Glutathione-mimetic D609 alleviates memory deficits and reduces amyloid-β deposition in an AβPP/PS1 transgenic mouse model

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Excessive extracellular deposition of amyloid-β-peptide (Aβ) in the brain is a pathological hallmark of Alzheimer's disease (AD). Oxidative stress is associated with the onset and progression of AD and contributes to Aβ generation. Tricyclodecan-9-yl-xanthogenate (D609) is a glutathione (GSH)-mimetic compound. Although the antioxidant properties of D609 have been well-studied, its potential therapeutic significance on AD remains unclear. In the present study, we used a mouse model of AD to investigate the effects and the mechanism of action of D609 on AD. We found that D609 treatment significantly improved the spatial learning and alleviated the memory decline in the mice harboring amyloid precursor protein (APP) and presenilin-1 (PS1) double mutations (AβPP/PS1 mice). D609 treatment also increased GSH level, GSH and oxidative glutathione ratio, and superoxide dismutase activity, whereas decreased malondialdehyde and protein carbonyl levels, suggesting that D609 alleviated oxidative stress in AβPP/PS1 mice. In addition, D609 reduced β-secretase 1 level and decreased amyloidogenic processing of AβPP, consequently reducing Aβ deposition in the mice. Thus, our findings suggest that D609 might produce beneficial effects on the prevention and treatment of AD. *NeuroReport* **29:833–838 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder and clinically characterized by cognitive dysfunction and memory loss. Major pathological hallmarks of AD include the presence of senile plaques, neurofibrillary tangles, and oxidative stress [1]. The major protein component of senile plaque core is amyloid-β peptide (Aβ). Increased Aβ is the key pathogenic factor of AD and a cause for neuronal loss [2]. Aβ plaques generate oxidative stress, which plays a key role in AD pathophysiology [1,3,4]. A number of studies have shown that oxidative damage in AD begins at an early stage of the disease [3]. Oxidative stress has also been found to contribute to Aβ generation [1]. It can create positive feedback on amyloid precursor protein (APP) proteolytic pathway and modulate the levels of β-secretase 1 (BACE1). Because oxidative stress-mediated toxicity is involved in neurodegenerative events, various experimental approaches aimed to stimulate antioxidant protection have been developed. It has been shown that numerous potential free-radical scavengers are neuroprotective, and they decrease AD risk and slow AD progression [5,6]. Glutathione (GSH) is one of the endogenous free-radical scavengers in brain and participates in major intracellular defense systems against free radicals. Reactive oxygen species (ROS)-induced oxidative stress and GSH homeostasis disorder in brain have been found to contribute to AD [7]. Thus, developing antioxidant compounds capable of 'mimicking' GSH function to protect the brain against oxidative stress has become an attractive strategy for treating AD.

Tricyclodecan-9-yl-xanthogenate (D609), an inhibitor for phosphatidylcholine-specific phospholipase C, exhibits diverse potent biological functions [8,9]. It has been shown that D609 can act as a GSH mimetic compound to scavenge hydrogen peroxide and hydroxyl free radicals [10]. D609 might also protect endogenous GSH, which plays an important role in defending neurons against oxidative stress. It has been reported that in-vivo loading with D609 has an antioxidant effect on the mitochondria of isolated brain and the synaptosomes exposed to Aβ (1–42) [11,12], suggesting that D609 might produce beneficial effects on the prevention and treatment of AD. However, the potential therapeutic effect of D609 on AD neuropathology *in vivo*, particularly on cognitive impairments of AD, and the mechanism of action remain unclear.

In this study, we investigated the effect of D609 on the behavior performance and Aβ deposition of a transgenic mouse model of AD.

Materials and methods

Animals and drug treatment

A AβPPswe/PS1-dE9 double transgenic mouse with C57BL/6 background harbors the mutant human genes APPswe (Swedish mutations K594N/M595L) and presenilin-1 with the exon-9 deletion (PS1-dE9) under the control of mouse prion protein promoter. All mice were males and were obtained from Beijing HFK Bio-Technology Co. Ltd., Institute of Laboratory Animal Science, Chinese 85 Academy of Medical Science (Beijing, China) and were housed in temperature and humidity controlled rooms on a 12/12 h light/dark cycle.

