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Full Paper

Effects of ZSET1446/ST101 on Cognitive Deficits and Amyloid *β* Deposition in the Senescence Accelerated Prone Mouse Brain

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Abstract. The senescence accelerated prone mouse strain 8 (SAMP8) develops age-related deficits in learning and memory. Effects of the azaindolizinone derivative ZSET1446/ST101, a newly synthesized cognitive enhancer, on cognitive impairment and deposition of amyloid *β* (A*β*) were assessed in the SAMP8. ZSET1446 was administered in drinking water at estimated doses of 0.002, 0.01, and 0.1 mg/kg per day from the age of 8 months. The SAMP8 at the age of 8 months showed cognitive impairment in a novel object recognition task compared with young SAMP8 at the age of 8 weeks. Further, grading scores were gradually increased from 9 to 12 months and A*β*-like immunoreactivity in the hippocampus was increased at the age of 10 months. ZSET1446 ameliorated cognitive deficits of SAMP8 after 4, 8, 12, and 16 weeks of treatment in a novel object recognition test. ZSET1446 also reduced grading scores of SAMP8 after 16 weeks of treatment. Further, 8-week treatment of ZSET1446 significantly reduced the total number of A*β*-positive granules in the hippocampus. These results suggest that ZSET1446 shows ameliorating effects on SAMP8 partly due to the suppression of an increase of A*β*-deposition in the hippocampus.

Keywords: ZSET1446, senescence accelerated prone mouse strain 8 (SAMP8), object recognition test, amyloid *β*, grading score

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by amyloid-*β* peptide (A*β*) deposition. A*β* is generated from a larger protein, the amyloid precursor protein (APP), and aggregates to form soluble oligomers, insoluble fibrils, and ultimately Alzheimer's plaques. The deposition of A*β* in the brain is thought to have a causal role in synaptic dysfunction, synaptic loss, neuronal death, and consequently, cognitive dysfunction (1). As the A*β* cascade hypothesis regarding AD pathogenesis is generally accepted, drugs reducing A*β* levels appear promising as therapeutic modalities (2). However, to date no drugs that reduce A*β* levels are clinically utilized.

Current mouse models of AD are mainly restricted to the AD-related pathology caused by transgenically-made specific mutations present in an early-onset familial AD, which accounts for only < 5% of total AD cases. On the other hand, the senescence accelerated prone mouse strain 8 (SAMP8) has been established through phenotypic selection from a common genetic pool of AKR/J strain of mice (3). The nucleotide sequence of APP in the hippocampus of SAMP8 does not have mutations similar to those that have been reported in human familial AD (4). Moreover, the SAMP8 PS1 cDNA sequence is identical to that of normal mice (5). The SAMP8 is characterized by an earlier onset of deficits in learning and memory than the normally aging mice, SAMR, in several tasks such as active and passive avoidance, T-maze, and water maze tasks (6 – 8). Further, immunocytochemical studies have shown that SAMP8 has an age-related increase in A*β*-like deposits in the hippocampus using an antibody against $A\beta_{1-40}$ and $A\beta_{1-42}$ (9 – 11). In addition, increases in hyperphosphorylated tau and cyclin-dependent kinase 5 (cdk5) expression and activation have been shown in SAMP8 (12). A major histopathological hallmark of AD is the presence of amyloid deposits and neurofibrillary tangles (NFT) in the brain, the latter of which are formed from abnormally hyperphosphorylated tau, and cdk5 is the putative kinase that

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phosphorylates the tau. Therefore, the SAMP8 strain may provide an excellent model to study the neurodegenerative changes associated with sporadic AD (13, 14).

ZSET1446/ST101, spiro[imidazo-[1,2-*a*]pyridine-3,2-indan]-2(3*H*)-one, has been reported to exert ameliorating effects on pharmacologically and operatively induced cognitive impairment in various animal models: by scopolamine or dizocilpine in the passive avoidance task (15); by a single intracerebroventricular injection of Aβ_{25-35} or nucleus basalis magnocellularis lesions by ibotenic acid in the passive avoidance task (16); by Aβ_{1-40} in the Y-maze, water maze, and passive avoidance tasks at the dose range of 0.01 – 1 mg/kg (17); by methamphetamine (18); and by olfactory bulbectomy (19). Recently, it has been reported that ZSET1446 induces cleavage of APP protein at a novel site, generating a 17-kDa C-terminal fragment, and robustly reduces brain Aβ in 3xTg AD mice and nonhuman primate (20).

