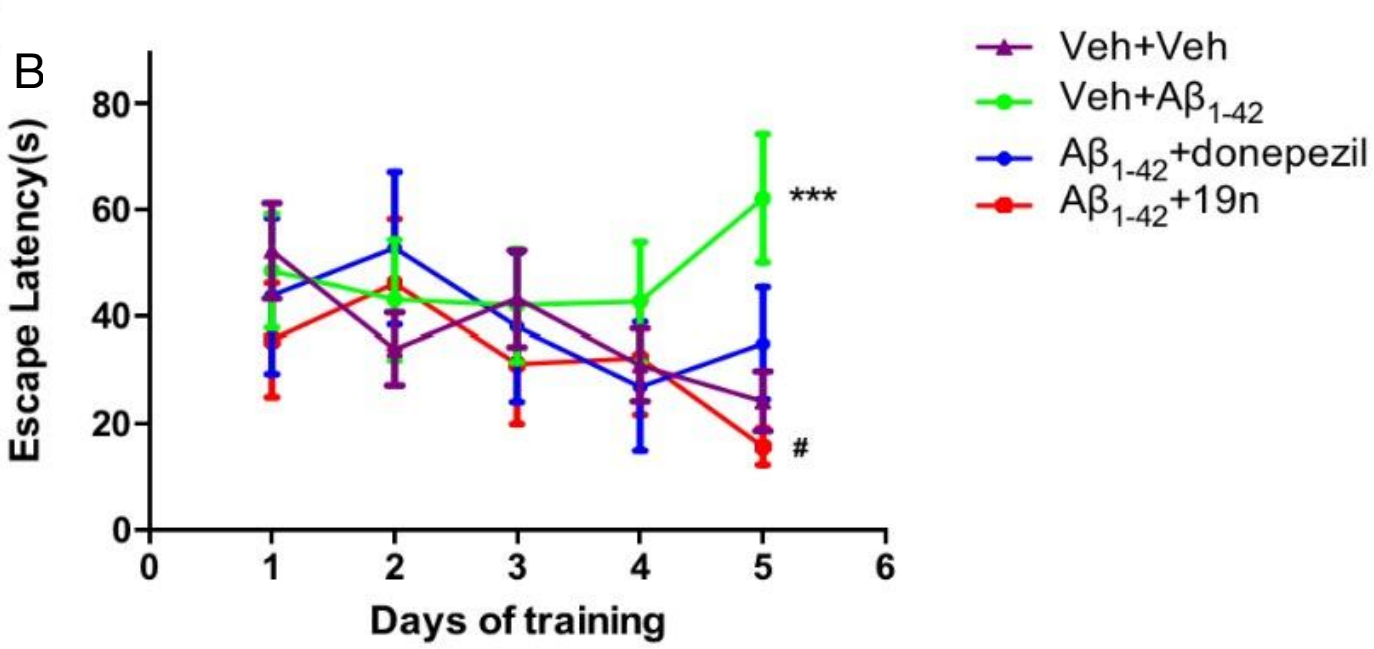
Figure 8: Design of animal experiments and results of Y maze. (A) Design of the animal experiment: Mice were separated into 4 groups: Vehicle (Veh) + Veh (control) (n = 8), veh + A$\beta_{1\text{-}42}$ (model) (n = 8), A$\beta_{1\text{-}42}$ + Don (n = 8), A$\beta_{1\text{-}42}$ + **19n** (n = 8). (B) Spontaneous alternative behaviour was tested in the Y maze. Data were illustrated as mean ± SD. Statistical significance was analyzed by Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$.

A





B



C

Group	Latency to target (s)	Distance to target (m)
control	32.56 ± 15.30	7.35 ± 3.43
model	71.89 ± 25.59##	16.93 ± 5.88##
19n	35.22 27.53*	8.05 ± 6.12*
donepezil	37.88 ± 32.10*	7.95 ± 7.07*

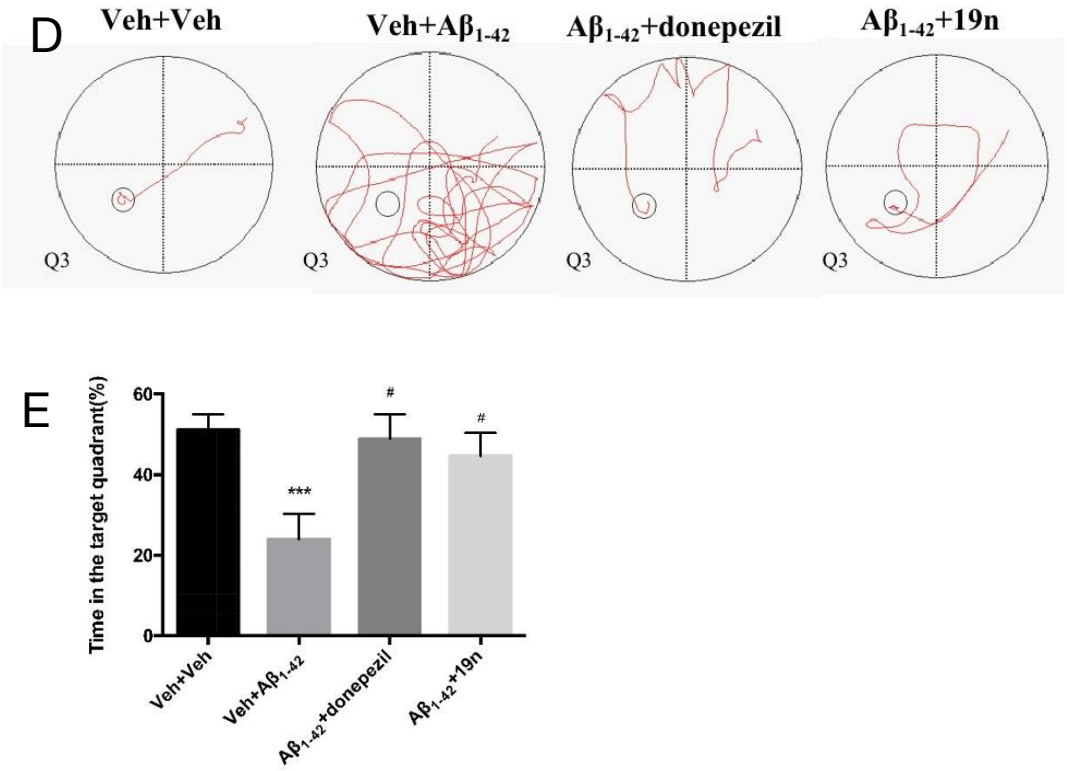


Figure 9: Results of MWM. (A) Distance and velocity were recorded on the first acquisition day. (B) Integral trends of the four groups during the five days of MWM training. (C) Escape latency and distance consumed by mice in the assessment. (D) Representative traces of each group. (E) The proportion of time consumed in the Q3. Data were illustrated as mean ± SD. Statistical significance was analyzed by Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. Veh + Veh in B, E, vs. model in C; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, vs. Veh + A$\beta_{1\text{-}42}$ in B, B, E, vs. control in C.

In the Cu^{2+} induced A$\beta_{1\text{-}42}$ aggregation experiment, **19n** showed a distinct effect in inhibiting aggregation. To determine whether it could relieve the symptoms of AD, we operated in vivo experiment. Thirty-two male ICR mice were stochastically allocated into 4 groups (n = 8 for each group): control, AD model, compound **19n** (15 mg/kg per day), and donepezil (5 mg/kg per day). Because donepezil is more toxic than 19n, so we adjust the dosages. The whole experiment lasted for sixteen days (Figure 8 A), with mice first undergoing a one-day modelling period, following one-day break mice. On the second day after I.C.V, drug administration started. The first etholoy

we prepared is the Y maze on the ninth day. It was used to evaluate short-term memory. The result showed that the percentage of correct alteration in the acute AD model group was visibly lower than in the control group (p less than 0.01). The donepezil, as well as, **19n** reversed the situation after treating the percentages of correct alterations in both of these two groups were elevated ($P < 0.05$) and the differences between them were hardly observed (Figure 8 B). Then another 6 days of training and examining in a water maze named MWM were followed. On the sixth day of this task, behavioural performance was evaluated. From the distance and velocity recorded on the first day, there are no significant differences between those four groups which means the swimming abilities of all the mice were the same (Figure 9 A). Following was the escape latency tendency during the whole training period, all the groups illustrated declining trends except the model group (Figure 9 B). Next, as shown in the table and bar charts there were dramatic differences between the model group and the wild-type group indicating that these mice model was successfully established. The latency of the target for the mice treated with donepezil ($p < 0.05$) and compound **19n** ($p < 0.05$) were remarkably improved compared to the AD model group. Meanwhile, the mice administered with donepezil ($p < 0.05$) and **19n** ($p < 0.05$) spent less distance to the target compared to the model group (Figure 9 C). From the track, it was also distinctly that donepezil and **19n** spent less time and journey finding the under-surface platform (Figure 9 D). The hidden platform was located in quadrant 3 (Q3) which was defined as the target quadrant. The column graph illustrated that the model group spent the least time in Q3 and the donepezil and **19n** treated groups were much better than it (Figure 9 E). To conclude, all the data in ethology demonstrated that **19n** could alleviate the cognitive deficit in mice induced by $A\beta_{1\text{-}42}$.

