Original Research

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The machinery of γ-secretase inhibitor alleviated cognitive deficits and neuropathology in APP/PS1 transgenic mice

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Abstract

γ-secretase inhibitor is associated with Alzheiner's disease (AD). Since crenigacestat is a gamma-secretase inhibitor, it has been extensively studied in preclinical models and clinical trials of cancer prevention due to it as a γ-secretase inhibitor. However, the possible involvement of crenigacestat in progression of AD remains unknown. This study found that crenigacestat treatment attenuated neurological impairment in AD mice. Moreover, treatment with crenigacestat decreased the level of insoluble and soluble Aβ and attenuated the apoptosis of neuron in hippocampus of AD mice. We also found that crenigacestat significantly inhibited the activation of microglia and neuron inflammation of hippocampus of AD mice. Finally, we explored the mechanism of crenigacestat suppressed the progression of AD, we found that crenigacestat significantly inhibited Notch signaling pathway, which suppressed the PI3K and mTOR signaling pathway. Collectively, these findings indicated that crenigacestat can suppressed the progression of AD by suppressing Notch signaling pathway, which blocking PI3K/PBK/mTOR signaling pathway.

Keywords: γ-secretase inhibitor; Alzheimer's disease; PI3K and mTOR signaling pathway

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that is the most frequent cause of dementia in the elderly, and it characterized by cognitive impairment, impaired learning and memory, and behavioral abnormalities[1, 2]. Previous studies shown that 47 million people in the world are now suffering from AD, with a new patient appearing every 3 seconds, and it is expected that by 2050, we will have more than 130 million people suffering from it[3]. However, AD still lacked an effective treatments method[4].

The pathological features of AD are mainly cortical atrophy over a large area, the appearance of β-amyloid (Aβ) plaques in the brain and hippocampus, the accumulation of extracellular age spots (SP), the abnormal accumulation of tau protein in brain nerve cells, the appearance of neurofibrillary tangles (NTF) composed of paired helical filaments (PHF), and the reduction of cortical neuronal cells[5, 6]. Aβ play an important role in neuropathology of AD, and it not only the initiator and main structural substance of SP, but also has a toxic effect on neuron, so it is considerate as an early trigger for the development of AD[7, 8]. Aβ is a kind of peptides of 39-43 amino acid in length, and Aβ is mainly composed of Aβ40 (40 amino acids) and Aβ42 (40 amino acids)[9]. Despite the small structural difference between Aβ40 and Aβ42, they display distinct difference function in AD[10]. Aβ42 has higher neurotoxin, and easy amyloidogenic than Aβ40, however Aβ40 can inhibit the aggregation of Aβ42[10]. Recent studies suggested that the ratio of Aβ42/Aβ40 was relative with the pathogenesis of AD, and decreased the level of Aβ42 alleviated the risk of AD[11]. Therefore, reducing the ratio of Aβ42/Aβ40 is one of the potential therapeutic approaches for AD. The Aβ40 and Aβ42 peptides are cleaved from the larger precursor amyloid precursor protein (APP)[12, 13]. According to the ability of form Aβ peptide, the process of APP cleaved can be divided into the amyloidogenic proteolytic pathway and the non-amyloidogenic pathway[13]. In non-amyloidogenic pathway, APP is cleaved to sAPPα by α-secretase, this pathway can't form complete Aβ due to the excision position is in the middle of Aβ[13]. In the amyloidogenic pathway, APP is cleaved to sAPPβ and C-terminal fragment C99 by β-secretase, after that C99 is cleaved to APP intracellular domain (AICD) and Aβ40 or Aβ42[13]. Inhibited the activated the γ-secretase decreased the secretion level of Aβ40 or Aβ42, hence, γ-secretase is an important target of AD therapeutics[14]. γ-secretase is a multi-subunit protease complex, it consists of four individual proteins: presenilin-1 (PSEN1), Nicastro, anterior pharynx-defective 1 (APH-1) and presenilin enhancer 2 (PEN-2)[15].

γ-secretase inhibitors (GSIs) are potential drugs that inhibit the enzyme γ-secretase. In order to suppress the γ-secretase activity for AD therapy, a variety of GSIs i.e., Semagacestat, Avagacestat, Crenigacestat have been development[16]. However, the results of clinical trials of these GSIs for AD were disappointing, such as Semagacestat was worsening the memory of AD patients, and increased the risk of a skin cancer due to inhibit Notch signaling pathway[17]. To date, no GSIs is available for clinical treatment of AD[18], it is urgent to development a new GSIs for AD therapy. Crenigacestat is a potent and competitive peptide aldehyde inhibitor of γ-secretase and notch, mainly used in cancer therapy[19]. However, the role of Crenigacestat in AD still unknown. Here, we investigated the function and mechanism of Crenigacestat in AD therapy.

