

Full-length article

Protective effects of compound FLZ on β -amyloid peptide-(25–35)-induced mouse hippocampal injury and learning and memory impairment¹Fang FANG, Geng-tao LIU²*Department of Pharmacology, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, China***Key words**FLZ; $A\beta_{25-35}$; intracerebroventricular injection; hippocampus; acetylcholinesterase; Bax; Bcl-2; malondialdehyde¹Project supported by a grant from the Major State Basic Research and Development Program of China (973 Program; No G2000057010).²Correspondence to Prof Geng-tao LIU.
Phn 86-10-6316-5178.
Fax 86-10-6301-7757.
E-mail liugt@imm.ac.cn

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Abstract

Aim: To study the protective effects of compound FLZ, a novel synthetic analogue of natural squamosamide, on learning and memory impairment and lesions of the hippocampus caused by icv injection of β -amyloid_{25–35} ($A\beta_{25-35}$) in mice. **Methods:** Mice were icv injected with the $A\beta_{25-35}$ (15 nmol/mouse), and then treated with oral administration of 75 mg/kg or 150 mg/kg of FLZ once daily for 16 consecutive days. The impairment of learning and memory in mice were tested using step-down test and Morris water maze test. The content of malondialdehyde (MDA) and the expressions of acetylcholinesterase (AChE), Bax, and Bcl-2 in the CA1 region of the mouse hippocampus were measured by biochemical and immunohistochemical analysis, respectively. The pathological damages of hippocampus were observed using a microscope. **Results:** FLZ (75 mg/kg, 150 mg/kg) significantly attenuated $A\beta_{25-35}$ -induced impairment of learning and memory in the step-down test and Morris water maze test. FLZ also reduced pathological damages to the hippocampus induced by $A\beta_{25-35}$. Furthermore, FLZ prevented the increase of AChE and Bax, and the decrease of Bcl-2 immunoreactive cells in the CA1 region of the hippocampus, and reduced the increase of MDA content in the hippocampus in mice injected with $A\beta_{25-35}$. **Conclusion:** FLZ has protective action against the impairment of learning and memory and pathological damage to the hippocampus induced by icv injection of $A\beta_{25-35}$ in mice.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that is clinically characterized by progressive memory loss and other cognitive impairments. The neuropathological hallmarks of AD include the formation of senile plaque of beta-amyloid ($A\beta$), neurofibrillary tangles, and synapse and neuron loss in several areas (hippocampus, cortex, amygdale, and others) of the brain^[1]. $A\beta$ has been thought to be a critical factor in the pathogenesis of AD. Many studies have found that the accumulation of $A\beta$ in the brain is associated with progressive neuronal death and cognitive deficits^[2–4]. Therefore, preventing the neurotoxicity induced by $A\beta$ might be an optimal strategy for treatment of AD. $A\beta_{25-35}$ is considered the shorter toxic fragment exerting neurotoxic effects

similar to those produced by $A\beta_{1-40/42}$, such as learning and memory impairment, neuronal apoptosis, cholinergic dysfunction, and oxidative stress^[5,6], thus $A\beta_{25-35}$ is usually used to establish the AD model for study of the neurotoxic properties of $A\beta$ and drug screening.

Because memory loss and cognitive dysfunction are the main clinical symptoms of AD patients, any therapy of AD requires identification of the factors that can confer protection against learning and memory impairment. Compound FLZ (Figure 1) is a novel synthetic cyclic analogue of natural squamosamide from a Chinese herb^[7]. Our previous studies have demonstrated that compound FLZ has a strong antioxidant property, and that FLZ protected against the damage and apoptosis of primary cultured rat brain neurons and PC12 cells exposed to hydrogen peroxide, glutamate,

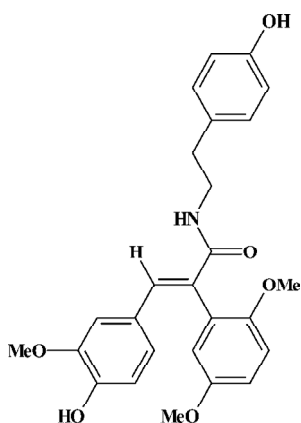


Figure 1. Chemical structure of FLZ.