In the present study, we examined the ameliorating effects of ZSET1446 on the cognitive deficits in a novel object recognition task in the sporadic AD model, SAMP8, and on an increase in grading scores and Aβ-like immunoreactivity in SAMP8.

Materials and Methods

Animals

Male SAMP8 strain (Japan SLC, Shizuoka), 8 weeks and 8 months of age, were used in the experiment. They were housed in a cage in a group of 3 or 4 mice, in a room maintained at around 22°C with a 12-h light/dark cycle. Food and water were available ad libitum. All animal care and treatments were conducted in accordance with the Guideline for the Care and Use of Laboratory Animals established at the Central Research Laboratory, Zenyaku Kogyo Co., Ltd.

Drug treatments

ZSET1446, synthesized in our Department of Organic Synthesis of Zenyaku Kogyo Co., Ltd., was added to drinking water. ZSET1446 solutions were made in tap water. The concentration of ZSET1446 was 0.01, 0.1, and 1 mg/L, to provide an estimated dose of 0.002, 0.01, or 0.1 mg/kg per day (Tables 1 and 2). Dose levels were estimated from the average daily fluid intake during the test period.

Grading score of senescence

Grading scores were designated to represent changes in the behavior and appearance of the mice that were considered to be associated with the aging process (3). Briefly, the 11 categories including reactivity, passivity, glossiness and coarseness of coat, hair loss, ulcers, periophthalamic lesions, cataract, corneal ulcers, corneal opacity, and lordokyphosis were measured. Each category has five grades of intensity of characteristics of changes. Each mouse was examined from 4 to16 weeks after the start of treatment with ZSET1446.

Novel object recognition test

The novel object recognition task was carried out from 4 to 16 weeks after the start of drug treatment. This model

Table 1. Estimated doses of ZSET1446 used in the experiment of object recognition task

Administration	Concentration (mg/L)	Mean daily intake (mL/kg per day)	Estimated dose[a] (mg/kg per day)
Tap water	—	174.0	—
ZSET1446	0.01	154.1	0.002
ZSET1446	0.1	143.5	0.01
ZSET1446	1	144.9	0.1

[a]Dose levels were estimated from the average daily fluid intake during the test period.

Table 2. Estimated doses of ZSET1446 in the experiment of Aβ deposition

Administration	Concentration (mg/L)	Mean daily intake (mL/kg per day)	Estimated dose[a] (mg/kg per day)
Tap water	—	173.8	—
ZSET1446	0.01	237.9	0.002
ZSET1446	0.1	143.3	0.01
ZSET1446	1	143.6	0.1

[a]Dose levels were estimated from the average daily fluid intake during the test period.

is based on the behavior of rodents which prefer a novel object to a familiar object and spend more time in exploring the novel object (21). The experimental apparatus consisted of a Plexiglas open-field box (25 × 41 × 17 high cm), the floor of which was covered with sawdust. The apparatus was located in a sound-attenuated room. The procedure for the novel object recognition task consisted of three different sessions: habituation, training, and retention sessions. Each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects (day 1). During the training session, two different novel objects were symmetrically fixed to the floor in the box, and each animal was allowed to explore in the box for 10 min (day 2). These objects were different in shape but similar in size. The mice were considered to be exploring the object when the head of the mouse was facing the object or the mouse was touching or sniffing the object. The time exploring each object was recorded. After training, mice were immediately returned to their home cages. In the training session, we also measured locomotor activity for a period of 10 min, using an Animex Auto (Muromachi Kikai Co., Ltd., Tokyo) placed under the cage. Twenty-four hours after the training session (day 3, retention session), one of the familiar objects used during training was replaced by a novel object. The animals were then allowed to explore freely for 5 min and the time spent exploring each object was recorded. A discrimination index, a ratio of the difference in time spent for exploring the novel and familiar object to the total time spent for exploring both objects, was used as a parameter of cognitive function.