All the procedures described in this study were in accordance with the Ethical Committee for Animal Experiments of Shandong University. All efforts were made to minimize animal suffering. D609 (J&K, Beijing, China) was dissolved in PBS and intraperitoneally administered at the dosage of 10 mg D609/kg body weight/mouse/day to 4.5-month-old male AβPP/PS1 transgenic mice for 10 weeks. Control male transgenic mice were injected with the same volume of PBS. We also carefully monitored the general health of the mice throughout the course of treatment and did not observe any adverse effects or significant changes in their weight gain.

Behavior test

After the treatment completed, we used the modified Morris water maze test to assess the spatial memory performance of the mice [13]. Fifteen mice were used in each group. Detailed methodology was described in our previous report [14]. In summary, in the spatial acquisition tests, mice were released into the pool and given 60 s to find the hidden platform. If a mouse did not find the platform within 60 s, it was guided to the platform. Animals were given 4 trials/day. The distal starting positions were semi-randomly selected. A single probe trial, in which the platform was removed, was performed after the hidden platform task had been completed. Mice were placed in a novel start position (northeast) in the maze, and each mouse was allowed to swim for 60 s. Mouse behavior was observed blindly.

Thioflavin S staining

AβPP/PS1 mice were killed after the behavior test. The mice were deeply anesthetized with chloral hydrate, and then were immediately cardiac perfused with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M PBS (pH: 7.4). After the perfusion, the brains of the mice were excised and bisected, and a hemibrain was postfixed overnight at 4°C. The brain tissue was then incubated in 30% sucrose at 4°C until equilibration (6 mice/group). Thirty micrometer coronal sections were cut by a freezing microtome (CM1850; Leica, Germany) and stored at − 20°C. For thioflavin S staining, six serial sections with an interval of 50 μm were taken from the cortex and the hippocampus per mouse. Brain sections were incubated in 0.5% Thioflavin S (Sigma-Aldrich; St. Louis, Missouri, USA) dissolved in 50% ethanol for 5 min, and then washed twice with 50% ethanol for 5 min each time. The brain sections were washed once with tap water for 5 min, and then mounted with mounting medium. The green fluorescence-stained plaques were observed under a fluorescent microscope. The staining was analyzed by the image analyzing system, Image pro plus 6 (Media Cybernetics; Rockville, Maryland, USA).

Aβ ELISA

Brain tissue samples were isolated from APP/PS1 mice of each group after behavioral testing. Hemibrains were flash frozen and stored at –80°C until homogenization. Aβ40 and Aβ42 enzyme-linked immunosorbent assays (ELISA) were performed using the ELISA kits (Invitrogen; Carlsbad, California, USA). ELISA was performed as described in our previous report [14].

Measurement of oxidative stress

Brain tissues were homogenized in nine volumes of ice-cold 0.9% saline (6 mice/group). The homogenates were centrifuged at 3000*g* for 10 min at 4°C to obtain the supernatant. The supernatant was diluted with the appropriate buffer solution to determine the relative biochemical index. GSH and oxidative glutathione (GSSG) were determined using the commercial kits (Beyotime Ins. Bio, Shanghai, China), according to the manufacturer's instructions [15]. Absorbance was read at 405 nm on a microplate reader. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx), and MDA and protein carbonyl levels were determined by using the assay kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions [16]. Protein contents in the supernatant were quantified using the bicinchoninic acid assay (Beyotime Ins. Bio).

Western blot analysis

Western blot (6 mice/group) was performed as described in our previous report [14]. The following primary antibodies were used: rabbit anti-APP C-terminus polyclonal antibody (rabbit, 1 : 10 000; Sigma, St. Louis, Missouri, USA), BACE1 (rabbit, 1 : 1000; Abcam, Cambridge, Massachusetts, USA), and β-actin (mouse, 1 : 400; Santa Cruz, Dallas, Texas, USA). The second antibodies used were as follows: goat anti-mouse IgG/HRP (1 : 5000; Golden Bridge International, Beijing, China) and goat anti-rabbit IgG/HRP (1 : 5000; Golden Bridge International). The intensity of the bands was quantified by using Image J software (National Institutes of Health; Bethesda, Maryland, USA).