N-methyl-*D*-aspartate (NMDA), dopamine, MPP⁺, and ischemia-reoxygenation. FLZ also improved abnormal behavior due to the functional disturbance of dopaminergic and cholinergic neurons in mice, indicating that FLZ possesses a neuroprotective property^[8]. It is very interesting to study whether FLZ can attenuate learning and memory impairment, and pathological and biochemical damages in the hippocampus induced by icv injection of A β ₂₅₋₃₅ in mice.

Materials and methods

Reagents Compound FLZ was kindly provided by Professor Xiao-tian LIANG in the Department of Pharmaceutical Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences. FLZ, a white powder with 99% purity, was suspended in 0.5% (*w/v*) sodium carboxymethyl cellulose (CMC-Na) for oral administration. Tacrine (Sigma Chemical, St Louis, MO, USA) was dissolved in saline. A β ₂₅₋₃₅ (Sigma) was dissolved in sterile double-distilled water at a concentration of 2 g/L, and incubated at 37 °C for 4 d for aggregation, and then stored at -20 °C for use^[9]. Anti-acetylcholinesterase (AChE) polyclonal antibody was purchased from Boster Biotechnology (Boster, Wuhan, China). Bcl-2 and Bax polyclonal antibodies, second antibody and diaminobenzidine tetrahydrochloride (DAB) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other reagents were of analytical grade from Beijing Chemical Company (Beijing, China).

Animals Male ICR mice weighing 23–27 g were obtained from the Center of Experimental Animals, Chinese Academy of Medical Sciences (Grade II, Certificate No SCXKJing2004-0001). The mice were housed 5 or 6 per cage in a thermoregulated environment (23±1 °C, 50%±5% humidity) with free access to food and water, under a 12 h light/dark cycle. All animal experiments followed the instructions of the Commit-

tee for Care and Welfare of Laboratory Animals in Chinese Academy of Medical Sciences and Peking Union Medical College.

A β ₂₅₋₃₅ injection and drug treatment The icv injection of A β ₂₅₋₃₅ was performed as described in a previous study^[6]. Mice were lightly anesthetized with ether. The aged A β ₂₅₋₃₅ or saline at a volume of 7.5 μ L was gradually injected into the right ventricle by a 28-gauge stainless-steel needle, with the following stereotaxic coordinates (in mm) from the bregma: A: -0.22, L: 1.0, V: 2.5. The injection point had already been confirmed at ventricle by injection of Indian ink in stead of A β ₂₅₋₃₅ peptide in preliminary experiments. The day after icv injection, mice were randomly divided into groups including: control, A β ₂₅₋₃₅ model, FLZ (75 mg/kg, 150 mg/kg), and tacrine (15 mg/kg). The FLZ, tacrine and vehicle (0.5% CMC-Na) were administered by gavage to mice once a day for 16 d. To observe the memory and learning function of mice, the step-down test was started on d 8 and the Morris water maze test on d 11 after the injection of A β ₂₅₋₃₅. The learning and memory tests were carried out between 9:00 AM and 18:00 PM.

Step-down test Amnesia in mice was examined through the step-down test on d 8 after the A β ₂₅₋₃₅ peptide injection. The apparatus (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China) was a transparent acrylic cage. An insulated platform was fixed in the center of the grid floor. During the training, each mouse was kept in the cage for 3 min to adapt to the environment before electricity was delivered to the grid floor for 5 min. The retention test was carried out 24 h after the above training test. The grid floor was delivered with 36 v electricity, individual mice were placed on the insulated platform and the step-down latency and the number of errors within 5 min was recorded.