Materials and methods

Animals and drug treatment

APP/PS1 transgenic mice (APPSWE, PSEN1dE9) were purchased from the Cyagen Biosciences (Guangzhou, China). And then APP/PS1 transgenic mice matched wild-type (WT) C57BL/6 mice were purchased from Animal Experimental Center of Kunming Medical University. All mice were housed under SPF conditions, 18-26℃ with 40-70% humidity under with free access to food and water. The work was approved by the Animal Ethics Committee of Kunming medical University.

Morris water maze (MWM) test

Morris water maze (MWM) test was used to detected learning and memory capacity of the mice follower the previous described[20]. MWM pool was a circular pool (120 cm diameter × 60 cm height) with a water depth of 30 cm. The MWM test consists of three parts: on the first day, the escape platform (10 cm diameter) was located at a fixed space position 1 cm above the water surface to detected the spatial learning ability, and on the second to fifth days, the escape platform was located 1 cm below the water surface to detected the spatial memory ability, on the sixth day, the escape platform was backout for spatial probe test. All data, including escape latency, swimming path, and target zone frequency of the mice were recorded by AnyMaze (Clever Sys, Inc.).

Modified neurological severity scores (mNSS)

The sensory, motor balance and reflex tests were detected using mNSS test at 1 week, 4 and 8 weeks. The score ranges from 0 to 14. When rats were unable to complete the task due to neurological dysfunction, 1 point was given for each task. Higher scores indicate more severe neurological dysfunction. The mNSS test was assessed as previously reported[21].

ELISA

The secretion of Aβ, TNF-α, IL-1β and IL-6 in brain tissue were detected using ELISA kits (abcam, China). Brain tissue lysates were centrifuged at 18,000 g for 20 min at 4 ℃, and the supernatants of brain tissue were collected. After that, 50 μl sample and 50 μl antibody cocktail was added into each well of 96-well plate. Sealed the plate and incubated for 1 h at room temperature sharked with 400 rpm. After washed with wash buffer PT for 3 times, 100 μl TMB development solution was added into each well and incubated for 10 min sharked with 400 rpm under dark. 100 μl stop solution were added into each well, and then sharked 1 min, the secretion will detect by spectrometry at 450 nm.

Immunofluorescence (IF)

Mice were euthanized and transcardially perfused with 4% paraformaldehyde (PFA) solution (pH 7.40, 4 °C). The samples were fixed with 4% PFA for 48 h, dehydration with 30% sucrose solution for 72 h. After that, the samples were frozen at -80 °C and sectioned into 8 μm thick slices. The slices were permeabilized with 3% Triton X-100 for 10 mins at room temperature, treated with 5% Bovine Serum Albumin (BSA) for 1 h at room temperature. After washed with PBS, the slices were incubated with primary antibody Iba1 (1:1000, abcam) overnight at 4 ℃. After washed with PBS, the cells were incubated with Alexa-488-conjugated secondary antibody (1:800, Jackson ImmunoResearch Inc.) for 2 h at room temperature. Nuclei were staining with DAPI. The samples were imaged under a fluorescence (Leica, German). And the relative fluorescence intensity was detected using image J software.

Thioflavin S (ThioS) Staining

ThioS Staining was used to detect β-amyloid (Aβ) plaques in brain of mice. The sections of brain were mounted onto slides, washed with PBS, after the brain sections were staining with 0.02% ThioS solution (Sigma, China) for 10 min. Next, brain sections were differentiated in 50% ethanol for 2 min, and washed with PBS. Finally, the images were viewed and taken using fluorescence microscopy.

TUNEL staining assay

The apoptosis was detected using terminal deoxytransferase-mediated dUTP-biotin nick end labelling (TUNEL) assay. Strictly following the manufacturer's instructions fo TUNEL kit (ThermoFisher, China).