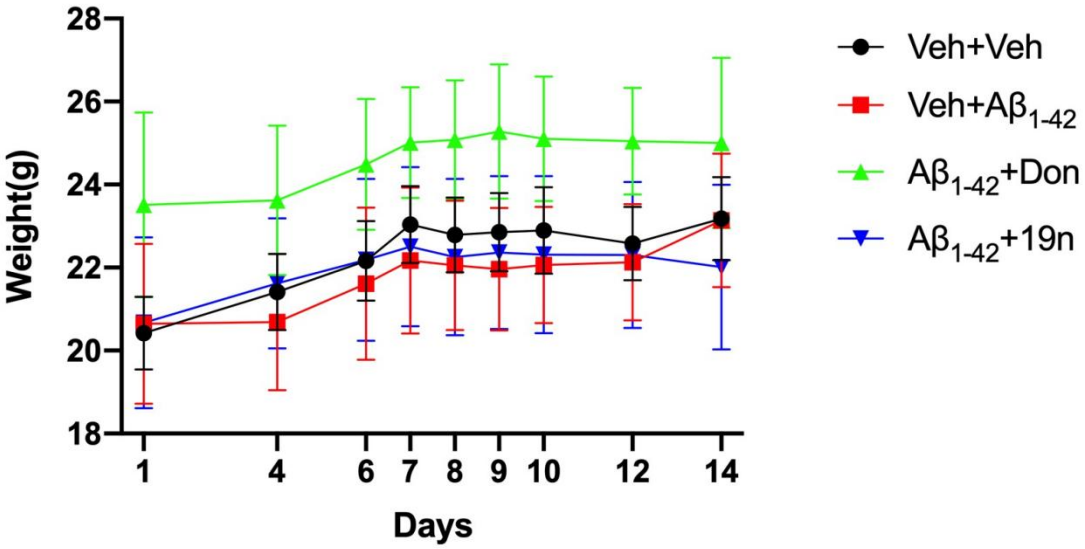


Figure 10: Variations of the mice's body weights in 14 days. Data were illustrated as mean ± SD.

The line chart demonstrated that the four groups of mice experienced wave trends and the holistic trend is increasing (Figure 10). Despite the last several days the mean weight in **19n** was decreasing, it was still higher than at the beginning of the test. Overall, the 15 mg/kg of **19n** for mice is a nontoxic dosage.

3.4 **19n** slightly decreased the amount of cytokines

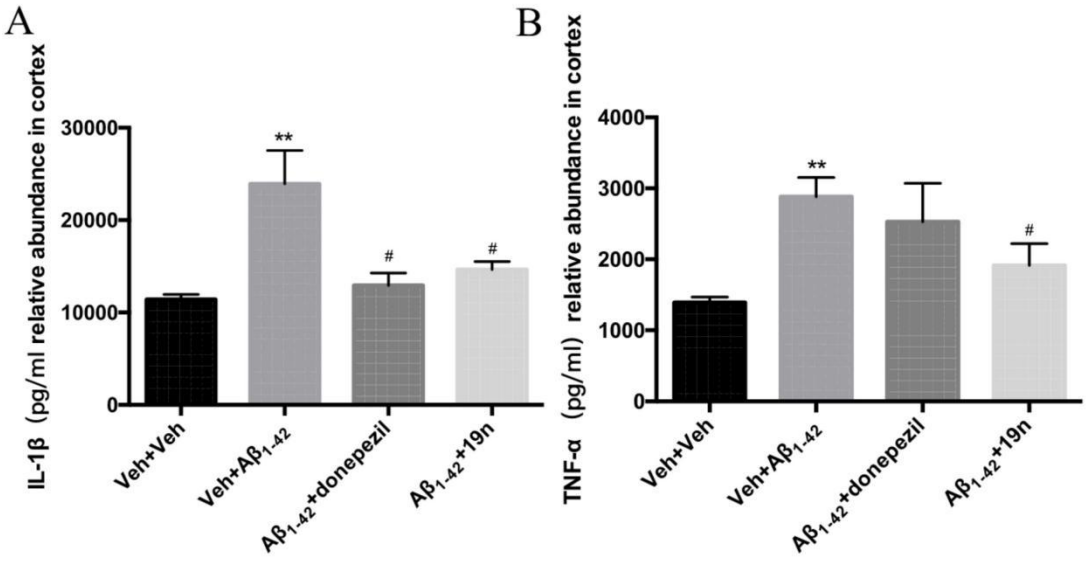
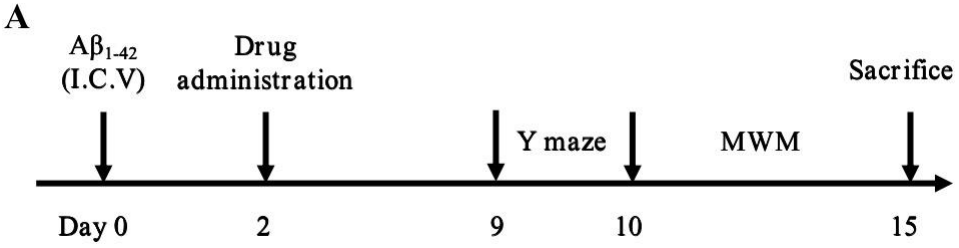


Figure 11: Concentration of IL-1β and TNF-α in the frontal cortex. (A) Concentration of IL-1β in the cortex. (B) Concentration of TNF-α in the cortex. Data were illustrated as mean ± SD. Statistical significance was analyzed by Student's t-test: * $p < 0.05$, ** $p < 0.01$, vs. Veh + Veh; # $p <$ vs. Veh + A$\beta_{1\text{-}42}$.

One research illustrated that A$\beta_{1\text{-}42}$ could induce neuroinflammation [109]. Here we next tested whether **19n** could alleviate neuroinflammation. From the two charts, it was clear that β amyloid stimulated the release of IL-1β and TNF-α in the AD model group compared to the wild-type group ($p < 0.01$). For IL-1β, both donepezil and **19n** could decrease the amount of it ($p < 0.05$). But for TNF-α, only **19n** could reduce it ($p < 0.05$), and donepezil hardly could lower the concentration of TNF-α (Figure 11). The results of ELISA proved that **19n** had the ability of anti-inflammation even though the ability was not dramatic.

3.5 MP could alleviate A$\beta_{1\text{-}42}$-induced cognitive impairment



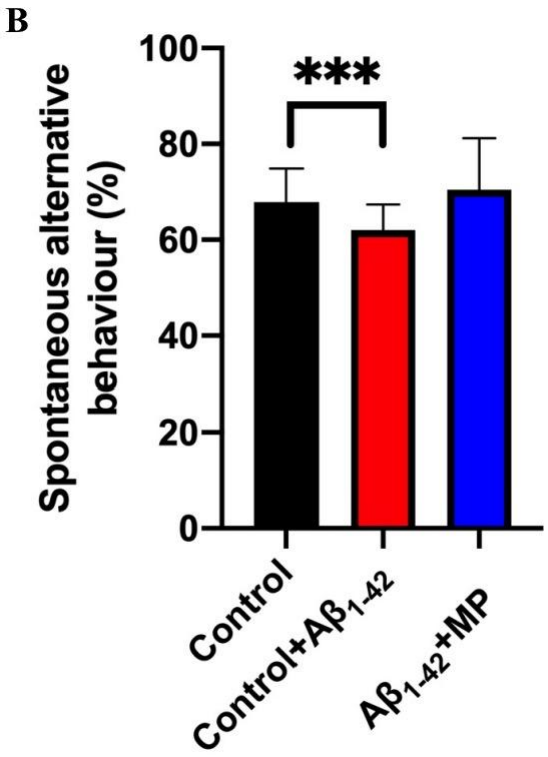
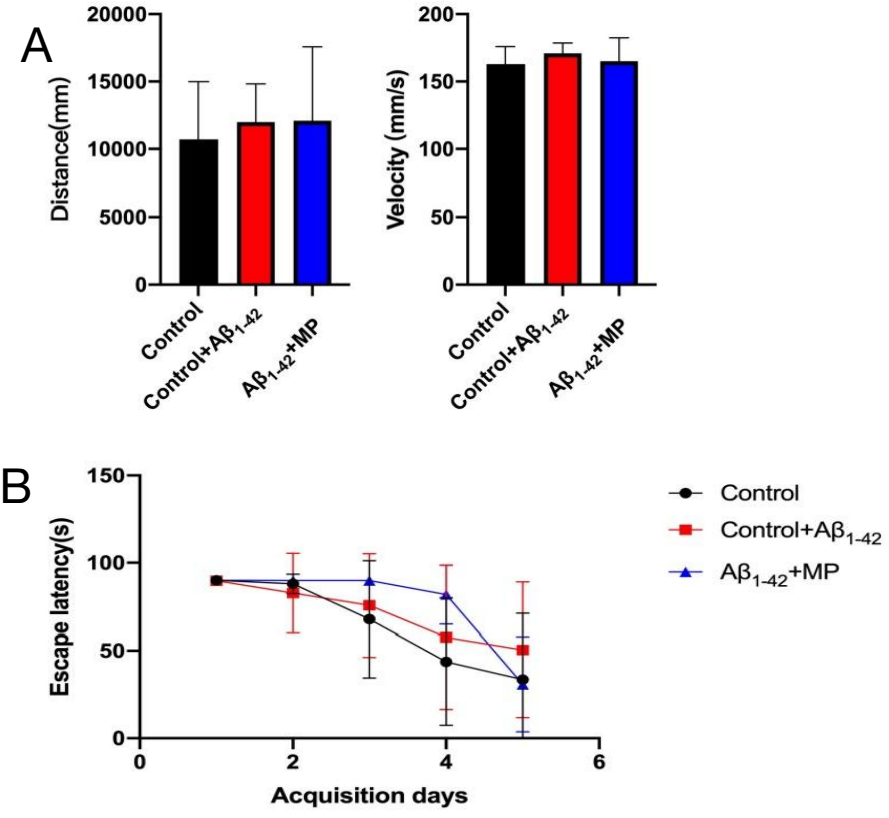
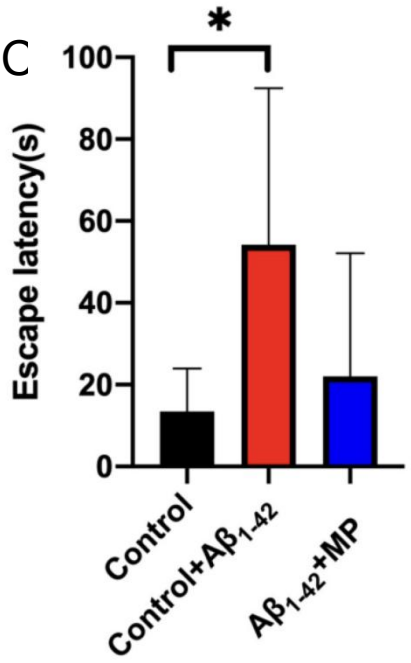