Morris water maze test The apparatus of Morris water maze (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China) test is a white circular pool, 120 cm diameter and 40 cm deep, randomly divided into 4 quadrants. Milk powder was put into the pool to render the water opaque. The water temperature was maintained at 23±1 °C. A transparent Plexiglas platform was placed 2 cm below the water surface in the middle of one quadrant. The position of the platform was unchanged during the training trials. Two training trials per day were conducted for 5 consecutive days from d 11 after the injection of A β ₂₅₋₃₅. In each trial, a mouse was placed in the water faced to the pool wall at one starting position. The latency to find the platform was recorded up to 2 min. Mice that found the platform were allowed to remain on the platform for 30 s, and were then returned to the home cage. If the mouse could not find the platform within 2 min, it was gently guided to find the platform by the

experimenter, and allowed to remain on the platform for 30 s, and the latency was recorded for 2 min. Data of each mouse behavior were collected by a video camera linked to a computer through an image analyzer. The total sum of latency in two trials of each mouse was counted as the individual result of a mouse per day. The mean latency was calculated by all tested mice per group per day.

At the end of the training period, mice were tested on a spatial probe trial in which the platform was removed, and they were allowed to swim freely for 2 min. The latency of the location of first crossing the platform and the number of crossings of the platform were recorded.

Malondialdehyde (MDA) measurement After completion of the Morris water maze behavior observation, 9–10 mice in every group were killed by decapitation. The hippocampus of the brain was rapidly removed and placed on ice. The dissected tissue was weighed and homogenized with cold 0.9% physiological saline. MDA content was determined by the thiobarbituric acid method. 1,1,3,3-tetra-methoxypropane was used as a standard^[10].

Pathology examination of brain tissues After completion of the above behavior tests, 4 mice in every group were anesthetized with pentobarbital sodium (50 mg/kg, ip), and the brains were transcardially perfused with phosphate buffer solution (PBS, pH 7.4), followed by 4% paraformaldehyde in PBS (pH 7.4). The brains were removed and kept overnight in PBS containing 4% paraformaldehyde at 4 °C, and embedded in paraffin. Sections of 5- μ m thickness were stained with hematoxylin and eosin. The lesions of brain tissues were observed with light microscope (NIKON E600, Japan) and the images were collected by image analysis system (Image Pro-Plus 7200, America, SONY 3CCD, Japan).

Immunohistochemistry assay of AChE, Bcl-2, and Bax After the brains were fixed as above, they were removed and kept overnight in PBS containing 20%–30% sucrose and 4% paraformaldehyde at 4 °C. Coronal sections of 35- μ m thickness were cut using a cryostat at the hippocampus. Immu-

nohistochemistry was performed as described in a previous study^[11–13]. The primary antibodies were AChE (1:500), Bcl-2, and Bax (1:200). Immunostaining was visualized by the peroxidase method with a biotinylated anti-rabbit secondary antibody and diaminobenzidine oxidation (ABC kit, Santa Cruz). The primary antibody was replaced with normal serum in negative control.

Four mice were taken from each group for quantitative immunohistochemistry. One in every four sections was taken from a continuous series of sections prepared from hippocampal tissue. Six sections were selected in each mouse, so 24 sections of each group were read under a $\times 10$ objective, and the number of positively stained cells in each group was counted. The mean of the number of positively stained cells was calculated from 24 sections of each group^[11].

Statistical analysis Data are expressed as mean \pm SEM. Statistical analysis was performed by one-way ANOVA followed by least significant difference test. In all tests, $P < 0.05$ was considered as statistically significant.

Results

Effect of FLZ on the learning and memory impairment induced by icv injection of $A\beta_{25-35}$ in mice In the step-down test, the icv injection of aged $A\beta_{25-35}$ (15 nmol/mouse) induced a significant decrease of the avoidance latency and increase of the number of errors, and also the step-down percentage of mice from the platform increased significantly ($P < 0.05$). Oral administration of FLZ (75 mg/kg, 150 mg/kg) and tacrine (15 mg/kg) significantly improved the above impairments of learning and memory induced by $A\beta_{25-35}$ in mice ($P < 0.05$, Table 1). The latency of FLZ treated groups was restored to near that of the normal control group. The number of errors and step-down percentage were also improved markedly.