Western blot

The western blot assay was performed to detected the expression levels of Notch1, PI3K, PKB, p-PKB, Wnt, mTOR, NF-κB, TLR4 in brain tissues of mice. Brain tissues were added with liquid nitrogen and grind to powder form, cold protein lysate with protease inhibitor were added and at 4 ℃ for 30 min. After that, the tissue lysates were centrifuged at 12000 rpm for 15 min, and the supernatant were collection. The protein concentrations were detected by Bicinchoninic Acid (BCA) kit (Beyotime, China) according to the instruction, and protein concentration was adjusted for all proteins. Subsequently, 25ug of protein was loaded on to 8% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) to separate, and then transfected to a polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with 5% non-fat milk for 1 h at room temperature. The membrane was rinsed with tris-buffered saline tween (TBST), and then incubated with primary antibodies i.e. Aβ (proteintech,1:1000), Notch1 (abcam, 1:1000), PI3K (proteintech,1:1000), PKB (protein-tech,1:1000), p-PKB (proteintech,1:1000), Wnt (proteintech,1:1000), mTOR (protein-tech,1:10000), NF-κB (protein-tech,1:1000), TLR4 (proteintech,1:1000), and then β-actin (ZSGB-BIO, 1:2000) overnight at 4 ℃. Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. The bands were detected by the enhanced chemiluminescence (ECL) system (Tanon, Shanghai, China). The β-actin were used as loading control, the protein expression levels were illustrated by the ratio of the gray value of the protein and the loading control.

Statistical analysis

Statistical analysis was performed using the GraphPad 8.0.0 software. All data were consistent with the normal distribution and presented as mean ± standard deviation. T-test were used for comparisons between two groups and one-way analysis of variance (ANOVA) was used to compare the differences among multiple groups. The difference was statistically significant at $p\text{-value} < 0.05$.

Results

Crenigacestat decreased the neurological impairment of AD mice

To value whether Crenigacestat affects the spatial learning and memory performance of AD mice, the Morris water maze (MWM) test and modified neurological severity scores (mNSS) were performed. In visible platform phase of MWM test, the escape latency, AD mice spend more time to reach the platform compared with ctrl mice, and the GSI remarkedly decreased the time to reach the platform in AD mice (figure1.AB). During hidden platform phase of MWM test, the results of the escape latency shown same trends as visible platform phase, and all mice in