Aβ-like immunoreactivity

Mice were treated with ZSET1446 at estimated doses of 0.002, 0.01, and 0.1 mg/kg per day from 8 months of age. Eight weeks later, mice were sacrificed. The brains were fixed with Methacarn solution (methanol : chloroform : acetic acid = 6:3:1), embedded in paraffin, and cut into 8-μm sections. The sections were immunostained by the streptavidin–biotin method with a commercial kit (VECTASTAIN ABC Kit; Vector Laboratories, Inc., Burlingame, CA, USA). After 1 h preincubation at room temperature in 10% normal goat serum in phosphate-buffered saline (PBS) and 0.3% Triton X-100, the slides were incubated overnight at 4°C with the Aβ-specific antibody (Immuno-Biological Laboratories Co., Ltd., Takasaki), which recognizes both Aβ_{1-40} and Aβ_{1-42}, at a dilution of 1:10 with PBS. On the following day, the sections were washed in PBS and incubated for 1.5 h at room temperature with biotinylated anti-rabbit IgG. After a further PBS wash, the sections were incubated with the peroxidase-conjugated streptavidin for 1.5 h at room temperature. The immunoreactions were visualized with 3,3-diaminobenzidine. Then the sections were washed in distilled water for 5 min, dehydrated through an ethanol series, cleared in xylene, and mounted.

For microscopic analysis, Aβ-like granules were counted under a 10 × objective through a calibrated eyepiece grid. The dark brown deposit in the cytoplasm was identified as the Aβ-immunoreactive granule in the hippocampus. One section at the position approximately 2.0-mm posterior from the bregma was analyzed in each animal.

Statistical analyses

Results were each expressed as the mean ± S.E.M. Data were analyzed by Dunnett's multiple comparison test. The criterion for significance was $P < 0.05$ in all statistical evaluations.

Results

Effect of ZSET1446 on grading score

Grading scores that reflect senescence are shown in Fig. 1. Increasing senescence scores with aging were found in the control group. However, the gradual increase in grading scores was completely suppressed by the treatment of ZSET1446 at the estimated dose of 0.1 mg/kg per day (Fig. 1). The grading scores of mice treated with ZSET1446 at estimated doses of 0.002, 0.01, and 0.1 mg/kg per day were significantly lower ($P < 0.05$) than the control at 16 weeks, but not from 4 to 12 weeks, after the start of the experiment (Fig. 1).

Effect of ZSET1446 on cognitive impairment in the object recognition task

We initially made comparisons between 8-week-old and 8-month-old SAMP8 since it is well known that cognitive function of SAMP8 is impaired by aging

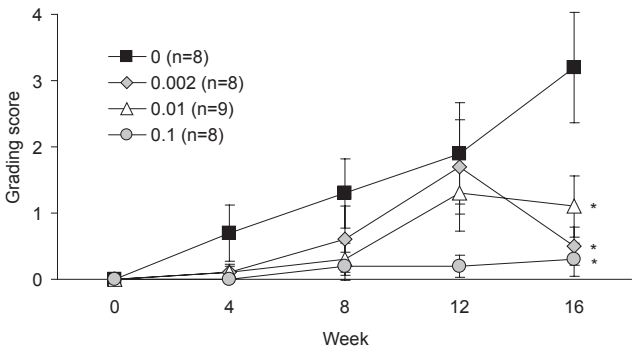

Fig. 1. Effects of oral administration of ZSET1446 on age-related increase in grading score every 4 weeks after the start of the treatment in SAMP8. Vertical bars show S.E.M. *$P < 0.05$, compared with the control (Dunnett's multiple comparison test).

(6 – 8). In the training session, there were no significant differences in total exploring time between 8-week-old and 8-month-old SAMP8. The total exploration time was significantly increased by treatment of ZSET1446 at estimated doses of 0.002 mg/kg per day at 4 ($P < 0.01$), 8 ($P < 0.01$), and 16 ($P < 0.05$) weeks, 0.01 mg/kg per day at 8 ($P < 0.05$) and 12 ($P < 0.05$) weeks, and 0.1 mg/kg per day at 4 ($P < 0.05$) weeks after the start of the experiment compared with SAMP8 drinking tap water (Fig. 2A). However, there were no significant differences in locomotor activity counts in the training sessions among all groups 4 to 16 weeks after the start of the treatment (Fig. 2B). The retention session was carried out 24 h after the training session. The 8-month-old SAMP8 showed a significant decrease in discrimination index compared with the young SAMP8 (Fig. 3, $P < 0.05$). Thus, 8-month-old SAMP8 showed cognitive impairment in a novel object recognition test compared to young SAMP8. ZSET1446 significantly increased the discrimination index at estimated doses of 0.002 mg/kg per day at 8 ($P < 0.01$) and 16 ($P < 0.05$) weeks, 0.01 mg/kg per day at 4 ($P < 0.05$), 8 ($P < 0.01$), 12 ($P < 0.01$), and 16 ($P < 0.01$) weeks and 0.1 mg/kg per day at 4 ($P < 0.05$), 8 ($P < 0.01$), 12 ($P < 0.05$), and 16 ($P < 0.01$) weeks after the start of treatment (Fig. 3). Thus, ZSET1446 ameliorated cognitive deficit of aged SAMP8 in a novel object recognition test.