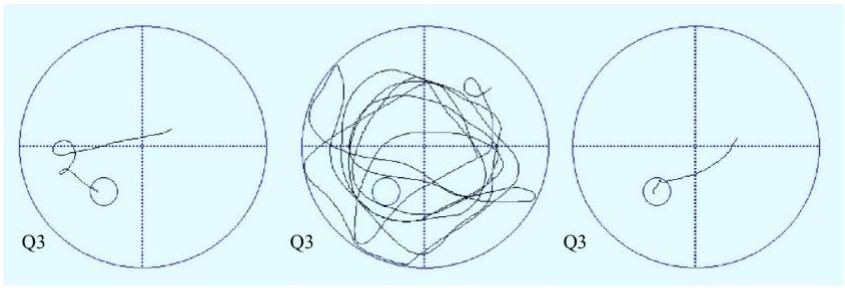
Figure 12: Design of animal experiments and results of Y maze. (A) Design of the animal experiment: the mice were grouped as followed: control (n = 10), control + A$\beta_{1\text{-}42}$ (n = 10) and A$\beta_{1\text{-}42}$ + MP (n = 10). (B) The number of spontaneous alterations in the Y maze. Data were illustrated as mean ± SD. Statistical significance was analyzed by Student's *t*-test: * p < 0.05, ** p < 0.01, *** p < 0.001, vs. Control



C



D



Control Control+$A\beta_{1-42}$ $A\beta_{1-42}$+MP

E

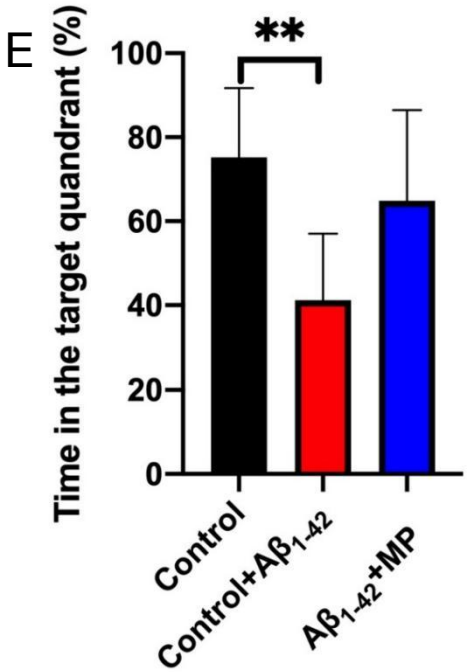


Figure 13: Results of MWM. (A) The distance and velocity were recorded in MWM on the first day of training. (B) Escape latency trends in the 5 training days. (C) The escape latency in the evaluation. (D) Representative trace of each group. (E) Time spent on the fifteenth day in quadrant 3 (Q3) where the platform was. Data were illustrated as mean ± SD. Statistical significance was analyzed by Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. Control.

From the testing of **19n**, the results illustrated that it had a good function in ameliorating AD. Then the MP was used to evaluate whether it could from the direction of anti-inflammation overcome A$\beta_{1\text{-}42}$-caused cognitive impairment. The experiment was designed similar to that when testing **19n** (Figure 12 A), the groups were control (n = 10), control + A$\beta_{1\text{-}42}$ (n = 10), and A$\beta_{1\text{-}42}$ + MP (n = 10), respectively. To evaluate the effect, we performed Y maze and MWM. Similarly, the effect of MP on short-term memory was measured by the Y maze (Figure 12 B). The result demonstrated that the correct spontaneous alterations in A$\beta_{1\text{-}42}$ administered group were dramatically less than in the untreated group, but MP could alleviate this circumstance. With the help of MP, more correct choices were recorded in A$\beta_{1\text{-}42}$ + MP group by comparing with the A$\beta_{1\text{-}42}$ treated group, which proved that MP could improve short-term memory of spatial cognition. Then, we next tested that long-term memory could be improved by MP by using MWM for continuous 6 days. On the first day, the velocity and distance of the three groups of mice were measured and analyzed by software, there are no differences between all the three groups which means the ability of these mice was the same (Figure 13 A). The escape latency is the time consumption before finding the hidden platform. On the first day of training, no differences could be observed between the three groups, as the training processed, a distinct decrease in escape latency could be found and the time cost in the mice after Aβ injection was more than the other two groups even though the performance of MP treated

group was not excellent in the first four days of acquisition (Figure 13 B). On the final day, the time consumption before finding the platform of the A$\beta_{1\text{-}42}$ treated group was dramatically higher than the other two groups (Figure 13 C). The trace in the A$\beta_{1\text{-}42}$ treated model group was rambling but for normal mice as well as MP treated groups were concise (Figure 13 D). In addition, in the hidden platform located in quadrant three (Q3) which was defined as the target quadrant, mice only treated with A$\beta_{1\text{-}42}$ spent the least time in this quadrant which means they may not memorise where the platform is, but MP ameliorated the situation (Figure 13 E). From the results of these two tests, we found that A$\beta_{1\text{-}42}$-induced cognitive impairment could be overcome by MP not only in short-dated memory but in long-range memory, and spatial cognition was improved by MP.

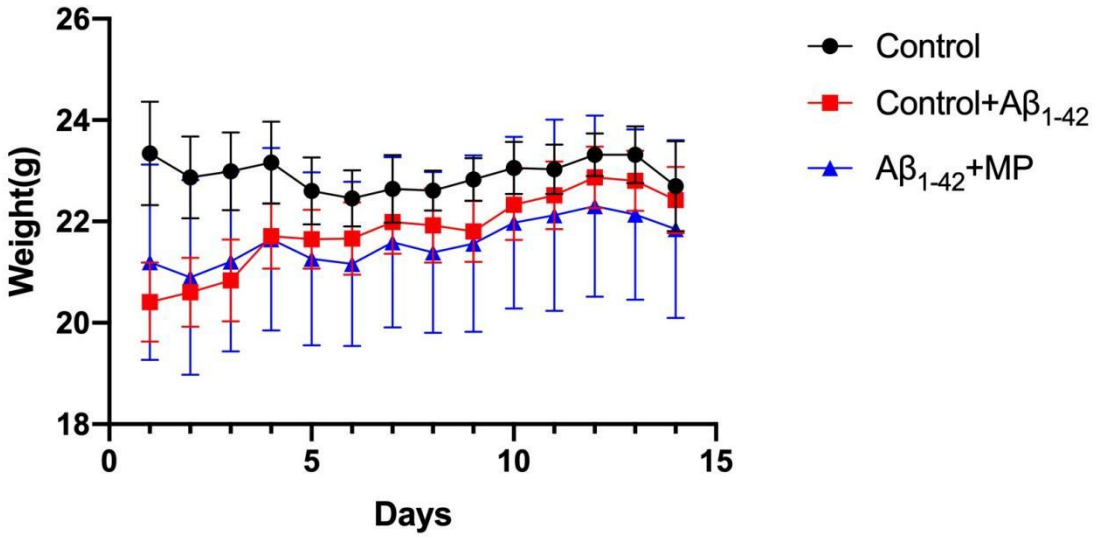
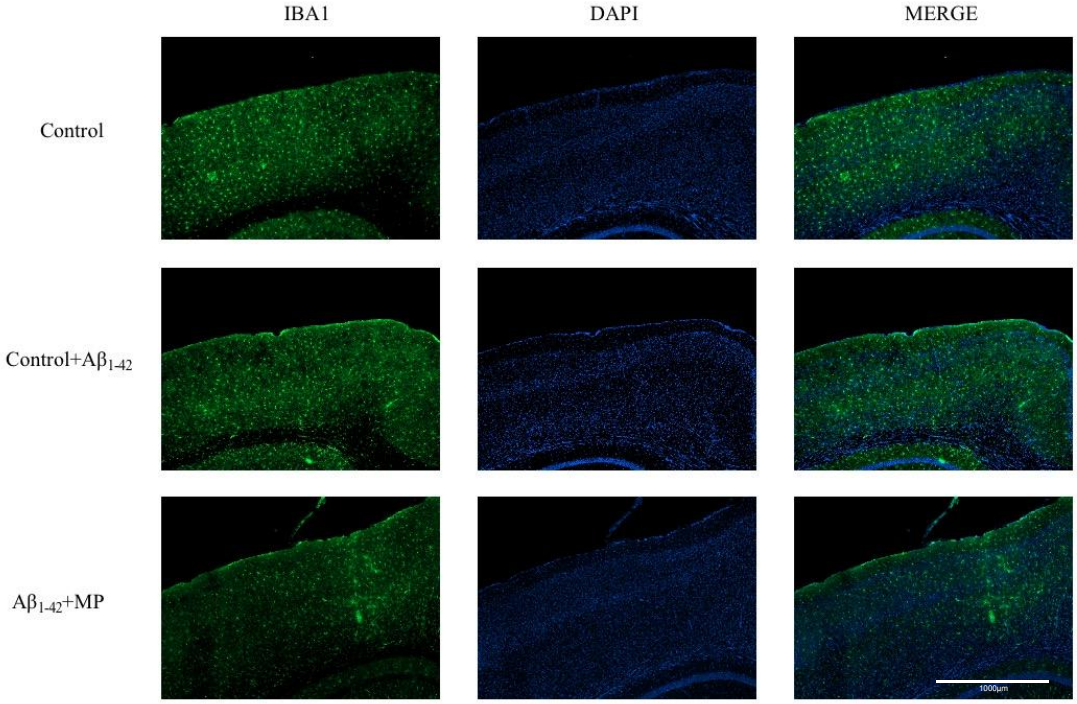


Figure 14: Changes in mice body weights in 14 days. Data were illustrated as mean ± SD.

We analyzed the body weights of those mice recorded during the 14 days. The line chart demonstrated that all three groups experience a fluctuant tendency, despite the integral weight of control experiencing a declining trend, both A$\beta_{1\text{-}42}$ treated and MP treated groups exhibited slightly increasing trends. The decrease in the control group was abnormal. We checked the padding in each cave

and excluded diarrhoea. This consequence was suspected that caused by the irritable instinct which promotes them to fight with each other. The other groups after modelling may decrease the desire of fighting. The mean weights of both of the I.C.V treated groups were lower than the untreated group (Figure 14). Overall, there is no influence exerted by MP on body weight, which means the dosage, 25 mg/kg, had no toxicity to mice.