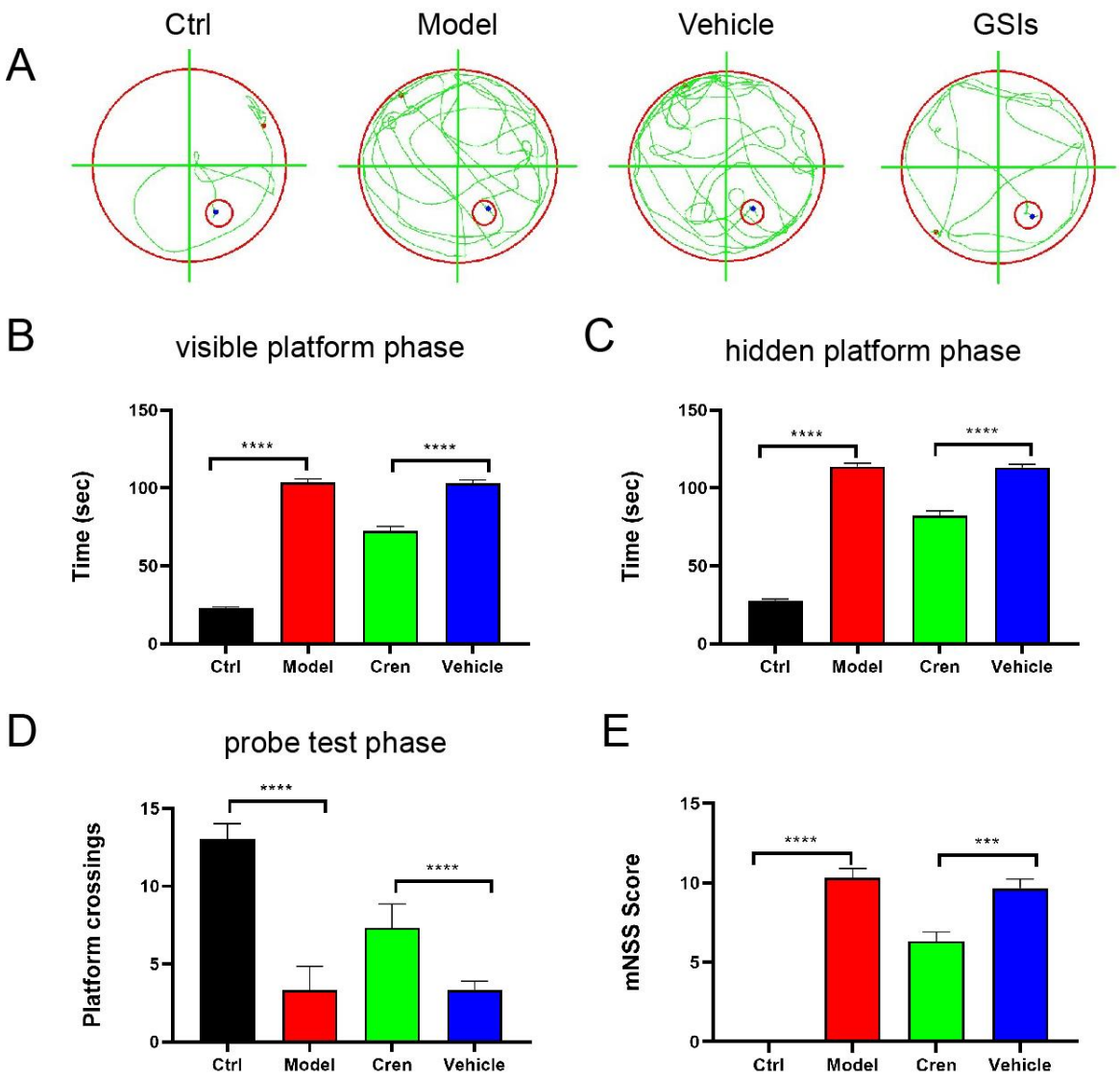

Figure 1 Protective effects of crenigacestat on cognitive function in AD mice. (A) Swimming trajectory of mice during three training trials on a visible platform water maze. (B) Analysis of mice find a visible platform. (C) Analysis of mice find a hidden platform. (D) Average numbers of platform crossings during probe test phase. (E) Quantitative analysis of mNss scores (***p<0.001, ****p<0.0001).

hidden platform phase took more time reached the platform compared to the visible platform phase (figure1.C). During the probe test phase of MWM test, the number of passes across the platform of AD mice significantly decreased compared with ctrl mice, and crenigacestat increased the time number of passes across the platform in AD mice (figure1.D). The result of MWM test suggested that crenigacestat alleviated the learning and memory capacity of AD mice.Furthermore, the results of mNSS demonstrated that the mNSS score was significantly increased in AD mice compared with ctrl mice, and GSI decreased mNSS score in AD mice. Taken together, these results indicated that GSI alleviated the spatial