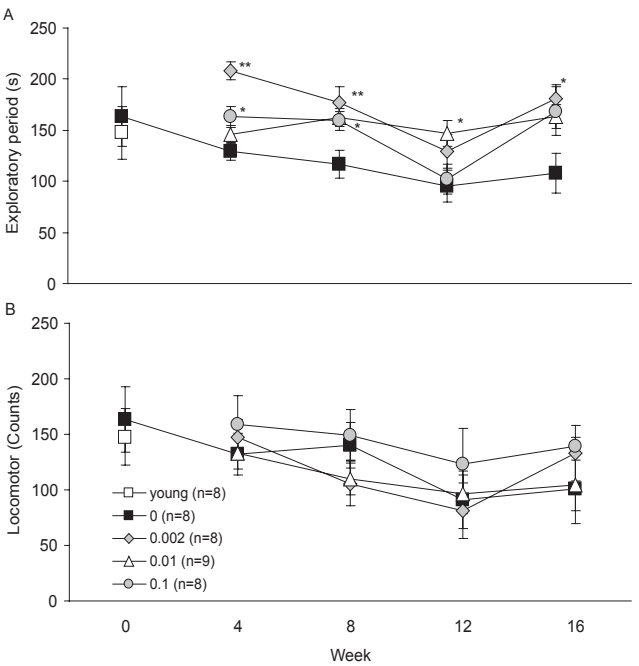

Fig. 2. Effects of oral administration of ZSET1446 on performance in a novel object recognition task every 4 weeks after the start of the treatment in SAMP8. Each symbol represents the mean total exploration time (A) and the mean locomotor activity counts (B) in the training sessions and vertical bars show S.E.M. $*P < 0.05$, $**P < 0.01$, compared with the control (Dunnett's multiple comparison test).

Effect of ZSET1446 on Aβ-like immunoreactivity

The hippocampi from 10-month-old SAMP8 that had been treated with ZSET1446 in drinking water for 8 weeks were analyzed by immunochemistry. The granular structures of Aβ-like immunoreactivity were extensively immunostained in the hippocampus of SAMP8 at the age of 10 months (Fig. 4A). However, treatment of the SAMP8 mice with ZSET1446 at estimated doses of 0.002, 0.01, and 0.1 mg/kg per day for 8 weeks significantly reduced the total number of the Aβ-positive granules in the hippocampus compared with the control group ($P < 0.01$, Fig. 4: B, C).

Discussion

In this study, the SAMP8 treated with ZSET1446 at all doses showed lower grading scores than those of control mice at 16 weeks after the start of the experiment. These results suggest that ZSET1446-treated SAMP8 were more youthful in appearance than the control. However, the SAMP8 treated with ZSET1446 showed no significant effect at 4 to 12 weeks. It is likely that the effect of

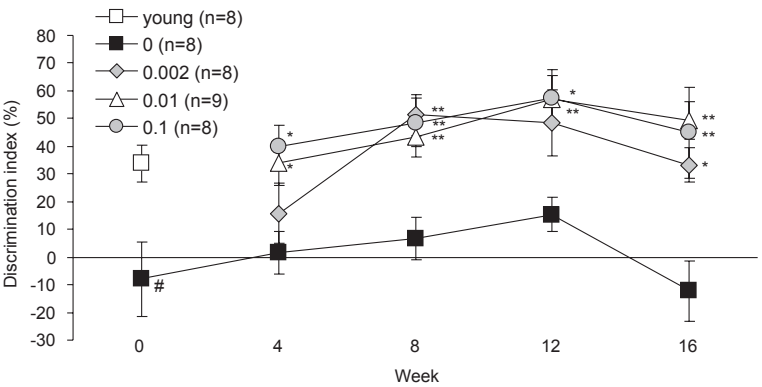

Fig. 3. Effects of ZSET1446 on the discrimination index in the retention session of a novel object recognition test. Vertical bars show S.E.M. $^{\#}P < 0.05$, compared with the control of 8-week-old SAMP8. $*P < 0.05$, $**P < 0.01$, compared with the control (Dunnett's multiple comparison test).