Furthermore, the Morris water maze was used to test the spatial learning and memory of each mouse from d 11 till d 16

Table 1. Effect of compound FLZ on learning and memory impairment of mice induced by the icv injection of aged $A\beta_{25-35}$ in step-down test. Mean \pm SEM. ^b $P < 0.05$ vs control. ^e $P < 0.05$, ^f $P < 0.01$ vs $A\beta_{25-35}$ model group.

Group	n	Latency (s)	Number of errors	Step-down percentage (%)
Control	16	260.8 \pm 19.8	0.4 \pm 0.1	37.5
$A\beta_{25-35}$ model	15	195.6 \pm 27.6 ^b	1.0 \pm 0.3 ^b	66.7 ^b
FLZ (75 mg/kg)+ $A\beta_{25-35}$	16	258.7 \pm 21.4	0.2 \pm 0.1 ^f	25.0 ^e
FLZ (150 mg/kg)+ $A\beta_{25-35}$	15	268.1 \pm 16.3 ^e	0.4 \pm 0.2 ^e	26.7 ^e
Tacrine (15 mg/kg)+ $A\beta_{25-35}$	16	243.5 \pm 27.9	0.3 \pm 0.1 ^f	25.0 ^e

after the icv injection of $A\beta_{25-35}$. There was no difference of the mean latency to find the platform for mice between all groups on the first training day (d 1 after the icv injection of $A\beta_{25-35}$). On the second day, the latency of mice in β -amyloid injected model group was longer than that of the control group, and still significantly longer on d 4 and d 5 ($P<0.05$). In comparison with $A\beta_{25-35}$ model group, the latency of mice treated with FLZ (75 mg/kg, 150 mg/kg) significantly shortened on d 4 and d 5 ($P<0.05$). The latency of mice treated with tacrine also significantly decreased on d 5 ($P<0.05$, Figure 2).

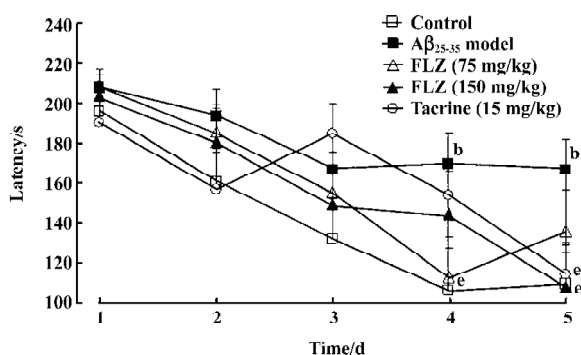


Figure 2. Effect of FLZ on spatial learning and memory impairment in mice by injection of aged $A\beta_{25-35}$ in Morris water maze test. The mean latency represents mice of every group trained to find the platform that was placed in a fixed location in the pool. The latency shown in the results is the sum of two trials per day per group. $n=15-16$. Mean \pm SEM. ^b $P<0.05$ vs control. ^e $P<0.05$ vs $A\beta_{25-35}$ model group.

The spatial probe trial was performed to examine whether the mice had remembered the position of the platform. In comparison with the control group, the latency of location for first crossing the platform for mice in the $A\beta_{25-35}$ model group increased ($P<0.05$), while the number of crossings of the platform for mice decreased ($P<0.05$). The treatment of mice with FLZ (75 mg/kg, 150 mg/kg) and tacrine (15 mg/kg) significantly shortened the latency of first-crossing the platform and increased the number of crossings of the platform within 2 min ($P<0.05$, Table 2).

All the results of the above two behavioral tests indicated that FLZ protected against the impairment of learning and memory function of mice induced by $A\beta_{25-35}$.

Effect of compound FLZ on the MDA level in the hippocampus of mice icv injected with $A\beta_{25-35}$ After completing the behavior test of Morris water maze test, the MDA level in the cerebral hippocampus of 9–10 mice per group was determined. The icv injection of $A\beta_{25-35}$ induced a significant increase in MDA level in the hippocampus ($P<0.05$). The

Table 2. Effect of FLZ on spatial learning and memory impairment of mice induced by the icv injection of aged $A\beta_{25-35}$ during the probe trial in Morris water maze test. Mean \pm SEM. ^b $P<0.05$ vs control. ^e $P<0.05$, ^f $P<0.01$ vs $A\beta_{25-35}$ model group.