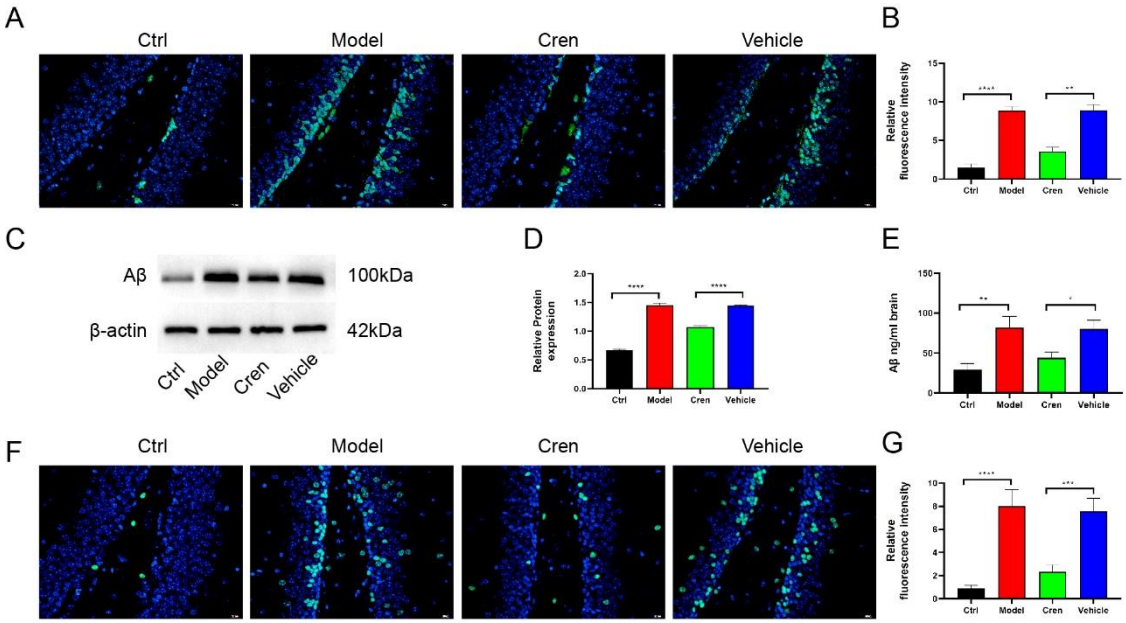

Figure 2 Crenigacestat treatment decreased Aβ and neuron apoptosis of hippocampus in mice. (A) Representative fluorescence images of ThioS staining, blue (DAPI), green (Aβ). (B) Analysis of the level of Aβ. (C) Representative western blot image of Aβ of hippocampus in mice. (D) Quantification of relative levels of Aβ in hippocampus of mice. (E) Aβ were analyzed by ELISA in the hippocampus of mice. (F) TUNEL staining in hippocampus of mice (DAPI: blue, TUNEL: green). (G) Quantitative analysis of TUNEL staining. (*p<0.05, ****p<0.005,***p<0.001, ****p<0.0001, original magnification, ×400).

learning and memory performance s in AD mice.

GSI attenuated the cerebral Aβ plaque burden and hippocampus apoptosis in AD mice

Previous study demonstrated that GSI improved spatial learning and memory performance in AD[16]. Crenigacestat was one of GSI, while whether crenigacestat participated in the progression of AD still unknown. To determine the effect of GSI on Aβ generated in AD mice, the insoluble and soluble Aβ were detected by ThioS stain, western blot and ELISA assay, respectively. The results of Thios stain demonstrated that cerebral Aβ plaque deposition in hippocampus was very low in the ctrl group, and crenigacestat decreased cerebral Aβ plaque deposition in hippocampus of AD mice (fig2. A-B). Similarly, the result of western blot demonstrated that the expression of Aβ was significantly upregulated in hippocampus of AD mice compared with normal mice, while crenigacestat significantly decreased the expression of Aβ in AD mice hippocampus (fig2. C-D). Moreover, the results of ELISA demonstrated that soluble Aβ in brain tissue was remarkedly increased in AD mice compared with normal mice, and crenigacestat significantly decreased the secretion of soluble Aβ of brain tissue in AD mice (fig2. E). These results demonstrated that crenigacestat remarkedly decreased the insoluble and soluble Aβ of hippocampus of AD mice. AD is a kind of neurodegenerative disorder, that induced by neuron death, hence we examined the apoptosis level of hippocampus. The results demonstrated that the apoptosis level of hippocampus was significantly increased in AD mice compared with normal mice, and crenigacestat decreased the level of apoptosis of hippocampus in AD mice (fig2. F, G). Taken together, these results indicated that GSI

