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Temsirolimus promotes autophagic clearance of amyloid-β and provides protective effects in cellular and animal models of Alzheimer's disease

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ABSTRACT

Accumulation of amyloid-β peptides (Aβ) within brain is a major pathogenic hallmark of Alzheimer's disease (AD). Emerging evidence suggests that autophagy, an important intracellular catabolic process, is involved in Aβ clearance. Here, we investigated whether temsirolimus, a newly developed compound approved by Food and Drug Administration and European Medicines Agency for renal cell carcinoma treatment, would promote autophagic clearance of Aβ and thus provide protective effects in cellular and animal models of AD. HEK293 cells expressing the Swedish mutant of APP_{695} (HEK293-APP_{695}) were treated with vehicle or 100 nM temsirolimus for 24 h in the presence or absence of 3-methyladenine (5 mM) or Atg5-siRNA, and intracellular Aβ levels as well as autophagy biomarkers were measured. Meanwhile, APP/PS1 mice received intraperitoneal injection of temsirolimus (20 mg/kg) every 2 days for 60 days, and brain Aβ burden, autophagy biomarkers, cellular apoptosis in hippocampus, and spatial cognitive functions were assessed. Our results showed that temsirolimus enhanced Aβ clearance in HEK293-APP_{695} cells and in brain of APP/PS1 mice in an autophagy-dependent manner. Meanwhile, temsirolimus attenuated cellular apoptosis in hippocampus of APP/PS1 mice, which was accompanied by an improvement in spatial learning and memory abilities. In conclusion, our study provides the first evidence that temsirolimus promotes autophagic Aβ clearance and exerts protective effects in cellular and animal models of AD, suggesting that temsirolimus administration may represent a new therapeutic strategy for AD treatment. Meanwhile, these findings emphasize the notion that many therapeutic agents possess pleiotropic actions aside from their main applications.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder in humans, which is characterized by the formation of extracellular amyloid plaques containing aggregated amyloid-β peptides (Aβ) [1]. According to the amyloid hypothesis, Aβ plays a critical pathogenic role in AD, as it initiates a deleterious cascade in brain and ultimately leads to cognitive impairment [2]. Recently, several lines of evidence indicates that impaired Aβ clearance rather than its overproduction is the central event in AD progression, as the clearance rates for Aβ are declined in AD patients while its production rates stay unchanged when compared with those in healthy controls [3,4]. Hence, promotion of Aβ clearance has now been considered as a promising therapeutic strategy for AD.

As a highly conserved catabolic process, autophagy is responsible for the clearance of aggregate-prone proteins and damaged cytoplasmic organelles within cells [5]. Morphologically, autophagy is initiated when a phagophore is formed. The membrane of phagophore undergoes elongation and sequestrates aggregate-prone proteins as well as damaged cytoplasmic

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organelles into autophagosomes. Subsequently, autophagosomes fuse with lysosomes for content degradation [6]. In recent years, several studies indicated that autophagy was involved in the clearance of Aβ under the physiological condition, thus maintaining Aβ homeostasis in healthy brain [7–9]. However, emerging evidence suggested that the activity of autophagy was reduced in brain of AD patients and animal models, and the reduction in autophagic activity led to the accumulation of Aβ in brain and subsequently contributed to the pathogenesis of AD [10–12]. Therefore, it seems that activating autophagy represents a viable approach for Aβ clearance in brain, and considerable efforts have been made to identify safe and effective pharmacological inducer of autophagy for AD treatment [13,14].

As a newly developed compound, temsirolimus has recently been approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of renal cell carcinoma (RCC) [15,16]. Apart from its main applications, temsirolimus was recently found to provide beneficial effects via induction of autophagy in animal models of Huntington's disease and spinocerebellar ataxia type 3, two neurodegenerative diseases caused by accumulation of aberrant proteins within brain [17,18]. To date, the therapeutic potentials of temsirolimus in AD have not been determined thus far. Considering the above evidence, in the present study, we investigated whether temsirolimus would promote Aβ clearance via induction of autophagy and thus exert a protective effect using in vitro and in vivo models of AD.

2. Methods

2.1. Reagents

Temsirolimus and 3-methyladenine (3-MA) were purchased from Sigma–Aldrich. For in vitro studies, temsirolimus was dissolved in 100% ethanol and adjusted to the final concentration with culture medium. For in vivo studies, temsirolimus was prepared in 50 mg/mL 100% ethanol. On the day of injection, the drug was diluted in vehicle (5% Tween-80 and 5% polyethylene glycol 400) up to a final concentration (20 mg/kg).