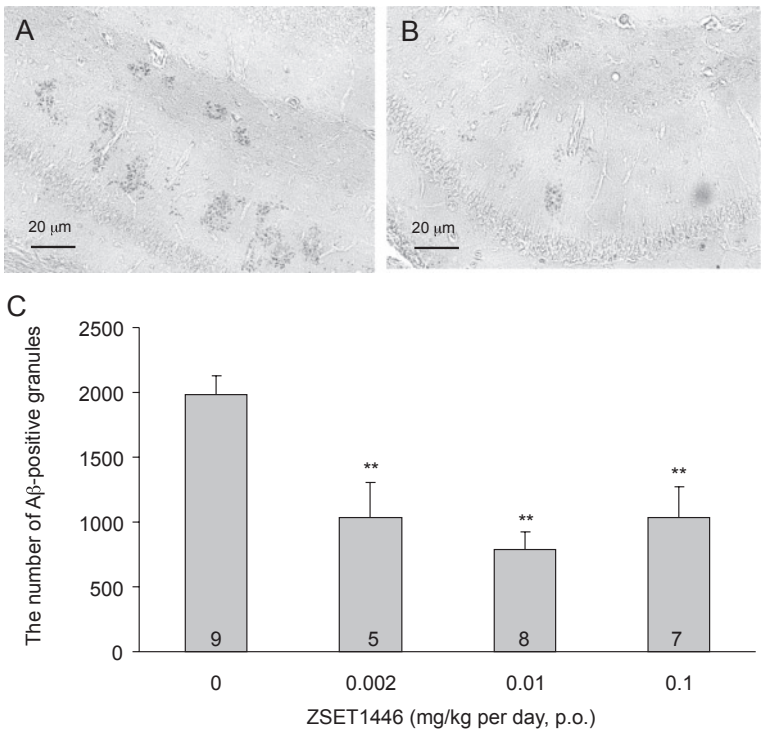

Fig. 4. Suppressive effects of ZSET1446 on *Aβ*-like deposits in the hippocampus of SAMP8. A*β*-positive granules were deposited in control SAMP8 (A), whereas they were markedly reduced in the SAMP8 treated with ZSET1446 at the estimated dose of 0.01 mg/kg per day for 8 weeks (B). C: Each column represents the mean number of A*β*-immunoreactive granules and vertical bars show S.E.M. The number within column indicates the number of animals. $^{**}P < 0.01$, compared with SAMP8 treated with vehicle.

ZSET1446 can not be detectable because of insufficiently increased grading scores of the control mice. Aging of SAMP8 was associated with the reduction in learning and memory ability in SAMP8 as well as the age-related increase in grading score. In the present study, oral administration of ZSET1446 at estimated doses of 0.002, 0.01, and 0.1 mg/kg per day ameliorated cognitive impairment of SAMP8 in the novel object recognition task. These ameliorative effects of ZSET1446 are not due to the changes in motor functions because locomotor activities did not differ between control and ZSET1446-treated mice. However, the changes in motivational functions may partly contribute to the ameliorative effects of ZSET1446 on novel object recognition performance because increased total exploratory time in the training sessions was observed at several time points, although not all time points, after treatment with ZSET1446. There are some fluctuations of the exploratory time in each training session. However the discrimination indexes of the ZSET1446-treated mice were consistently higher than that of the control mice. Therefore, increase in the discrimination index by ZSET1446 is thought to be caused mainly by the amelioration of learning. In addition, treatment with ZSET1446 at the estimated dose of 0.002 mg/kg per day for 4 weeks did not significantly affect the discrimination index. These results suggest that more than 4 weeks of treatment with this dose of ZSET1446 is required for improving cognitive function of SAMP8.

Central cholinergic dysfunction has been observed in the brain of SAMP8. [^{3}H]QNB binding [muscarinic acetylcholine (ACh) receptors] and [^{3}H]pirenzepine binding (M_1 ACh receptors) are decreased in the hippocampus of SAMP8 at the ages of 9 and 12 months, respectively (22, 23). Additionally, choline acetyltransferase (ChAT) activity is decreased in the hippocampus of SAMP8 at the age of 12 months (24), and the Pearson correlation test shows that cognitive function is positively correlated with the level of ChAT in the cerebral cortex, hippocampus, and forebrain of SAMP8 (25). Further, high potassium–stimulated ACh release of SAMP8 is lower than that of age-matched SAMR1 at the ages of 9 and 12 months (26). Therefore, these cholinergic dysfunctions may be related to the impairment of learning and memory in SAMP8.