Group	<i>n</i>	Latency to cross the platform/s	Average number of crossing platform
Control	16	47.4 \pm 11.7	1.8 \pm 0.4
$A\beta_{25-35}$ model	15	82.1 \pm 8.4 ^b	0.7 \pm 0.2 ^b
FLZ (75 mg/kg)+ $A\beta_{25-35}$	16	63.4 \pm 12.4	1.4 \pm 0.3
FLZ (150 mg/kg)+ $A\beta_{25-35}$	15	65.4 \pm 11.3	1.5 \pm 0.3 ^e
Tacrine (15 mg/kg)+ $A\beta_{25-35}$	16	55.7 \pm 11.0	2.5 \pm 0.5 ^f

treatment with FLZ (75 mg/kg, 150 mg/kg) and tacrine (15 mg/kg) markedly decreased MDA levels in the hippocampus of $A\beta_{25-35}$ -treated mice (Table 3).

Table 3. Effect of compound FLZ on the MDA content in cerebral hippocampus of mice injected (icv) with aged $A\beta_{25-35}$. Mean \pm SEM. ^b $P<0.05$ vs control. ^e $P<0.05$, ^f $P<0.01$ vs $A\beta_{25-35}$ model group.

Group	<i>n</i>	MDA/nmol·g ⁻¹ brain weight
Control	9	358.6 \pm 37.3
$A\beta_{25-35}$ model	9	461.0 \pm 21.1 ^b
FLZ (75 mg/kg)+ $A\beta_{25-35}$	9	273.8 \pm 42.4 ^f
FLZ (150mg/kg)+ $A\beta_{25-35}$	10	224.7 \pm 27.9 ^f
Tacrine (15mg/kg)+ $A\beta_{25-35}$	10	351.2 \pm 27.0 ^e

Effect of FLZ on the pathological injury of the hippocampus induced by icv injection of $A\beta_{25-35}$ in mice No remarkable neuronal abnormalities in the hippocampus from mice of the normal control group were observed, while all examined brains of $A\beta_{25-35}$ model group mice showed degeneration of neurons in the hippocampus and disorder of the array of neurons. The body of neuron became short and deeply stained with dye. Some neurons were shrunk and necrosed. Whereas the neurons in the FLZ (75 mg/kg, 150 mg/kg) and tacrine (15 mg/kg) group mice were close to that of normal control group, indicating that FLZ protected against the injuries of the hippocampus induced by $A\beta_{25-35}$ injection (Figure 3).

Effect of FLZ on AChE, Bel-2, and Bax immunoreactivity in the hippocampus cells of mice icv injected with $A\beta_{25-35}$ There were few AChE and Bax immunoreactive neuronal cells

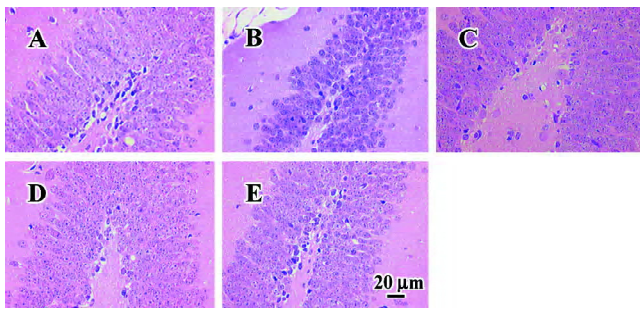


Figure 3. Histopathological observation of the mouse brain hippocampus (HE stain). (A) Control group (with the vehicle injection); (B) Aβ₂₅₋₃₅ injected group; (C) FLZ (75 mg/kg) group; (D) FLZ (150 mg/kg) group; (E) Tacrine (15 mg/kg) group.

in the hippocampal CA1 region of control group mice (Figure 4, 5). The staining was light and the immunoreactive cells had few processes. In the hippocampal CA1 region of Aβ₂₅₋₃₅-icv injected mice the number of AChE ($P<0.05$) and Bax ($P<0.01$) immunoreactive cells increased significantly in comparison with control group mice. The immunoreactive cells had long and darkly stained processes. Compared with Aβ₂₅₋₃₅-treated mice, the number of AChE and Bax immunoreactive cells in the hippocampal CA1 region of FLZ (150 mg/kg) and tacrine-treated group mice was significantly reduced ($P<0.05$ or $P<0.01$), the staining was light, and the marked immunoreactive cells had few processes.