3.6 MP could suppress the activation of microglial cell



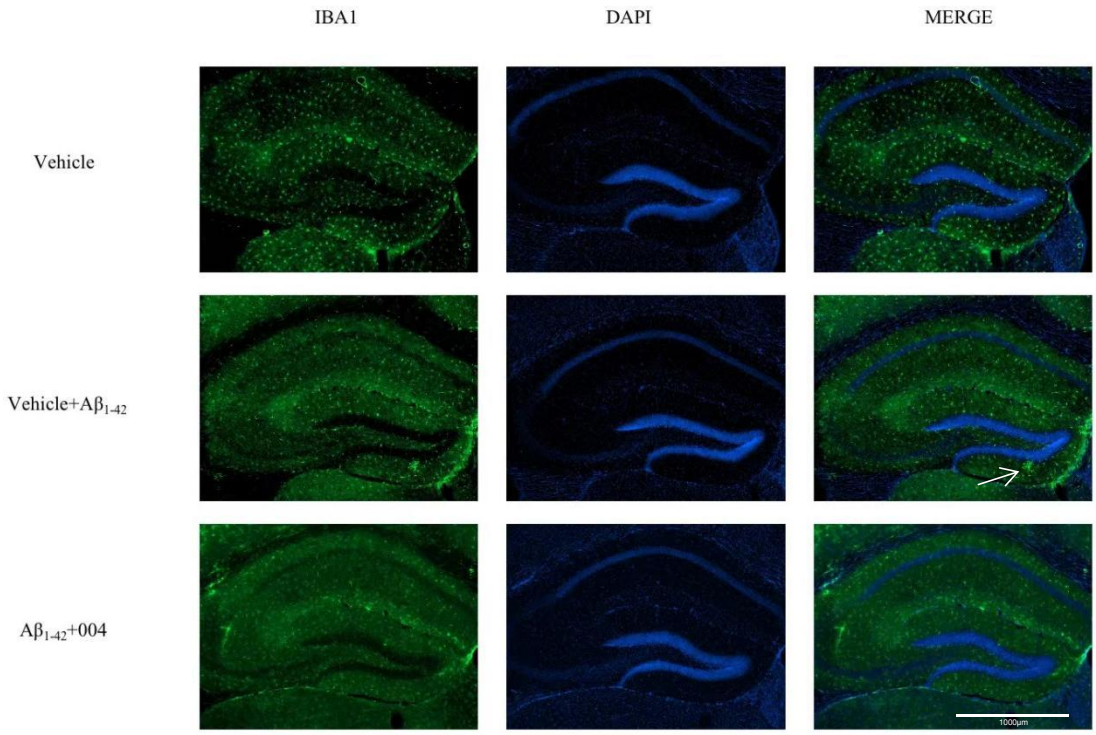


Figure 15: MP ameliorated neuroinflammation. Representative images of activated microglial cells in hippocampal regions: CA1, CA3, dentate gyrus (DG) and frontal cortex. Scare bar = 1000 μm.

Recent research proved the relationship between inflammation and AD, the symbol of this event is the activation of microglial cells [110]. The ionized Ca^{+2}-binding adapter protein 1 (Iba1) has always been treated as the cellular marker of microglial cells [111]. Hippocampus is an important region of the brain which associated with memory. Here, we evaluated the activation of microglial cells in some regions of the hippocampus: CA1, CA2 and DG, and a part of the frontal cortex. From the image, what we could find was by comparing with the control group, micorglial cells (the green dot) in the model group were differentiated. The differentiated microglial cell would not be a round light dot, but a dot with tentacles. This means the inflammation occurred, however, MP therapy slightly alleviated the circumstance (Figure 15). To conclude, MP could limitedly attenuate neuroinflammation.

4. Discussion

Globally, about 50 million individuals suffered from dementia, AD contributes to nearly 50 - 70% of cases of it [112]. Besides, as a progressive neurodegenerative disease, it may exacerbate as the age increasing, from mild cognitive impairment to symptoms that interfere with most of the day-to-day activities [113]. To solve the great challenge, scientists are dedicated to finding effective therapy. Nowadays, there are only five legal drugs proved by FDA which could be divided into two categories AChEI and NMDA antagonists [114], but AChEI cannot reveal better rescue in the treatment of slight to middling AD [115] and memantine didn't reveal better efficacy in the enhancement of cognitive impairment [116]. Based on the pessimistic research outcome, it was necessary to develop more effective drugs or therapies to confront AD. In this research, we focused on cholinergic dysfunction, metal ions and neuroinflammation these three parts, the two compounds which were based on these three hypotheses were tested. Firstly, we proved that **19n** was a dual target compound. We set up corresponding conditions to induce amyloid aggregation and evaluated the function of how **19n** dealt with the situation. Based on the results of Cu^{2+} induce Aβ aggregation and AChE to induce Aβ aggregation, be compared with the positive drug, clioquinol and donepezil, respectively, it was obvious that **19n** could target the metal ion and play the role of AChEI. Because of the mechanism was verified, we assumed whether it might ameliorate AD. Then we established an acute AD mouse model to further proved in vivo function of **19n**. We used Y maze and MWM to evaluate how they manifested during testing. As the testing finished, we firstly analyzed whether the models were successful. The results illustrated that the Aβ treated group revealed various degrees of cognitive impairment

and after **19n** treated, not only short-term memory but also long-term memory as well as spatial recognition was improved in contrast with the model group whether the difference between donepezil was not obvious. Following this, we managed the brain of each group and tested the levels of cytokines to evaluate whether **19n** could regulate the neuroinflammation induced by Aβ. Both two mentioned cytokines, TNF-α and IL-1β play a crucial role in the process of AD, the appearance of them means the activation of microglia which also could be considered as the beginning of inflammation. They also could induce series of inflammatory responds and Aβ aggregation. Thus reducing the concentrations of them could attenuate the process of AD. It was obvious that the amount of TNF-α and IL-1β decreased in the cortex, despite the trends were not remarkably. Then, we considered whether the anti-inflammation drugs could alleviate AD. We next tested MP, a kind of glucocorticoids, by using Y maze and MWM. Y maze illustrated that short-term cognitive impairment was improved by MP and the time consumption in finding platform decreased and time staying in the target quadrant increased, which highlighted long-term memory and ability of learning were improved. Finally, we used immunofluorescence to stain microglia cells in the hippocampus as well as the cortex and found the activation was suppressed by MP.

Next, we thought that for AD treatment, the lack of effect of AChEI is on account AD is a disease which influences by many factors, and it is difficult to block all targets. But from the result we could find that by targeting multiple targets, AChEI and metal ion chelator, the outcome was better than a single target, AChEI, despite the significant difference was not obvious. Initially, we anticipated that 19n should be much better than donepezil because it was based on its function of

it and carried the ability of a metal chelator. What we considered about the results was the targets we focused on were not the most serious. It might be more useful if we combined secretase inhibitor and A*β* aggregation inhibitors, such as *β*-secretase (verobecestat [117], atabecestat [118]) or *γ*-secretase (semagasestat [119], avagacestat [120]) with clioquinol, though in phase Ⅲ clinical trials, thus compounds failed because of the lack of efficiency in alleviating cognitive impairment [112]. Furthermore, excepted combined several anti-amyloid strategies, hyperphosphorylation of tau protein as another biomarker of AD was also considered as a potential target. The literature illustrated possible ways to regulate kinase inhibitors which could regulate and modify the phosphorylation of tau protein. For CDK5, selective inhibitors were reported in cancer studies, such as roscovitine and flavopiridol, which were tested in animal models that the former held tau phosphorylated and the latter could improve cognitive function [121, 122]. For GSK3*β* inhibitor, tideglusib had mentioned in a phase II trial that at the lowest dose could process the pharmacological function [123]. Yao *et al.* raised a compound named **27g** that hybridized the functional skeleton of tacrine and pyrimidone which performed as a dual AChE and GSK3*β* inhibitor [124]. In vitro experiments illustrated a good phosphorylation function in different phosphorylated sites and *in vivo* testing proved that **27g** could enhance cognitive decline. This made us believe that it is viable of changing a target for dual target compound design.

Combination therapy is also an effective method. It could also target numerous targets which achieve the same effect as MTDL compounds. Based on the support of the FDA, AChEI + memantine had become a classic combination therapy for treating AD, which was better than the effect of monotherapies revealed by them alone. One study

provided by Youn *et al.* investigated the function of combining memantine and AChEI, and the consequence demonstrated that the combination therapy dramatically enhanced the manner and mental symptoms of dementia compared with the monotherapy elicited by AChEI in moderate patients [125], whereas the research proved by Choi *et al.* held the opposite perspective that the outcome of memantine plus rivastigmine didn't illustrate a significant advantage compared with the group treated by rivastigmine alone [126]. Here we considered that the targets of the classic combination therapy were not satisfied, even though the memantine, as well as the several kinds of AChEI, were frequently used as the first line of treating AD. Our results inspired us that MP could be based on reducing inflammation responses to alleviate AD which was a potential therapy. The combination therapy composed of **19n** + MP could be from three targets to ameliorate AD simultaneously, which convinced us that it was possible. The body weight changes proved that the two compounds were well tolerated *in vivo* making us believe that it could be a latent trial. Except for AD, combination therapy was also widely used in treating cancer, such as fusing chemotherapy and target therapy in treating HER2-positive breast carcinoma, which made it more reliable.

Besides, MP is a positive drug for treating inflammation and it illustrated its role in confronting AD. But the results of immunofluorescence were not satisfactory for us. The number of activated microglial cells were not significantly decreased by using MP. What we thought was more detailed about the activation of microglia cells was the signal pathway. The classical glial activation is related to the triggering of the receptor expressed on myeloid cell 2 (TREM2) recognizes ApoE [127]. Zhong *et al*. proved that sTREM2 could reduce

the concentration of Aβ in the brain and improve memory deficit in the 5 × FAD mouse model which proved the positive effect of TREM2 [128]. AL002, an artificial monoclonal antibody was developed as a TREM2 regulator. A variant of AL002, AL002a, was tested in a 5 × FAD mouse model, results highlighted that amyloid deposition and the expression of Aβ-associated genes were reversed by AL002a, it also facilitated microglia cells clustered to plaques [129]. Another variant, AL002c, also could activate TREM2 and recruit microglia cells for Aβ elimination in the 5 × FAD mouse model [130]. Several references proved that TREM2 is a positive target for regulating AD, thus for combination therapy, it is possible to select TREM2 regulators for the target of inflammation.