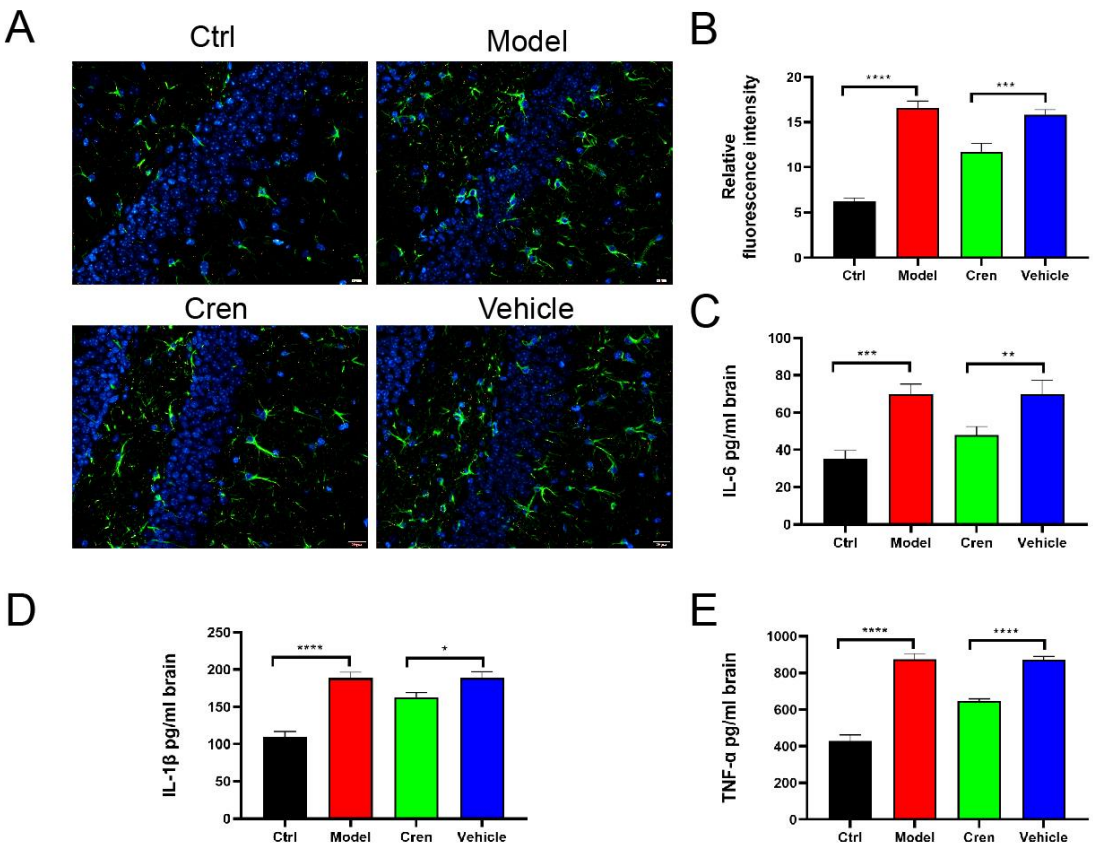

Figure 3 Crenigacestat treatment attenuate microglia activation and reduce neuroinflammatory responses. (A) Representative microscopy images of lba-1 labeled microglia in the hippocampus of mouse (DAPI: blue, lba-1: green). (B) Quantification of the immunoactivity of Iba-1. (C) ELISA of IL-6 proteins in brain homogenates. (D) ELISA of IL-1β proteins in brain homogenates. (F) ELISA of TNF-α proteins in brain homogenates. (*p<0.05, ****p<0.005, ***p<0.001, ****p<0.0001, original magnification, ×400).

attenuated the hippocampal apoptosis via decreased Aβ plaque burden in AD mice.

Crenigacestat regulated activity microglia and inflammation in brain tissue of AD mice

Aβ binds to microglia and activates microglia in brain tissue[22]. Activated microglia produce large amounts of inflammatory factors in the brain tissue of AD patients, which inducing a central neuroinflammatory response or directly damaged neurons[22]. Therefore, we assessed whether crenigacestat regulated the activity microglia and inflammation in brain tissue of AD mice. The microglial activation was assessed by immunohistochemistry using the specific microglial marker, Iba1. In the ctrl group, the microglial cells were ramified with multiple branches, in the AD group and vehicle group, the microglial cells were hypertrophied and shortened branches, and the microglial in GSI group the degreed of hypertrophied and branched was decreased (fig3.AB). Simultaneous, the secretion of IL-6, IL-1β and TNF-α was increased in brain tissue of AD mice compared with normal mice, and crenigacestat decreased the secretion of IL-6, IL-1β and TNF-α in AD mice. Altogether, these results indicated that crenigacestat decreased the microglial activation and secretion of inflammasome.

Crenigacestat reduce Notch protein expression and directly participate in the Notch signaling pathway