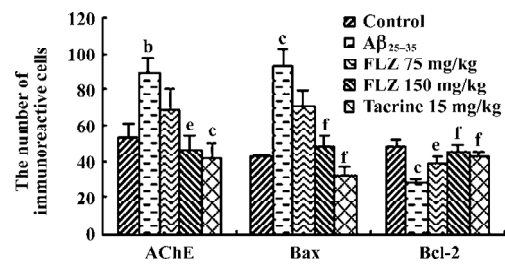


Figure 5. The number of AChE, Bax, and Bcl-2 immunoreactive cells in the hippocampal CA1 region from four mouse brains of every group. Four mice were taken from each group. One in every four sections was taken from a continuous series of section prepared from hippocampal tissue. Six sections were selected in each mouse, so 24 sections of each group were read under an objective, and the number of positively stained cells was counted. Mean±SEM. ^b $P<0.05$, ^c $P<0.01$ vs control. ^e $P<0.05$, ^f $P<0.01$ vs Aβ₂₅₋₃₅ model group.

The results of Bcl-2 immunochemical staining showed that the hippocampal CA1 region of control group mice contained abundant and darkly stained Bcl-2 immunoreactive cells (Figure 4, 5). The marked cells had long processes. In the hippocampal CA1 region of Aβ₂₅₋₃₅-treated mice, the number of Bcl-2 immunoreactive cells was less than that of control group mice ($P<0.05$), the staining was light, and the marked cells had few processes. There were more Bcl-2 immunoreactive cells in the hippocampal CA1 region of FLZ (150 mg/kg)