Statistical analysis

All data were expressed as mean ± SE. The Student's *t*-test was used to compare the two groups. In two-variable

experiments, two-way repeated-measures analysis of variance was used to evaluate the significance of differences between the group means. *P* values less than 0.05 were considered statistically significant. Data were analyzed with SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

D609 rescues learning and memory deficits in AβPP/PS1 mice

Figure 1a shows the mean escape latencies during the acquisition training phase. D609-treated mice showed significantly improved learning compared with the control group. The D609-treated mice also had significantly higher number of platform location crosses and longer time spent in the target quadrant than the PBS-treated groups (Fig. 1b and c), suggesting that the D609 treatment improved the memory of the mice. Similar swimming speeds (Fig. 1d) between the two groups suggested that the improved performance in D609-treated mice was a result from cognitive processes but not noncognitive behavioral components. Thus, our data indicated that D609 treatment could rescue learning memory impairments in AβPP/PS1 mice.

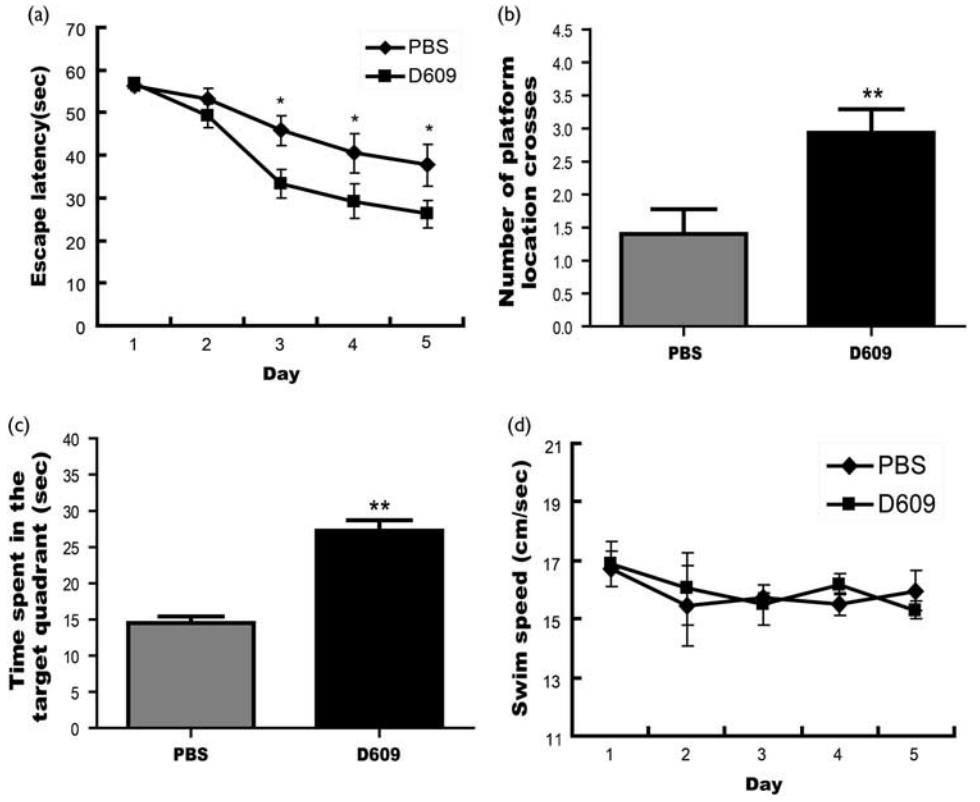
D609 significantly reduces Aβ deposition and soluble Aβ levels

To investigate the effect of D609 on Aβ pathology, we analyzed Aβ deposition in the mice by thioflavin S staining after the behavioral test. Interestingly, D609 treatment dramatically reduced Aβ deposition in both cortex and hippocampus of the mice (Fig. 2a and b). We examined cerebral soluble Aβ40 and Aβ42 levels by ELISA assay. D609 treatment significantly decreased Aβ40 and Aβ42 levels in the mice (Fig. 2c and d). These data clearly supported that D609 treatment decreased Aβ level in the brain of AβPP/PS1 mice.