On the other hand, we have previously demonstrated that administration of a single high oral dose of ZSET1446 increases the extracellular ACh in the hippocampus (15) and causes an increase in nicotine-stimulated ACh release in the hippocampus (17). Moreover, we have demonstrated that decrease in both nicotine-stimulated ACh release and ChAT activity is recovered by repeated administration of ZSET1446 in the hippocampus of A*β*-infused rats (17). Since ZSET1446 has no inhibitory action on AChE activity (17), ZSET1446 might either directly or indirectly enhance the release of ACh to increase extracellular ACh and thereby to be effective in memory tests. Thus, it is likely that ameliorating effects

of ZSET1446 on learning impairment in SAMP8 result at least in part from the stimulation of the cholinergic neuronal system in the hippocampus.

In the present study, granular structures of Aβ-like immunoreactivity were extensively immunostained in the hippocampus of SAMP8 at the age of 10 months. Although the Aβ deposits in SAMP8 could not be the same as that in the brains of AD patients, the Aβ-immunoreactive granules in the hippocampus of SAMP8 may however pathologically relate to the Aβ deposits observed in humans. Transgenic mice that express high level of the human APP gene (27) or mutated APP genes (28) have been found to develop cognitive deficits progressively with AD-like neuropathology, such as formation of plaques and deposition of Aβ. On the other hand, SAMP8 spontaneously develops Aβ-immunoreactive deposition (9 – 11). It has been reported that SAMP8 exhibits age-related deficits in learning and memory (6, 7). Therefore, the SAMP8, which spontaneously exhibits both amyloid-like deposition and cognitive deficits in an age-related manner, could be an animal model for assessing the therapeutic potential of the compounds aimed at preventing the development of AD. The SAMP8 may substantially reflect the pathology of at least some type of AD, which stands on a genetically heterogeneous background.

In the present study, ZSET1446 treatment markedly reduced the number of Aβ-positive granular structures in the hippocampus of the SAMP8. These results are consistent with the findings that ZSET1446 reduces the level of Aβ in the brain by inducing an alternate pathway of APP cleavage (20). Recently, Moriguchi et al. (29) reported that a T-type voltage-gated calcium channel inhibitor, mibefradil, completely blocks ZSET1446-induced enhancement of long-term potentiation in the cortex and that ZSET1446 stimulates the voltage-gated Ca^{2+} current in neuro2A cells with over-expressed $Ca_V3.1$, a T-type voltage gated calcium channel. It has been also reported T-type calcium channel and the K_V4 complex ($K_V4.2$-KChIP3-DPP10c) form a signaling complex that allows calcium-dependent regulation of K_V4 inactivation (30). KChIP3 is also known as calsenilin which regulates γ-secretase activity (31). Therefore, it is possible that ZSET1446 regulates γ-secretase activity by calsenilin through T-type calcium channel to reduce Aβ in the brain. Further study is required to clarify the possible effect of ZSET1446 on calsenilin through T-type voltage gated calcium channels.

It has been reported that cognitive deficits observed in aged SAMP8 mice were significantly ameliorated by down-regulating the expression of APP using antisense oligonucleotide specific to APP mRNA (32). Moreover, anti-Aβ antibodies administered intracerebrally or intravenously to the SAMP8 mice improve cognitive function (33, 34). These results suggest that decrease in Aβ level in the brain is associated with amelioration of cognitive function in SAMP8 mice. Therefore, ameliorating effects of ZSET1446 on cognitive deficits in SAMP8, as well as the above-mentioned stimulating effects on cholinergic system, may arise from the reduction of Aβ deposition in the hippocampus of SAMP8.

In conclusion, ZSET1446 alleviated the senescent appearance and cognitive impairment of the aged SAMP8 mouse, a putative AD model, probably via stimulation of cholinergic neuronal systems and/or inhibition against Aβ deposition. These results, together with the previous findings in various AD models, suggest that ZSET1446 is promising as a therapeutic drug for the treatment of AD.

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