Next, we considered the stability of the AD mouse mode. Here we considered the APP/PS1, especially the 5 × FAD mouse model could be a good replacement which could express cognitive impairment and the Aβ plaques were detected in the early stage. However, the mechanism performed by those models was simple. It is essential to use various models which could present different pathogenic mechanisms. A portion of the AD mouse model was designed based on tauopathy which carried human P301L or P301S mutations [131, 132]. JNPL3 mouse was the first transgenic AD mouse model which carried P301L mutation and express NFTs in 6.5 months. The neuronal loss was also observed in this model [91]. PS19 mouse carried P301S mutation generated by Yoshiyama's group, revealing microgliosis and synaptic dysfunction which were two symbols of processing tauopathy [133]. Those models could help us determine whether the new compounds through intervening tau protein alleviate AD.

Finally, we consider that AD researches that are based on several origin signal pathways are less valued, in other words, which hints to us that it is important and necessary to find some other process to elucidate the question. It highlights that the cGAS-STING pathway is crucial in cancer research and it is also important in neurodegenerative diseases. It mainly confronts external viral infection [134]. Cyclic GMP-AMP (cGMP-AMP) synthase (cGAS) is the beginning of this pathway which is activated by several factors such as viral dsDNA or ssRNA, besides, mitochondrial DNA (mtDNA) also could activate this process. After the signal is caught by cGAS, a cyclic dinucleotide named cGAMP is produced which finds and targets a stimulator of interferon genes (STING). It situates on the endoplasmic reticulum (ER) and then transfers to the Golgi apparatus after combination with cGAMP. STING activates tank binding kinase 1 (TBK1) and is phosphorylated by it. Then phosphorylated STING is recruited by interferon regulatory factor 3 (IRF3) leading to its translocation to the nucleus and promoting the expression of interferon or cytokines [135]. CGAS as a DNA sensor, not only responds to foreign DNA but also to internal DNA, such as mtDNA. One remarkable mechanism in AD is mitochondrial dysfunction which may lead to mtDNA diffuses into cytoplasm and trigger cGAS, leading to the activation of the cGAS-STING pathway and finally inducing interferon or interferon stimulating genes (ISGs) overexpression. This process always happens in microglial cells, excessive interferon and ISGs lead to neuroinflammation [33]. One research illustrated that Tau protein could activate the cGAS-STING pathway with the help of polyglutamine binding protein 1 (PQBP1) which facilitates Tau trigger cGAS [136]. Another study holds the perspective that the promoter of STING, cGAMP, could ameliorate the progress of AD by up-regulating TREM2 from activation of the cGAMP-STING-TBK1-IRF3 pathway in

the APP/PS1 mouse model[137]. Although there is no STING modulator for treating AD, the strong relationship between them hints to us that it is available from this direction for developing AD therapy.

5. Future works

Based on our results, we further try to combine 19n and MP in the acute AD model to evaluate the effect. Then we would use the combination therapy in different AD mouse models, such as 5 × FAD and JNPL3, to evaluate whether it is useful in various pathological mice. The groups are allocated as control, 5 × FAD (or JNPL3), control + compounds and 5 × FAD (or JNPL3) + compounds. Compounds are given by intragastric administration. Similarly, short-term and long-term cognitive functions are evaluated by the Y maze and MWM. Besides, the Barnes maze is also a good add-on evaluation which is designed based on the instinct of rodent animals that preferring to darkness rather than light. After sacrifice, the level of some vital proteins, such as Aβ, Tau and some other inflammation-associated markers could be detected by western blot. Following, the concentrations of cytokines would be tested by ELISA, and qPCR would use to check whether the combination therapy could interfere with the transcription level of cytokines and chemokines. Besides, MP is not a good choice for regulating microglia cells as well as neuroinflammation. Variants of AL002 are appropriate substitutors of MP. Then, the new compounds could be designed based on fusing secretase inhibitors and AChEI, followed by using the same experimental design to evaluate the effect.

For the research of the cGAS-STING pathway, it is necessary to establish a cGAS or STING knockout cell model in the BV2 cell line by Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/ CRISPR associated protein (Cas) 9 which for detection that the activation of microglial cells after Aβ stimulating could be regulated by

this pathway. Following, the in vivo experiment would hold in wild type, APP/PS1, STING$^{-/-}$, APP/PS1/STING$^{-/-}$, cGAS$^{-/-}$, and APP/PS1/cGAS$^{-/-}$ mouse models. Similarly, the same behavioural tests are performed to evaluate the function of STING in AD. Based on the outcome, we could confirm whether cGAS and STING are both potential targets which have the same importance for regulating AD.

6. References

[1] Eratne D.; Loi S. M.; Farrand S.; Kelso W.; Velakoulis D.; Looi J. C. , Alzheimer's disease: clinical update on epidemiology, pathophysiology and diagnosis. *Australas Psychiatry* **2018,** 26(4), 347-357.

[2] Naj A. C.,; Schellenberg G.D.; Alzheimer's Disease Genetics Consortium (ADGC). Genomic variants, genes, and pathways of Alzheimer's disease: An overview. *Am J Med Genet B Neuropsychiatr Genet*. **2017**, 174(1):5-26.

[3] Scheltens P.; De Strooper B.; Kivipelto M.; Holstege H.; Chételat G.; Teunissen C. E.; Cummings J.; van der Flier W. M., Alzheimer's disease. *The Lancet* **2021**, 397(10284), 1577-1590.

[4] Scheltens P.; Blennow K.; Breteler M. M.; de Strooper B.; Frisoni G. B.; Salloway S.; Van der Flier W. M., Alzheimer's disease. *Lancet* **2016**, 388(10043), 505-17.

[5] Tan C. C.; Yu J.T.; Tan L., Biomarkers for preclinical Alzheimer's disease. *J Alzheimers Dis* **2014**, 42(4), 1051-69.

[6] Kametani F.; Hasegawa M., Reconsideration of Amyloid Hypothesis and Tau Hypothesis in Alzheimer's Disease. *Front Neurosci* **2018**, 12, 25.

[7] Bowen D. M.; Smith C. B.; White P.; Davison A. N., Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. *Brain* **1976**, 99(3), 459-96.

[8] Whitehouse P. J.; Price D. L.; Clark A. W.; Coyle J. T.; DeLong M. R., Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann Neurol* **1981**, 10(2), 122-6.

[9] Drachman D. A.; Leavitt J., Human memory and the cholinergic system. A relationship to aging? *Arch Neurol* **1974**, 30(2), 113-21.

[10] Hampel H.; Mesulam M. M.; Cuello A. C.; Farlow M. R.; Giacobini E.; Grossberg G. T.; Khachaturian A. S.; Vergallo A.; Cavedo E.; Snyder P. J.; Khachaturian Z. S., The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. *Brain* **2018**, 141(7), 1917-1933.
[11] Ferreira-Vieira T. H.; Guimaraes I. M.; Silva F. R.; Ribeiro F. M., Alzheimer's disease: Targeting the Cholinergic System. *Curr Neuropharmacol* **2016**, 14(1), 101-15.
[12] Fine A.; Hoyle C.; Maclean C. J.; Levatte T. L.; Baker H, F.; Ridley R. M., Learning impairments following injection of a selective cholinergic immunotoxin, ME20.4 IgG-saporin, into the basal nucleus of Meynert in monkeys. *Neuroscience* **1997**, 81(2), 331-43.
[13] Dunnett S. B.; Everitt B. J.; Robbins T. W., The basal forebrain-cortical cholinergic system: interpreting the functional consequences of excitotoxic lesions. *Trends Neurosci* **1991**, 14(11), 494-501.
[14] Sarter M,; Bruno J. P., Cognitive functions of cortical acetylcholine: toward a unifying hypothesis. *Brain Res Brain Res Rev* **1997**, 23(1-2), 28-46.
[15] Potter L. T., Synthesis, storage and release of [14C]acetylcholine in isolated rat diaphragm muscles. *J Physiol* **1970**, 206(1), 145-66.
[16] Picciotto M. R.; Higley M. J.; Mineur Y. S., Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior. *Neuron* **2012**, 76(1), 116-29.
[17] Augustinsson K. B.; Nachmansohn D., Distinction between Acetylcholine-Esterase and Other Choline Ester-splitting Enzymes. *Science* **1949**, 110(2847), 98-9.
[18] Haam J.; Yakel J. L., Cholinergic modulation of the hippocampal region and memory function. *J Neurochem* **2017**, 142 Suppl 2(Suppl 2), 111-121.

[19] Hampel H.; Mesulam M. M.; Cuello A. C.; Khachaturian A. S.; Vergallo A.; Farlow M. R.; Snyder P. J.; Giacobini E.; Khachaturian Z. S., Revisiting the Cholinergic Hypothesis in Alzheimer's Disease: Emerging Evidence from Translational and Clinical Research. *J Prev Alzheimers Dis* **2019**, 6(1), 2-15.
[20] Ehret M. J.; Chamberlin K. W., Current Practices in the Treatment of Alzheimer Disease: Where is the Evidence After the Phase III Trials? *Clin Ther* **2015**, 37(8), 1604-16.
[21] Huat T. J.; Camats-Perna J.; Newcombe E. A.; Valmas N.; Kitazawa M.; Medeiros R., Metal Toxicity Links to Alzheimer's Disease and Neuroinflammation. *J Mol Biol* **2019**,431(9), 1843-1868.
[22] Colomina M. T.; Peris-Sampedro F., Aluminum and Alzheimer's Disease. *Springer International Publishing AG* **2017**.
[23] Candy J. M.; Oakley A. E.; Klinowski J.; Carpenter T. A.; Perry R. H.; Atack J. R.; Perry E. K.; Blessed G.; Fairbairn A.; Edwardson J. A., Aluminosilicates and senile plaque formation in Alzheimer's disease. *Lancet* **1986**, 1(8477), 354-7.
[24] White A. R.; Barnham K. J.; Bush A. I., Metal homeostasis in Alzheimer's disease. *Expert Rev Neurother* **2006**, 6(5), 711-22.
[25] Ayton S.; Lei P.; Bush A. I., Metallostasis in Alzheimer's disease. *Free Radic Biol Med* **2013**, 62, 76-89.
[26] Swerdlow R. H.; Khan S. M., A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. *Med Hypotheses* **2004**, 63(1), 8-20.
[27] Kabir M. T.; Uddin M. S.; Zaman S.; Begum Y.; Ashraf G. M.; Bin-Jumah M. N.; Bungau S. G.; Mousa S, A.; Abdel-Daim M, M., Molecular Mechanisms of Metal Toxicity in the Pathogenesis of Alzheimer's Disease. *Mol Neurobiol* **2021**, 58(1), 1-20.