D609 alleviates oxidative stress in AβPP/PS1 mice

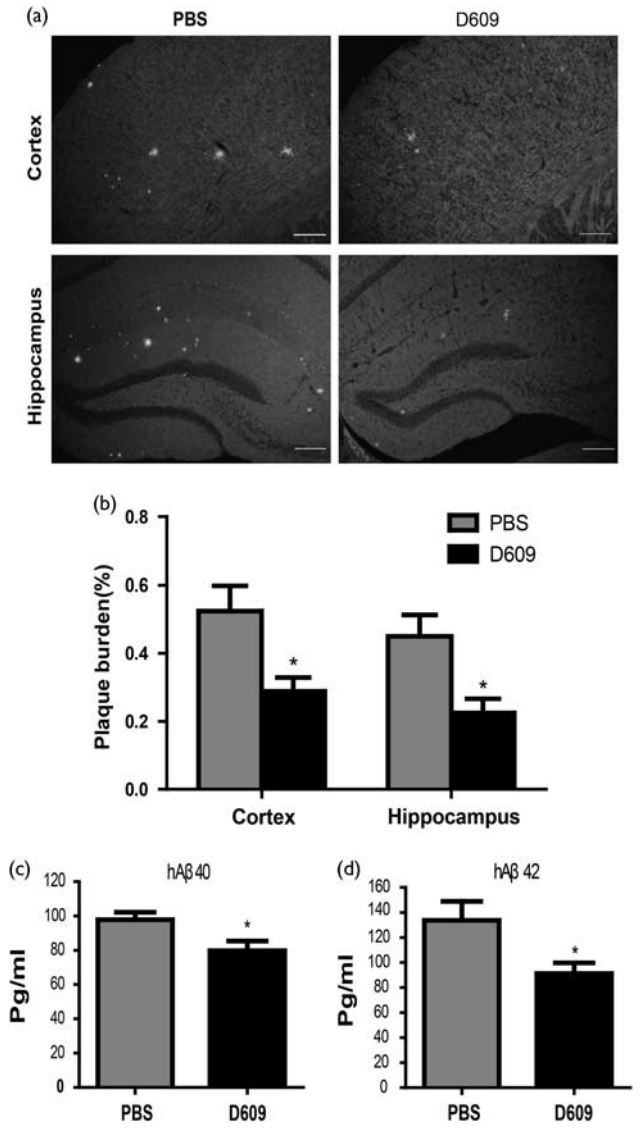
Because D609 has been shown to be able to act as an antioxidant, we determined GSH and GSSG levels in the mice. As illustrated in Fig. 3a, GSH level in the D609-treated mice was significantly increased compared with that in the control group, whereas GSSG level was

Fig. 1



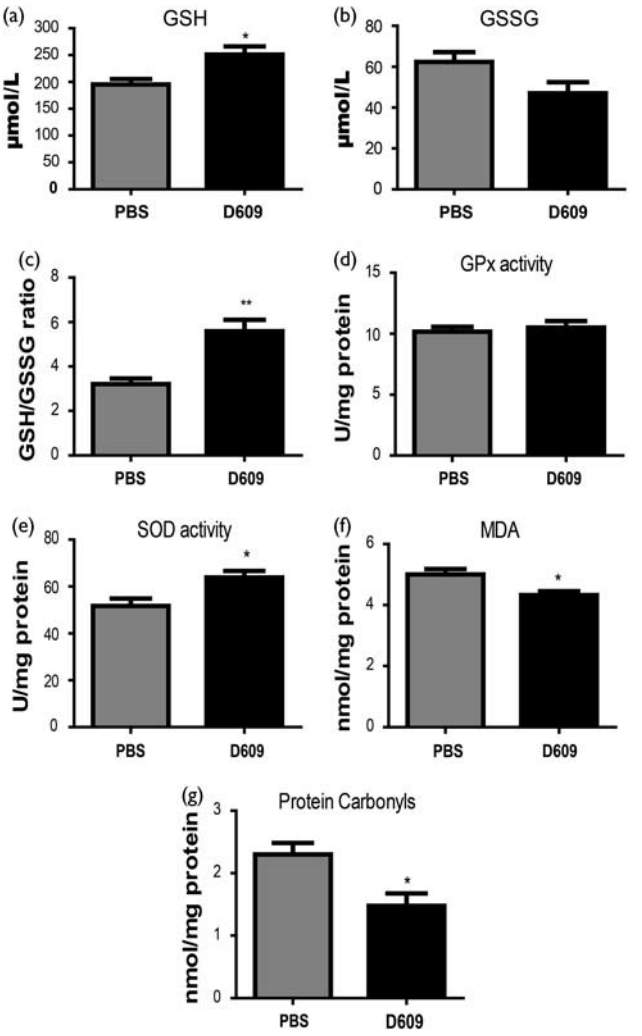
D609 improved spatial learning and alleviated memory impairments in AβPP/PS1 mice. (a) D609 treatment improved spatial learning in Alzheimer's disease (AD) mice. Spatial learning was measured as escape latencies per day. Fifteen mice were used in each group. (b, c) D609 treatment restored spatial memory in the AD mice. Spatial memory was evaluated by the probe trial performed 24 h after the last training session and quantified as the number of platform location crosses (b) and the time spent in the target quadrant (c). (d) Swimming speed was not significant different between the groups. Data are presented as mean ± SE; $*P < 0.05$, $**P < 0.01$, D609-treated group versus PBS-treated group.