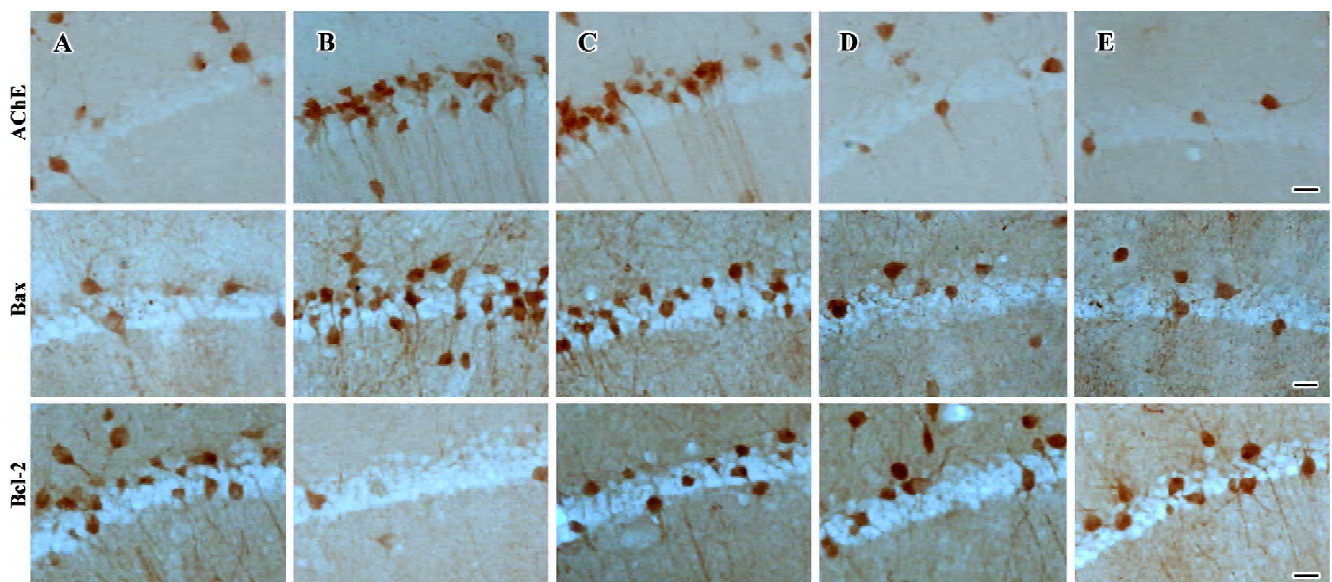


Figure 4. Effect of FLZ on Aβ₂₅₋₃₅-induced AChE, Bax, and Bcl-2 immunoreactive cells in the hippocampal CA1 region of mice (bar=20 μm). (A) Control group (with the vehicle injection): the immunoreactive cells are few and light-stained; (B) Aβ₂₅₋₃₅ injection group: the immunoreactive cells are abundant and dark-stained; (C, D, E) FLZ (75 mg/kg) group, FLZ (150 mg/kg) group, and Tacrine (15 mg/kg) group: the immunoreactive cells are few and light-stained. The representative figure is one of four mice in each group.

and tacrine-treated mice in contrast to A β_{25-35} -treated mice ($P < 0.01$), and the immunoreactive cells had long processes. The staining was near to that of control group mice.

Discussion

Animal models are playing a critical role in ongoing attempts to understand the pathology and screen therapeutics of AD. Although no available model can meet all the full pathologic spectrum of AD disease, the injection of A β into the brain was shown to impair learning and memory, and elicit a degree of Alzheimer's-type neurodegeneration^[2-6]. The important point of A β_{25-35} -induced amnesia is the influence of the physical state of the peptide, aggregated or soluble, at the time of administration. The *in vitro* incubation of A β_{25-35} leads to the formation of stable oligomeric aggregates, which contain an increased proportion of β -sheet structure that appears to be an important feature of the β -amyloid-induced neurotoxicity. This aggregation mimics *in vitro* the slow aging process that *in vivo* leads to the formation of the senile plaques^[14,15]. In the present study, a single icv injection of aged A β_{25-35} to mice induced a significant impairment of learning and memory in step-down test and Morris water maze test, and obvious pathological lesions of the hippocampus. In mammals, the hippocampus is a critical neural structure in the early stage of memory formation. The impairment of memory formation is caused by damage in the hippocampus and associated areas of the temporal cortex. The deposition of A β first forms in temporal cortical regions including the hippocampus^[16]. Some researchers reported that A β -induced injury of the hippocampus was associated with impairment in learning and memory beside biochemical changes and neuronal degeneration^[12,17,18]. Compound FLZ was shown to prevent the neurotoxicity of A β_{25-35} , as it significantly attenuated the learning and memory impairment and pathological injury of hippocampus in mice injected with A β_{25-35} .

Apoptosis is considered as the main cause of the loss of cholinergic neurons in AD. Several authors reported that A β_{25-35} induced the neuronal degeneration through an apoptosis pathway *in vitro* and *in vivo*^[19,20]. Bcl-2 family proteins play a pivotal role in regulating apoptotic cell death, some of which members, such as Bcl-2 and Bcl-x_L, inhibit apoptosis and others such as Bax induces cell apoptosis. The relative ratio of proapoptotic and antiapoptotic proteins is important to determine cell survival or death^[21]. The authors found that A β_{25-35} increased the expression of proapoptotic protein Bax and decreased the expression of anti-apoptotic protein Bcl-2 in the CA1 region of hippo-

campus, indicating that A β_{25-35} -induced pathological injury of the hippocampus was related to cell apoptosis. Compound FLZ exerted a significant anti-apoptotic effect through modulating the expression of Bcl-2 and Bax protein in the CA1 region of the hippocampus, which resulted in protection against pathological injury of the hippocampus and impairment of learning and memory.