2.2. Cell culture, drug treatment and siRNA transfection

HEK293 cells stably expressing the Swedish mutant of APP_{695} (HEK293-APP_{695}) were a generous gift from Dr. Jian-Quan Shi (Department of Neurology, Nanjing First Hospital, Nanjing Medical University, Nanjing, China). Cells were propagated to passage number 3, and then were treated with vehicle (ethanol) or temsirolimus (100 nM) in the presence or absence of 3-MA (5 mM) for 24 h. As demonstrated by Fig. S1A and S1B, 100 nM temsirolimus significantly enhanced autophagic activity whilst it did not markedly affect the viability of HEK293-APP_{695} cells. Hence, this dose was selected for the subsequent in vitro experiments. When indicated, vehicle or temsirolimus were added to medium at 48 h after transfection and incubated for another 24 h. For siRNA transfection, control siRNA (Cell Signaling Technology) and autophagy-related protein 5 (Atg5) siRNA (Cell Signaling Technology) were transfected into target cells by using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacture's protocol. The knockdown efficiency was determined at 48 h after transfection.

2.3. Animals and drug administration

Five-month-old male APP/PS1 (expressing Mo/$HuAPP_{695}$ and PS1dE9) transgenic mice and their age-matched Non-Tg mice were purchased from Model Animal Research Center of Nanjing University. They were housed in a standard animal room with a 12 h light/dark cycle and given free access to food and water. Animal Care and Management Committee of Qingdao Municipal Hospital approved the whole study protocol (permit No.QMHEC-130219). All animal experiments were conducted in accordance with Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were reported in accordance with the ARRIVE guidelines [19–21].

Mice were injected intraperitoneally with vehicle or temsirolimus (20 mg/kg) every 2 days for 60 days. The dose and route of administration for temsirolimus were chosen according to previous studies from Rubinsztein's group [17,18]. The frequency of temsirolimus administration was determined based on the results from the initial experiment, which showed that LC3-II, an autophagy biomarker, was significantly enhanced by a single dose of temsirolimus (20 mg/kg) and lasted for more than 48 h in APP/PS1 transgenic mice (Fig. S1C). During the course of treatment, we carefully monitored the general health of mice and did not observe any adverse effects or significant changes in their body weight (Fig. S2) or food intake (control group vs. temsirolimus group: 3.46 ± 0.29 vs. 3.34 ± 0.39 g/day, $P > 0.05$; data were obtained during last 10 days of treatment).

2.4. Morris water maze test

The Morris water maze test was conducted by technicians who were blinded to the experimental groups during last 6 days of treatment period as described previously [22,23]. The mice ($n = 12$ per group) were given 4 training trials per day for 5 consecutive days. The path length to the submerged platform was recorded, and the average time of 4 trials was calculated. If a mouse failed to find the platform within 60 s, it was picked up and placed on the platform for 15 s. Twenty-four hours after the last trial, mice were subjected to a probe test in which the platform was removed, and their swimming paths were recorded for 60 s by a computer-controlled system (Beijing Sunny Instruments Inc.).

2.5. Brain tissue preparation

At the end of treatment, mice were anesthetized and were handled as follows:

(1) For western blotting, enzyme-linked immunoabsorbent assay (ELISA), and colorimetric assay, mice ($n = 6$ per group) were perfused transcardially with 0.9% saline (pH 7.4) only. The brains were removed rapidly and stored in liquid nitrogen.
(2) For immunohistochemistry analysis and terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL), mice ($n = 6$ per group) were perfused transcardially with 0.9% saline (pH 7.4). The brains were removed and fixed in a solution containing 4% paraformaldehyde (PFA) in 0.9% saline (pH 7.4) at 4 °C.
(3) For transmission electron microscopic examination, mice were perfused transcardially with 0.9% saline (pH 7.4), followed by a fixative solution containing 4% PFA and 0.25% glutaraldehyde. The brains were removed and fixed in a solution containing 2% PFA and 2.5% glutaraldehyde at 4 °C.

2.6. Western blotting

Western blotting was performed by technicians who were blinded to the experimental groups. Cells and brain tissues were lysed in extraction buffer (Beyotime Inc.) containing complete protease inhibitor cocktail (Roche). Different samples with an equal amount of protein were separated on 8–12% SDS polyacrylamide gels, transferred to PVDF membranes, and then blocked with 5% bovine serum albumin (BSA) for 2 h. Membranes were incubated overnight at 4 °C with the primary antibodies (see Table S1), then