Fig. 2



D609 reduced amyloid-β (Aβ) deposition and soluble Aβ levels. (a) Thioflavin S staining for senile plaques of cerebral cortex and hippocampus in the mice. Scale bar, 200 μm. (b) Quantification of Thioflavin S staining ($n = 6$ in each group). (c, d) ELISA assay for soluble Aβ40 (c) and Aβ42 (d) in the cortex and hippocampus ($n = 6$ in each group). Data were presented as mean ± SE, $^{*}P < 0.05$, D609-treated group versus PBS-treated group.

Fig. 3



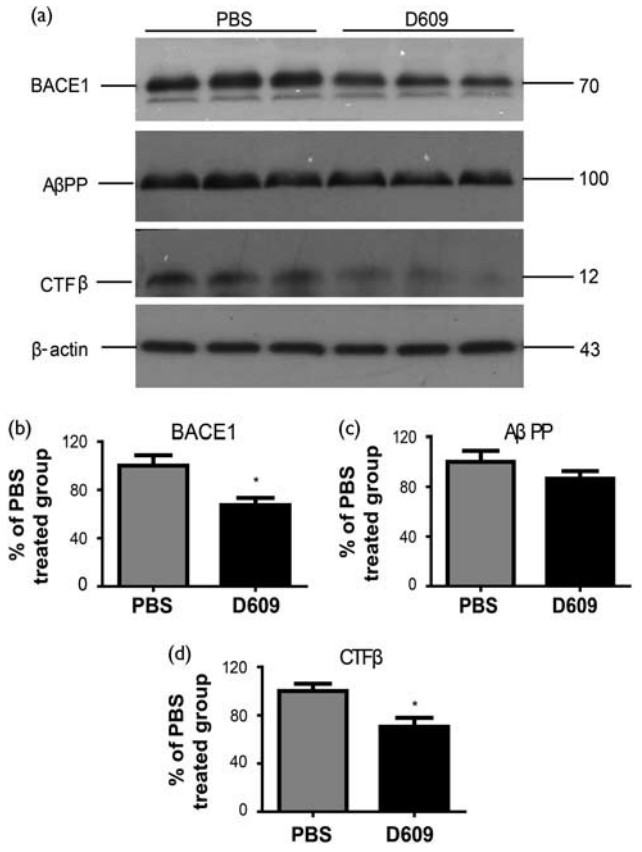
D609 alleviated oxidative stress in AβPP/PS1 mice. (a) glutathione (GSH), (b) oxidized glutathione (GSSG), (c) GSH/GSSG ratio, (d) glutathione peroxidase (GPx) activity, (e) superoxide dismutase (SOD) activity (f) MDA, and (g) protein carbonyls. The assay was performed using the commercial kits as described in the Materials and Methods section. Data are presented as mean ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$, D609-treated group versus PBS-treated group.

slightly decreased by D609 treatment (Fig. 3b). Consequently, the GSH/GSSG ratio in the D609-treated group was significantly increased compared with that in the control group (Fig. 3c). We then analyzed the activity of antioxidative enzymes, including SOD and GPx. D609 treatment did not alter GPx activity (Fig. 3d), but significantly increased SOD activity in the mice (Fig. 3e). D609 also significantly decrease MDA and protein carbonyl levels, which are indicators for lipid peroxidation and protein oxidation, respectively (Fig. 3f and g). These results suggested that D609 treatment significantly reduced oxidative stress in AβPP/PS1 mice.