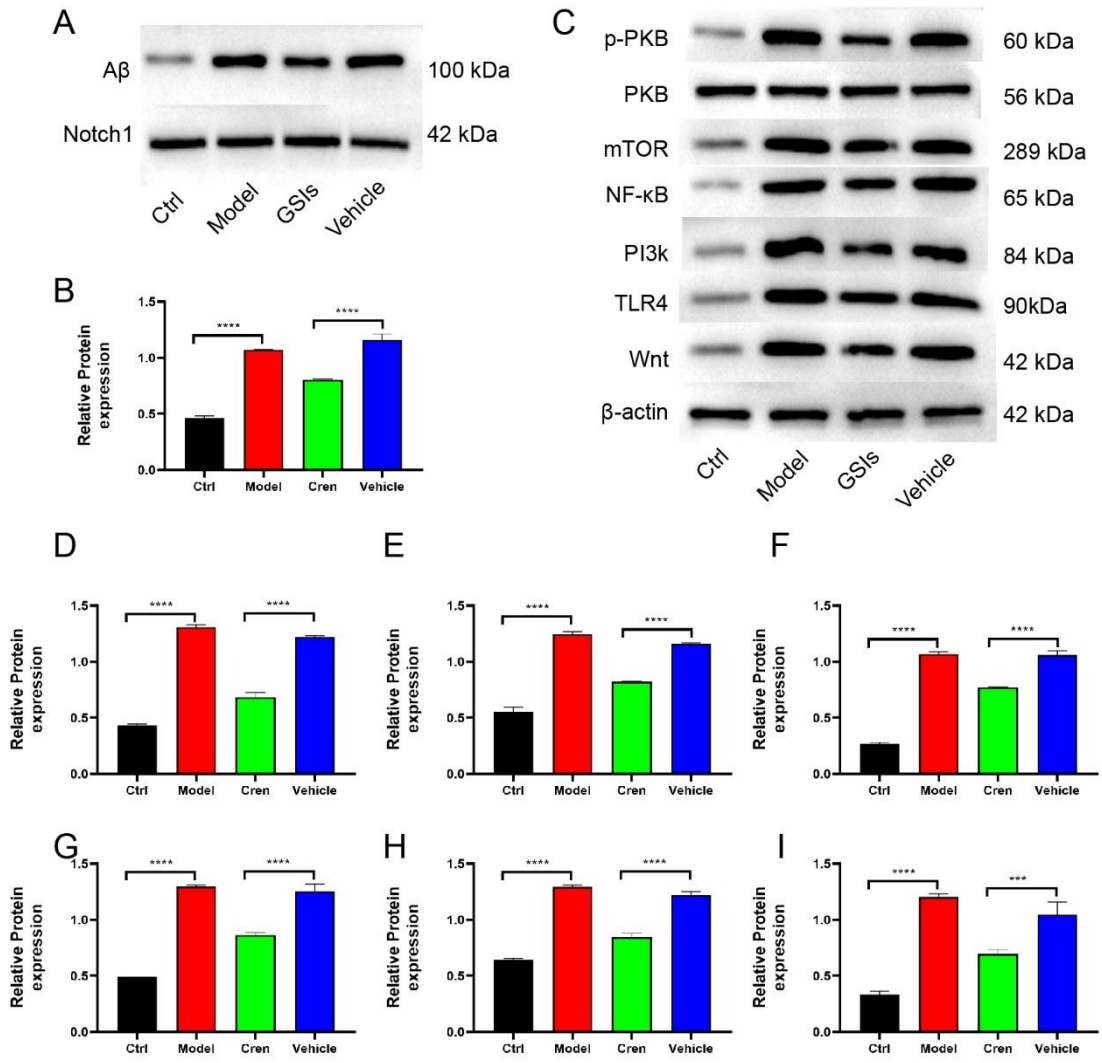

Figure 4 Crenigacestat inhibits the activation of Notch signaling pathway. (A) The protein levels of Notch1 in brain of mice were determined by western blot. (B) Quantification of the protein levels of Notch1 in brain of mice. (C) The protein levels of p-PLB, PKB, mTOR, NF-kB, PI3k, TLR4 and Wnt in brain of mice were determined by western blot. (D) Quantification of the protein levels of p-PKB in brain of mice. (E) Quantification of the protein levels of mTOR in brain of mice. (F) Quantification of the protein levels of NF-kB in brain of mice. (G) Quantification of the protein levels of PI3k in brain of mice. (H) Quantification of the protein levels of TLR4 in brain of mice. (I) Quantification of the protein levels of Wnt in brain of mice.

Previous studies demonstrated that Notch1 could mediate neurodegenerative progress including AD, and Notch1 protein expression was significantly increased in the brains of AD patients and Notch1 was deposited in Aβ1-42-positive plaques[23]. Crenigacestat is a potent inhibitor of small molecule Notch cleavage that reduces Notch signaling and its downstream biological effects. Hence, we assessed whether crenigacestat alleviated AD progression by Notch signaling pathway. The results of western blot demonstrated that Notch 1 was significantly increased in brain tissue of AD mice compared with normal mice, and crenigacestat attenuated the expression of Notch 1 in AD mice. Previous studies demonstrated that Notch signaling pathway participated in multiple disease progression by activity PI3K/PBK/mTOR signaling pathway[24], hence, we detected PI3K/PBK/ mTOR signaling pathway relative protein i.e. PI3K, PKB, p-PKB, Wnt, mTOR, NF-κB expression.

We found that most PI3K/PBK/mTOR signaling pathway relative protein in AD mice were higher than those in the ctrl group and that the GSI treatment decreased the expression of PI3K/PBK/mTOR signaling pathway relative protein in AD mice. Collectively, these results suggest that GSI might reduce the cerebral Aβ plaque burden, inflammasome, neuron apoptosis by regulated PI3K/PBK/mTOR signaling pathway.