[28] Calsolaro V.; Edison P., Neuroinflammation in Alzheimer's disease: Current evidence and future directions. *Alzheimers Dement* **2016**, 12(6), 719-32.
[29] Stephenson J.; Nutma E.; van der Valk P.; Amor S., Inflammation in CNS neurodegenerative diseases. *Immunology* **2018**, 154(2), 204-219.
[30] Hansen D. V.; Hanson J. E.; Sheng M., Microglia in Alzheimer's disease. *J Cell Biol* **2018**, 217(2), 459-472.
[31] Hu X.; Leak R. K.; Shi Y.; Suenaga J.; Gao Y.; Zheng P.; Chen J., Microglial and macrophage polarization—new prospects for brain repair. *Nat Rev Neurol* **2015**, 11(1), 56-64.
[32] Leng F.; Edison P., Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? *Nat Rev Neurol* **2021**, 17(3), 157-172.
[33] Li T.; Lu L.; Pember E.; Li X.; Zhang B.; Zhu Z., New Insights into Neuroinflammation Involved in Pathogenic Mechanism of Alzheimer's Disease and Its Potential for Therapeutic Intervention. *Cells* **2022**, 11(12), 1925.
[34] Guerreiro R.; Wojtas A.; Bras J.; Carrasquillo M.; Rogaeva E.; Majounie E.; Cruchaga C.; Sassi C.; Kauwe J. S.; Younkin S.; Hazrati L.; Collinge J.; Pocock J.; Lashley T.; Williams J.; Lambert J. C.; Amouyel P.; Goate A.; Rademakers R.; Morgan K.; Powell J.; St George-Hyslop P.; Singleton A.; Hardy J.; Alzheimer Genetic Analysis Group., TREM2 variants in Alzheimer's disease. *N Engl J Med* **2013**, 368(2), 117-27.
[35] Wang C.; Fan L.; Khawaja R. R.; Liu B.; Zhan L.; Kodama L.; Chin M.; Li Y.; Le D.; Zhou Y.; Condello C.; Grinberg L. T.; Seeley W. W.; Miller B. L.; Mok S. A.; Gestwicki J. E.; Cuervo A. M.; Luo W.; Gan L., Microglial NF-κB drives tau spreading and toxicity in a mouse model of tauopathy. *Nat Commun* **2022**, 13(1), 1969.

[36] Mangan M. S. J.; Olhava E. J.; Roush W. R.; Seidel H. M.; Glick G. D.; Latz E., Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat Rev Drug Discov* **2018**, 17(9), 688.
[37] Calsolaro V.; Edison P., Neuroinflammation in Alzheimer's disease: Current evidence and future directions. *Alzheimers Dement* **2016**, 12(6), 719-32.
[38] Karch C. M.; Cruchaga C.; Goate A. M., Alzheimer's disease genetics: from the bench to the clinic. *Neuron* **2014**, 83(1), 11-26.
[39] Sun L.; Zhou R.; Yang G.; Shi Y., Analysis of 138 pathogenic mutations in presenilin-1 on the in vitro production of Aβ42 and Aβ40 peptides by γ-secretase. *Proc Natl Acad Sci U S A* **2017**, 114(4), E476-E485.
[40] Karran E.; Mercken M.; De Strooper B., The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat Rev Drug Discov* **2011**, 10(9), 698-712.
[41] Busche M. A.; Hyman B. T., Synergy between amyloid-β and tau in Alzheimer's disease. *Nat Neurosci* **2020**, 23(10), 1183-1193.
[42] Miranda A.; Montiel E.; Ulrich H.; Paz C., Selective Secretase Targeting for Alzheimer's Disease Therapy. *J Alzheimers Dis* **2021**, 81(1), 1-17.
[43] Suh J.; Choi S. H.; Romano D. M.; Gannon M. A.; Lesinski A. N.; Kim D. Y.; Tanzi R. E., ADAM10 missense mutations potentiate β-amyloid accumulation by impairing prodomain chaperone function. *Neuron* **2013**, 80(2), 385-401.
[44] Hampel H.; Hardy J.; Blennow K.; Chen C.; Perry G.; Kim S. H.; Villemagne V. L.; Aisen P.; Vendruscolo M.; Iwatsubo T.; Masters C. L.; Cho M.; Lannfelt L.; Cummings J. L.; Vergallo A., The Amyloid-β Pathway in Alzheimer's Disease. *Mol Psychiatry* **2021**, 26(10), 5481-5503.

[45] Zhang Y. W.; Thompson R.; Zhang H.; Xu H., APP processing in Alzheimer's disease. *Mol Brain* **2011**, 4:3.

[46] Martin L.; Latypova X.; Wilson C. M.; Magnaudeix A.; Perrin M. L.; Yardin C.; Terro F. Tau protein kinases: involvement in Alzheimer's disease. *Ageing Res Rev.* **2013** 12(1):289-309.

[47] Guo T.; Noble W.; Hanger D. P. Roles of tau protein in health and disease. *Acta Neuropathol.* **2017** 133(5):665-704.

[48] Wang D.; Huang X.; Yan L.; Zhou L.; Yan C.; Wu J.; Su Z.; Huang Y., The Structure Biology of Tau and Clue for Aggregation Inhibitor Design. *Protein J* **2021**, 40(5), 656-668.

[49] Brunello C. A.; Merezhko M.; Uronen R. L.; Huttunen H. J., Mechanisms of secretion and spreading of pathological tau protein. *Cell Mol Life Sci* **2020**, 77(9), 1721-1744.

[50] Avila J.; Lucas J. J.; Perez M.; Hernandez F., Role of tau protein in both physiological and pathological conditions. *Physiol Rev* **2004**, 84(2), 361-84.

[51] Tapia-Rojas C.; Cabezas-Opazo F.; Deaton C. A.; Vergara E. H.; Johnson G. V. W.; Quintanilla R. A., It's all about tau. *Prog Neurobiol* **2019**, 175, 54-76.

[52] Ding Y.; Lei L.; Lai C.; Tang Z., Tau Protein and Zebrafish Models for Tau-Induced Neurodegeneration. *J Alzheimers Dis* **2019**, 69(2), 339-353.

[53] Krüger L.; Mandelkow E. M., Tau neurotoxicity and rescue in animal models of human Tauopathies. *Curr Opin Neurobiol* **2016**, 36, 52-8.

[54] Gao Y.; Tan L.; Yu J. T.; Tan L. Tau in Alzheimer's Disease: Mechanisms and Therapeutic Strategies. *Curr Alzheimer Res.* **2018** 15(3):283-300.

[55] Johnson G. V.; Stoothoff W. H. Tau phosphorylation in neuronal cell function and dysfunction. *J Cell Sci.* **2004** 117(Pt 24):5721-9.
[56] Lucas J. J.; Hernández F.; Gómez-Ramos P.; Morán M. A.; Hen R.; Avila J. Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. *EMBO J.* **2001** 20(1-2):27-39.
[57] Buée L.; Bussière T.; Buée-Scherrer V.; Delacourte A.; Hof P. R. Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res Brain Res Rev.* **2000** 33(1):95-130.
[58] Baumann K.; Mandelkow E. M.; Biernat J.; Piwnica-Worms H.; Mandelkow E. Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5. *FEBS Lett.* **1993** 336(3):417-24.
[59] Liu F.; Grundke-Iqbal I.; Iqbal K.; Gong C. X. Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. *Eur J Neurosci.* **2005** 22(8):1942-50.
[60] Hou H.; Sun L.; Siddoway B. A.; Petralia R. S.; Yang H.; Gu H.; Nairn A. C.; Xia H. Synaptic NMDA receptor stimulation activates PP1 by inhibiting its phosphorylation by Cdk5. *J Cell Biol.* **2013** 203(3):521-35.
[61] Martin L.; Magnaudeix A.; Esclaire F.; Yardin C.; Terro F. Inhibition of glycogen synthase kinase-3beta downregulates total tau proteins in cultured neurons and its reversal by the blockade of protein phosphatase-2A. *Brain Res.* **2009** 1252:66-75.
[62] Serrano-Pozo A.; Das S.; Hyman B. T. APOE and Alzheimer's disease: advances in genetics, pathophysiology, and therapeutic approaches. *Lancet Neurol.* **2021** 20(1):68-80.
[63] Chen Y.; Strickland M. R.; Soranno A.; Holtzman D. M. Apolipoprotein E: Structural Insights and Links to Alzheimer Disease Pathogenesis. *Neuron.* **2021** 109(2):205-221.

[64] Corder E. H.; Saunders A. M.; Strittmatter W. J.; Schmechel D. E.; Gaskell P. C.; Small G. W.; Roses A. D.; Haines J. L.; Pericak-Vance M. A. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science.* **1993** 261(5123):921-3.
[65] Holtzman D. M.; Herz J.; Bu G. Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease. *Cold Spring Harb Perspect Med.* **2012** 2(3):a006312.
[66] Castaño E. M.; Prelli F.; Pras M.; Frangione B. Apolipoprotein E carboxyl-terminal fragments are complexed to amyloids A and L. Implications for amyloidogenesis and Alzheimer's disease. *J Biol Chem.* **1995** 270(29):17610-5.
[67] Sanan D. A.; Weisgraber K. H.; Russell S. J.; Mahley R. W.; Huang D.; Saunders A.; Schmechel D.; Wisniewski T.; Frangione B.; Roses A. D. *et al.* Apolipoprotein E associates with beta amyloid peptide of Alzheimer's disease to form novel monofibrils. Isoform apoE4 associates more efficiently than apoE3. *J Clin Invest.* **1994** 94(2):860-9.
[68] Veitch D. P.; Weiner M. W.; Aisen P. S.; Beckett L. A.; Cairns N. J.; Green R. C.; Harvey D.; Jack C. R.; Jr.; Jagust W.; Morris J. C.; Petersen R. C.; Saykin A. J.; Shaw L. M.; Toga A. W.; Trojanowski J. Q.; Alzheimer's Disease Neuroimaging Initiative. Understanding disease progression and improving Alzheimer's disease clinical trials: Recent highlights from the Alzheimer's Disease Neuroimaging Initiative. *Alzheimers Dement.* **2019** 15(1):106-152.
[69] Mosconi L.; Berti V.; Glodzik L.; Pupi A.; De Santi S.; de Leon MJ. Pre-clinical detection of Alzheimer's disease using FDG-PET, with or without amyloid imaging. *J Alzheimers Dis.* **2010** 20(3):843-54.
[70] Beal M. F. Mitochondrial dysfunction in neurodegenerative diseases. *Biochim Biophys Acta.* **1998** 1366(1-2):211-23.