D609 decreases amyloidogenic AβPP processing by down-regulating BACE1 levels

Our western blot results showed that BACE1 level in the D609-treated mice was significantly reduced compared with that in the PBS-treated mice (Fig. 4a and b). Aβ is derived from AβPP by β-secretase and γ-secretase cleavages. BACE1 is the predominant endogenous β-secretase and produces C-terminal fragment β (CTFβ). To determine the effect of BACE1 reduction on AβPP processing, we analyzed the protein levels of AβPP and CTFβ in brain tissue.

Fig. 4



D609 significantly decreased BACE1and CTFβ levels. (a) Representative western blots for BACE1, AβPP, and CTFβ. Sample in triplicate was loaded into the gel. (b–d) Quantification of the western blot for BACE1 (b), AβPP (c), and CTFβ (d). Data are presented as mean ± SE, $^*P < 0.05$, D609-treated group versus PBS-treated group.

The expression level of AβPP was not reduced by D609 (Fig. 4a and c). However, CTFβ level was markedly decreased in the D609-treated mice compared with that in the control mice (Fig. 4a and d).

Discussion

A number of studies have demonstrated that D609 had protective effect on Alzheimer's Aβ (1–42)-induced oxidative stress in cultured neurons and in synaptosomes of animal models [11,17]. We examined the effect of D609 on cognitive behavioral performance and Aβ deposition of an AD mouse model. Our data showed that D609 significantly improved cognitive performance and reduced Aβ deposition in the AD mouse through the alleviation of oxidative stress and down-regulation of BACE1.

Oxidative stress is one of the most important mechanisms involved in Aβ-mediated neurotoxicity and has been proposed to play a key role in the pathogenesis of AD [3,4]. Oxidative stress in AD is caused by an imbalance between oxidant and antioxidant systems. In the AD brain, increased oxygen content has been demonstrated using protein oxidation markers and lipid peroxidation [18]. The AD brain has relatively low levels of antioxidants including nonenzymatic antioxidants GSH and antioxidant enzymes, GPx and SOD [19]. GSH, which is the most abundant endogenous antioxidant, plays a significant role in combating oxidative stress. Elevation of GSH is a therapeutic strategy in AD [7]. SOD and GPx are important antioxidant enzymes that play a significant role in scavenging free radicals. Many studies have shown that treatment with exogenous antioxidant reverses learning and memory deficits in AD mice [20,21]. Thus, one of the rational strategies for the prevention or treatment of AD is to decrease oxidative stress to restore GSH homeostatis and stimulate the activity of antioxidant enzymes. D609 has antioxidant/GSH mimetic properties owing to the presence of a free thiol group [10]. Our results showed that D609 increased GSH level, elevated GSH/GSSG ratio, and increased SOD activity in the AD mice. In addition, we also found that D609 treatment decreased MDA and carbonyl levels, indicating that D609 may repair the oxidative damage on lipids and protein in APP/PS1 mice. These results suggested that D609 could be an effective approach to alleviate in AD.

We proposed that the molecular mechanism of D609-mediated Aβ reduction was associated with the reduction of BACE1 expression by D609. BACE1 is the predominant endogenous β-secretase, and produces a 99-aminoacid C-terminal fragment of APP (CTFβ) during the amyloidogenic processing of APP. We observed a significant reduction of BACE1 and CTFβ levels in the D609-treated AD mice, which contributed to the decrease of Aβ deposition in the mice. The BACE1 reduction might be related to the D609-mediated alleviation of oxidative stress in the mice, because it has been found that oxidative stress has significant effect on BACE1 activity [22].

Conclusion

Our study demonstrated that D609 improved the behavioral performance and reduced Aβ deposition in the AβPP/PS1 transgenic mouse model of AD. The molecular mechanism of D609-mediated beneficial effects was related to the alleviation of oxidative stress and down-regulation of BACE1. Thus, long-term treatment with D609 may produce beneficial effects on the prevention and treatment of AD.

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Conflicts of interest

There are no conflicts of interest.

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