Dissuasion

Accumulated study demonstrated that the γ-secretase play an important regulated role in the process of Aβ plaques form in Alzheimer's disease (AD)[16]. Crenigacestat is a kind of γ-secretase inhibitors (GSIs), that has potent inhibition of γ-secretase activation in cancer[19]. In this study, we reported that Crenigacestat alleviated the cognitive deficits and neuropathology of AD mice by inhibiting the Notch signaling pathway, thereby reduce the form of Aβ plaques.

AD is a neurodegenerative disease, which lead to cognitive and neurological impairment[1]. γ-secretase is a multi-component protease complex that catalyzes the intramembranous cleavage of APP[12]. Extensive bibliography demonstrated that the capacity of γ-secretase inhibitors to prevent Aβ formation and aggregation both in human and in transgenic mouse models[3, 12, 16]. Crenigacestat is a kind of GSIs, which play an important role in ameliorating the progression of cancer by inhibited the decreased the produce of γ-secretase[19, 25]. γ-secretase was upregulated in AD, which leaded to the form of Aβ plaques[3]. However, the relationship between Crenigacestat and Aβ plaques from remains unclear in AD. Herein, in the present work, we investigate that the function and mechanism of Crenigacestat in AD transgenic mice (APP/PS1). Morris water maze (MWM) is a test of spatial learning and memory of rodents[20], and the severity of neurological deficits such as hemiparesis and motor coordination problems in rodents was detected by modified neurological severity scores (mNSS) is a test[21]. MWN test and mNSS score were using to detect the level of cognitive and neurological impairment. AD always company with cognitive and neurological impairment, Crenigacestat decreased the level of cognitive and neurological impairment. The beneficial effects of Crenigacestat on neurological protection suggest that Crenigacestat can be developed as a new therapy strategy for the treatment of AD.

Emerging evidences has shown that cerebral Aβ plaque burden may contribute to AD progression, which leaded to neuron apoptosis[7, 9]. Accumulating evidence has revealed that γ-secretase inhibitors inhibited the activity of γ-secretase, which decreased the level of cerebral Aβ plaque, ultimate alleviated cognitive deficits, and neuropathology[3]. Therefore, we hypothesized that crenigacestat might be effective by decreased the level of cerebral Aβ plaque in an AD mouse model. Expectedly, crenigacestat significantly decreased the cerebral insoluble Aβ plaque deposition, and decreased the secretion of soluble Aβ. In addition, crenigacestat treatment decreased the apoptosis level of brain tissue in AD mice. These results suggested crenigacestat might be a novel therapeutic medicine for decreased the level of Aβ in AD model mouse.
The age-related neurodegenerative disease, AD, is characterized by a robust growth of morphologically reactive microglia[21, 22]. In addition, reactive microglia are often associated with Aβ plaque deposits[22]. Microglia are the main producers of pro-inflammatory cytokines in the brain, neuro-inflammation is an important driver of AD[22], therefore, we investigated the pro-

inflammatory cytokines and reactive microglia. Consistent with previous study, pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 was up-regulated in APP/PS1 AD model mouse, and crenigacestat decreased the secretion of pro-inflammatory cytokines. In addition, knockdown of APP/PS1 promoted microglia activity, and crenigacestat suppressed the activity of microglia.

Notch signaling pathway play an important role in the developmental processes and glial cell activation[26]. Previous studies reported that Notch1 protein was increased in the brain of AD patient and Notch1 was deposited in Aβ plaque[27]. In addition, crenigacestat are inhibited the activity of γ-secretion by Notch signaling pathway in cancer[19]. However, whether crenigacestat alleviated cognitive deficits and neuropathology via Notch1 has never been explored in AD. The level of Notch1 in the brain of AD mice was increased suggested, and crenigacestat reduced the expression of Notch1, the results demonstrated that crenigacestat might be participated in AD by Notch signaling pathway[24]. Notch signaling pathway is associated with several signaling pathway and recent study shown that Notch signal pathway foster MTOR and PI3K signaling pathway[24]. In addition, our data shown that crenigacestat alleviated the expression of marker of MTOR and PI3K signaling pathway. In our study, we first found that crenigacestat could alleviated the cognitive deficits and neuropathology in AD by Notch signaling pathway.

In conclusion, our results suggested the evident effect of crenigacestat on suppressing the progression of AD in vivo. The mechanisms are included in inhibited the activation of Notch signaling pathway and suppressed the activetion of γ-secretion, Aβ plaque deposits. These results provide an important basis for a further exploration toward understanding the action mechanisms of crenigacestat.

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