Several studies with AD models demonstrated that A β increases AChE expression and AChE activity not only in cell culture but also in the intact brain^[22,23]. AChE may promote beta-amyloid plaque formation and also increase the A β toxicity as reported by Rees *et al*^[24]. Also, there is evidence to indicate that AChE is an exacerbating factor in the apoptosis of neurons, and plays a key role in the procession of apoptotic cell death. The apoptotic neurons can then secrete higher levels of AChE into the brain. This cascade amplification leads to progressive neuronal loss, which is the hallmark of AD^[25,26]. In addition, cholinergic system dysfunction of AD is correlated with cognitive impairment. It was reported that acetylcholine (ACh) levels in the cerebral cortex and hippocampus were significantly decreased by centrally administered A β in animals^[9,27]. AChE is a key enzyme in the catabolic metabolism of ACh. A β_{25-35} -enhanced AChE expression and AChE activity might decrease the ACh level. In the present study, the icv injection of A β_{25-35} resulted in a significant increase of AChE expression, and compound FLZ markedly attenuated the increase of the number of AChE immunopositive cells in the CA1 region of the mouse hippocampus induced by icv injection of A β_{25-35} . So, we considered that the inhibition of FLZ on AChE expression may be involved in anti-apoptosis and improvement of amnesia in mice injected with A β_{25-35} . We also speculated that the inhibitory effect of FLZ on AChE expression might lead to an increase in acetylcholine level through inhibition of acetylcholine hydrolysis, which thereby enhanced the cholinergic function and improved the impairment of learning and memory. To confirm this speculation, direct measurement of hippocampal acetylcholine level and AChE activity is needed in future experiments.

Multiple lines of evidence indicated that oxygen free radicals (ROS) were involved in A β -induced neuronal apoptosis and death. A β can induce intracellular ROS production, which causes peroxidation of protein and lipid in neurons^[28]. A β itself can transform into a radical state and further interacts with neuronal membranes^[29]. MDA is an end product of lipid peroxidation of biomembranes, and the MDA content usually reflects the level of lipid peroxidation and indirectly reflects the extent of injury. The icv injection of A β_{25-35} induced a significant increase of MDA content in the mouse

brain hippocampus, indicating that A β_{25-35} induced lipid peroxidation of neurons. FLZ was shown to have obvious antioxidant property, and also protected against oxidative damage and apoptosis of neurons induced by many toxins^[8]. In the present study, we confirmed that FLZ significantly decreased the MDA content in the hippocampus of A β_{25-35} -treated mice, suggesting that FLZ has a protective action against oxidative damage of neurons induced by A β_{25-35} . Some antioxidants such as vitamin E were reported to eliminate A β -induced apoptosis and slow the progression of AD^[30,31]. Xiao *et al* reported that the protective action of huperzine A and B and tacrine (AChE inhibitor) against A β toxicity to PC-12 cells was not cholinesterase-dependent but might be through an antioxidant pathway^[32]. So, it appears that therapeutic efforts aiming at the removal of free radicals formation or preventing their damage to related neurons may be beneficial in pharmacol-therapy of AD.

The reason why tacrine, an AChE inhibitor, was selected as a positive control in the present study should be made clear. This was because tacrine was the first drug approved to treat AD by the FDA in the USA^[33], and tacrine was known to attenuate the impairment of leaning and memory induced by icv injection of A β_{25-35} ^[9]. Tacrine also showed neuroprotective action against A β_{25-35} -induced oxidative injury and H₂O₂-induced apoptosis^[32,34]. The present results showed that both tacrine and FLZ have similar neuroprotective effect on icv injection of the A β_{25-35} model in mice. However, tacrine has been known to cause liver toxicity in AD patients by approximately 50% and has peripheral side effects^[35]. Compound FLZ of the studied dosages had no such effects in mice. The acute oral LD₅₀ of FLZ was over 5 g/kg body, indicating FLZ is a low toxicity compound. Moreover, not like tacrine, FLZ is not an inhibitor of AChE activity^[8].

In summary, FLZ significantly attenuated learning and memory deficits as well as pathological and biochemical injuries of hippocampus in mice induced by icv injection of aged A β_{25-35} through the neuroprotective pathway.

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