[71] Perez Ortiz J. M.; Swerdlow R. H. Mitochondrial dysfunction in Alzheimer's disease: Role in pathogenesis and novel therapeutic opportunities. *Br J Pharmacol.* **2019** 176(18):3489-3507.
[72] Islam M. T. Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders. *Neurol Res.* **2017** 39(1):73-82.
[73] Jones D. P. Redefining oxidative stress. *Antioxid Redox Signal.* **2006** 8(9-10):1865-79.
[74] Tönnies E.; Trushina E. Oxidative Stress, Synaptic Dysfunction, and Alzheimer's Disease. *J Alzheimers Dis.* **2017** 57(4):1105-1121.
[75] Sokoloff L. Energetics of functional activation in neural tissues. *Neurochem Res.* **1999** 24(2):321-9.
[76] Chen Z.; Zhong C. Oxidative stress in Alzheimer's disease. *Neurosci Bull.* **2014** 30(2):271-81.
[77] Praticò D. Oxidative stress hypothesis in Alzheimer's disease: a reappraisal. *Trends Pharmacol Sci.* **2008** 29(12):609-15.
[78] Nunomura A.; Castellani R. J.; Zhu X.; Moreira PI.; Perry G.; Smith M. A. Involvement of oxidative stress in Alzheimer disease. *J Neuropathol Exp Neurol.* **2006** 65(7):631-41.
[79] Markesbery W. R. The role of oxidative stress in Alzheimer disease. *Arch Neurol.* **1999** 56(12):1449-52.
[80] Wang X.; Wang W.; Li L.; Perry G.; Lee H. G.; Zhu X. Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochim Biophys Acta.* **2014** 1842(8):1240-7.
[81] Anderson A. J.; Su J. H.; Cotman C. W. DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. *J Neurosci.* **1996** 16(5):1710-9.

[82] Mullaart E.; Boerrigter M. E.; Ravid R.; Swaab D. F.; Vijg J. Increased levels of DNA breaks in cerebral cortex of Alzheimer's disease patients. *Neurobiol Aging.* **1990** 11(3):169-73.
[83] Nunomura A.; Perry G.; Pappolla M. A.; Wade R.; Hirai K.; Chiba S.; Smith M. A. RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *J Neurosci.* **1999** 19(6):1959-64.
[84] Kim T. S.; Pae C. U.; Yoon S. J.; Jang W. Y.; Lee N. J.; Kim J. J.; Lee S. J.; Lee C.; Paik I. H.; Lee C. U. Decreased plasma antioxidants in patients with Alzheimer's disease. *Int J Geriatr Psychiatry.* **2006** 21(4):344-8.
[85] De Strooper B.; Karran E. The Cellular Phase of Alzheimer's Disease. *Cell.* **2016** 164(4):603-15.
[86] McAllister B. B.; Lacoursiere S. G.; Sutherland R. J.; Mohajerani M. H. Intracerebral seeding of amyloid-β and tau pathology in mice: Factors underlying prion-like spreading and comparisons with α-synuclein. *Neurosci Biobehav Rev.* **2020** 112:1-27.
[87] Wu X.; Lv YG.; Du Y. F.; Chen F.; Reed M. N.; Hu M.; Suppiramaniam V.; Tang S. S.; Hong H. Neuroprotective effects of INT-777 against Aβ1-42-induced cognitive impairment, neuroinflammation, apoptosis, and synaptic dysfunction in mice. *Brain Behav Immun.* **2018** 73:533-545.
[88] Meyer-Luehmann M.; Coomaraswamy J.; Bolmont T.; Kaeser S.; Schaefer C.; Kilger E.; Neuenschwander A.; Abramowski D.; Frey P.; Jaton A. L.; Vigouret J. M.; Paganetti P.; Walsh D. M.; Mathews P. M.; Ghiso J.; Staufenbiel M.; Walker L. C.; Jucker M. Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host. *Science.* **2006** 313(5794):1781-4.
[89] Kane M. D.; Lipinski W. J.; Callahan M. J.; Bian F.; Durham R. A.; Schwarz R. D.; Roher A. E.; Walker L. C. Evidence for seeding of beta -amyloid by intracerebral infusion of Alzheimer brain extracts in beta

-amyloid precursor protein-transgenic mice. *J Neurosci.* **2000** 20(10):3606-11.
[90] Games D.; Adams D.; Alessandrini R.; Barbour R.; Berthelette P.; Blackwell C.; Carr T.; Clemens J.; Donaldson T.; Gillespie F. *et al.* Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature.* **1995** 373(6514):523-7.
[91] Chin J. Selecting a mouse model of Alzheimer's disease. *Methods Mol Biol.* **2011** 670:169-89.
[92] Esquerda-Canals G.; Montoliu-Gaya L.; Güell-Bosch J.; Villegas S. Mouse Models of Alzheimer's Disease. *J Alzheimers Dis.* **2017** 57(4):1171-1183.
[93] Weller J.; Budson A. Current understanding of Alzheimer's disease diagnosis and treatment. *F1000Res.* **2018** 7:F1000 Faculty Rev-1161.
[94] Mufson E. J.; Counts S. E.; Perez S. E.; Ginsberg S. D. Cholinergic system during the progression of Alzheimer's disease: therapeutic implications. *Expert Rev Neurother.* **2008** 8(11):1703-18.
[95] Johnson J. W.; Kotermanski S. E. Mechanism of action of memantine. *Curr Opin Pharmacol.* **2006** 6(1):61-7.
[96] Cummings J. L.; Tong G.; Ballard C. Treatment Combinations for Alzheimer's Disease: Current and Future Pharmacotherapy Options. *J Alzheimers Dis.* **2019** 67(3):779-794.
[97] Kennedy M. E.; Stamford A. W.; Chen X.; Cox K.; Cumming J. N.; Dockendorf M. F.; Egan M.; Ereshefsky L.; Hodgson R. A.; Hyde L. A.; Jhee S.; Kleijn H. J.; Kuvelkar R.; Li W.; Mattson B. A.; Mei H.; Palcza J.; Scott J. D.; Tanen M.; Troyer M. D.; Tseng J. L.; Stone J. A.; Parker E. M.; Forman M. S. The BACE1 inhibitor verubecestat (MK-8931)

reduces CNS β-amyloid in animal models and in Alzheimer's disease patients. *Sci Transl Med.* **2016** 8(363):363ra150.
[98] Egan M. F.; Kost J.; Tariot P. N.; Aisen P. S.; Cummings J. L.; Vellas B.; Sur C.; Mukai Y.; Voss T.; Furtek C.; Mahoney E.; Harper Mozley L.; Vandenberghe R.; Mo Y.; Michelson D. Randomized Trial of Verubecestat for Mild-to-Moderate Alzheimer's Disease. *N Engl J Med.* **2018** 378(18):1691-1703.
[99] Bareggi S. R.; Cornelli U. Clioquinol: review of its mechanisms of action and clinical uses in neurodegenerative disorders. *CNS Neurosci Ther.* **2012** 18(1):41-6.
[100] Bílková E.; Imramovský A.; Sedlák M. Recent advances in the design and synthesis of prednisolone and methylprednisolone conjugates. *Curr Pharm Des.* **2011** 17(32):3577-95.
[101] Jaturapatporn D.; Isaac M. G.; McCleery J.; Tabet N. Aspirin, steroidal and non-steroidal anti-inflammatory drugs for the treatment of Alzheimer's disease. *Cochrane Database Syst Rev.* **2012** 15;(2):CD006378.
[102] Aisen P. S.; Marin D.; Altstiel L.; Goodwin C.; Baruch B.; Jacobson R.; Ryan T.; Davis K. L. A pilot study of prednisone in Alzheimer's disease. *Dementia.* **1996** 7(4):201-6.
[103] Liu Y.; Li M.; Zhang Z.; Ye Y.; Zhou J. Role of microglia-neuron interactions in diabetic encephalopathy. *Ageing Res Rev.* **2018** 42:28-39.
[104] Adcock I. M.; Mumby S. Glucocorticoids. *Handb Exp Pharmacol.* **2017** 237:171-196.
[105] Jiang N.; Ding J.; Liu J.; Sun X.; Zhang Z.; Mo Z.; Li X.; Yin H.; Tang W.; Xie S. S. Novel chromanone-dithiocarbamate hybrids as multifunctional AChE inhibitors with β-amyloid anti-aggregation properties for the treatment of Alzheimer's disease. *Bioorg Chem.* **2019** 89:103027.

[106] Ohno M.; Sametsky E. A.; Younkin L. H.; Oakley H.; Younkin S. G.; Citron M.; Vassar R.; Disterhoft J. F. BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. *Neuron.* **2004** 41(1):27-33.
[107] Bromley-Brits K.; Deng Y.; Song W. Morris water maze test for learning and memory deficits in Alzheimer's disease model mice. *J Vis Exp.* **2011** (53):2920.
[108] Wang Z.; Wang Y.; Wang B.; Li W.; Huang L.; Li X. Design, Synthesis, and Evaluation of Orally Available Clioquinol-Moracin M Hybrids as Multitarget-Directed Ligands for Cognitive Improvement in a Rat Model of Neurodegeneration in Alzheimer's Disease. *J Med Chem.* **2015** 58(21):8616-37.
[109] Kim J.; Lee H. J.; Park S. K.; Park J. H.; Jeong H. R.; Lee S.; Lee H.; Seol E.; Hoe H. S. Donepezil Regulates LPS and Aβ-Stimulated Neuroinflammation through MAPK/NLRP3 Inflammasome/STAT3 Signaling. *Int J Mol Sci.* **2021** 22(19):10637.
[110] Baik S. H.; Kang S.; Lee W.; Choi H.; Chung S.; Kim J. I.; Mook-Jung I. A Breakdown in Metabolic Reprogramming Causes Microglia Dysfunction in Alzheimer's Disease. *Cell Metab.* **2019** 30(3):493-507.e6.
[111] Lituma P. J.; Woo E.; O'Hara B. F.; Castillo P. E.; Sibinga N. E. S.; Nandi S. Altered synaptic connectivity and brain function in mice lacking microglial adapter protein Iba1. *Proc Natl Acad Sci U S A.* **2021** 118(46):e2115539118.
[112] Yu, T. W.; Lane, H. Y.; Lin, C. H. Novel Therapeutic Approaches for Alzheimer's Disease: An Updated Review. *Int J Mol Sci.* **2021**, 22(15):8208
[113] 2021 Alzheimer's disease facts and figures. *Alzheimers Dement.* **2021** 17(3):327-406.

[114] Kevadiya B. D.; Ottemann B. M.; Thomas M.B.; Mukadam I.; Nigam S.; McMillan J.; Gorantla S.; Bronich T. K.; Edagwa B.; Gendelman H. E. Neurotheranostics as personalized medicines. *Adv Drug Deliv Rev.* **2019** 148:252-289.
[115] Blanco-Silvente L.; Castells X.; Saez M.; Barceló M. A.; Garre-Olmo J.; Vilalta-Franch J.; Capellà D. Discontinuation, Efficacy, and Safety of Cholinesterase Inhibitors for Alzheimer's Disease: a Meta-Analysis and Meta-Regression of 43 Randomized Clinical Trials Enrolling 16 106 Patients. *Int J Neuropsychopharmacol.* **2017** 20(7):519-528.
[116] Blanco-Silvente L.; Capellà D.; Garre-Olmo J.; Vilalta-Franch J.; Castells X. Predictors of discontinuation, efficacy, and safety of memantine treatment for Alzheimer's disease: meta-analysis and meta-regression of 18 randomized clinical trials involving 5004 patients. *BMC Geriatr.* **2018** 18(1):168.
[117] Egan M. F.; Kost J.; Voss T.; Mukai Y.; Aisen P. S.; Cummings J. L.; Tariot P. N.; Vellas B.; van Dyck C. H.; Boada M.; Zhang Y.; Li W.; Furtek C.; Mahoney E.; Harper Mozley L.; Mo Y.; Sur C.; Michelson D. Randomized Trial of Verubecestat for Prodromal Alzheimer's Disease. *N Engl J Med.* **2019** 380(15):1408-1420.
[118] Henley D.; Raghavan N.; Sperling R.; Aisen P.; Raman R.; Romano G. Preliminary Results of a Trial of Atabecestat in Preclinical Alzheimer's Disease. *N Engl J Med.* **2019** 380(15):1483-1485.
[119] Doody R. S., Raman R., Farlow M., Iwatsubo T., Vellas B., Joffe S., Kieburtz K., He F., Sun X., Thomas R. G., Aisen P. S. Alzheimer's Disease Cooperative Study Steering Committee; Siemers E., Sethuraman G., Mohs R. Semagacestat Study Group. A phase 3 trial of semagacestat for treatment of Alzheimer's disease. *N Engl J Med.* **2013** 369(4):341-50.

[120] Coric V.; Salloway S.; van Dyck CH.; Dubois B.; Andreasen N.; Brody M.; Curtis C.; Soininen H.; Thein S.; Shiovitz T.; Pilcher G.; Ferris S.; Colby S.; Kerselaers W.; Dockens R.; Soares H.; Kaplita S.; Luo F.; Pachai C.; Bracoud L.; Mintun M.; Grill J. D.; Marek K.; Seibyl J.; Cedarbaum J. M.; Albright C.; Feldman H. H.; Berman R. M. Targeting Prodromal Alzheimer Disease With Avagacestat: A Randomized Clinical Trial. *JAMA Neurol.* **2015** 72(11):1324-33.
[121] Khalil H. S.; Mitev V.; Vlaykova T.; Cavicchi L.; Zhelev N. Discovery and development of Seliciclib. How systems biology approaches can lead to better drug performance. *J Biotechnol.* **2015** 202:40-9.
[122] Leggio G. M.; Catania M. V.; Puzzo D.; Spatuzza M.; Pellitteri R.; Gulisano W.; Torrisi S. A.; Giurdanella G.; Piazza C.; Impellizzeri A. R.; Gozzo L.; Navarria A.; Bucolo C.; Nicoletti F.; Palmeri A.; Salomone S.; Copani A.; Caraci F.; Drago F. The antineoplastic drug flavopiridol reverses memory impairment induced by Amyloid-ß1-42 oligomers in mice. *Pharmacol Res.* **2016** 106:10-20.
[123] Lovestone S.; Boada M.; Dubois B.; Hüll M.; Rinne J. O.; Huppertz H. J.; Calero M.; Andrés M. V.; Gómez-Carrillo B.; León T.; del Ser T.; ARGO investigators. A phase II trial of tideglusib in Alzheimer's disease. *J Alzheimers Dis.* **2015** 45(1):75-88.
[124] Yao H.; Uras G.; Zhang P.; Xu S.; Yin Y.; Liu J.; Qin S.; Li X.; Allen S.; Bai R.; Gong Q.; Zhang H.; Zhu Z.; Xu J. Discovery of Novel Tacrine-Pyrimidone Hybrids as Potent Dual AChE/GSK-3 Inhibitors for the Treatment of Alzheimer's Disease. *J Med Chem.* **2021** 64(11):7483-7506.
[125] Youn H.; Lee K. J.; Kim S. G.; Cho S. J.; Kim W. J.; Lee W. J.; Hwang J. Y.; Han C.; Shin C.; Jung H. Y. The Behavioral Effects of Combination Therapy of Memantine and Acetylcholinesterase Inhibitors Compared with Acetylcholinesterase Inhibitors Alone in

Patients with Moderate Alzheimer's Dementia: A Double-Blind Randomized Placebo-Controlled Trial. *Psychiatry Investig.* **2021** 18(3):233-240.
[126] Choi S. H.; Park K. W.; Na D. L.; Han H. J.; Kim E. J.; Shim Y. S.; Lee J. H.; Expect Study Group. Tolerability and efficacy of memantine add-on therapy to rivastigmine transdermal patches in mild to moderate Alzheimer's disease: a multicenter, randomized, open-label, parallel-group study. *Curr Med Res Opin.* **2011** 27(7):1375-83.
[127] Kinney J. W.; Bemiller S. M.; Murtishaw A. S.; Leisgang A. M.; Salazar A. M.; Lamb B. T. Inflammation as a central mechanism in Alzheimer's disease. *Alzheimers Dement (N Y).* **2018** 4:575-590.
[128] Zhong L.; Xu Y.; Zhuo R.; Wang T.; Wang K.; Huang R.; Wang D.; Gao Y.; Zhu Y.; Sheng X.; Chen K.; Wang N.; Zhu L.; Can D.; Marten Y.; Shinohara M.; Liu C. C.; Du D.; Sun H.; Wen L.; Xu H.; Bu G.; Chen X. F. Soluble TREM2 ameliorates pathological phenotypes by modulating microglial functions in an Alzheimer's disease model. *Nat Commun.* **2019** 10(1):1365.
[129] Price B. R.; Sudduth T. L.; Weekman E. M.; Johnson S.; Hawthorne D.; Woolums A.; Wilcock D. M. Therapeutic Trem2 activation ameliorates amyloid-beta deposition and improves cognition in the 5XFAD model of amyloid deposition. *J Neuroinflammation.* **2020** 17(1):238.
[130] Wang S.; Mustafa M.; Yuede C. M,; Salazar S. V.; Kong P.; Long H.; Ward M.; Siddiqui O.; Paul R.; Gilfillan S.; Ibrahim A.; Rhinn H.; Tassi I.; Rosenthal A.; Schwabe T.; Colonna M. Anti-human TREM2 induces microglia proliferation and reduces pathology in an Alzheimer's disease model. *J Exp Med.* **2020** 217(9):e20200785.
[131] Götz J.; Ittner L. M. Animal models of Alzheimer's disease and frontotemporal dementia. *Nat Rev Neurosci.* **2008** 9(7):532-44.

[132] Rossi G.; Conconi D.; Panzeri E.; Paoletta L.; Piccoli E.; Ferretti M. G.; Mangieri M.; Ruggerone M.; Dalprà L.; Tagliavini F. Mutations in MAPT give rise to aneuploidy in animal models of tauopathy. *Neurogenetics.* **2014** 15(1):31-40.
[133] Yoshiyama Y.; Higuchi M.; Zhang B.; Huang S. M.; Iwata N.; Saido T. C.; Maeda J.; Suhara T.; Trojanowski J. Q.; Lee V. M. Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. *Neuron.* **2007** 53(3):337-51.
[134] Paul B. D.; Snyder S. H.; Bohr V. A. Signaling by cGAS-STING in Neurodegeneration, Neuroinflammation, and Aging. *Trends Neurosci.* **2021** 44(2):83-96.
[135] Guo Y.; Jiang F.; Kong L.; Li B.; Yang Y.; Zhang L.; Liu B.; Zheng Y.; Gao C. Cutting Edge: USP27X Deubiquitinates and Stabilizes the DNA Sensor cGAS to Regulate Cytosolic DNA-Mediated Signaling. *J Immunol.* **2019** 203(8):2049-2054.
[136] Jin M.; Shiwaku H.; Tanaka H.; Obita T.; Ohuchi S.; Yoshioka Y.; Jin X.; Kondo K.; Fujita K.; Homma H.; Nakajima K.; Mizuguchi M.; Okazawa H. Tau activates microglia via the PQBP1-cGAS-STING pathway to promote brain inflammation. *Nat Commun.* **2021** 12(1):6565.
[137] Xu Q.; Xu W.; Cheng H.; Yuan H.; Tan X. Efficacy and mechanism of cGAMP to suppress Alzheimer's disease by elevating TREM2. *Brain Behav Immun.* **2019** 81:495-508.