Journal of Alzheimer's Disease 64 (2018) 447–457
DOI 10.3233/JAD-180241
IOS Press

A Small Molecule Inhibitor of Plasminogen Activator Inhibitor-1 Reduces Brain Amyloid-β Load and Improves Memory in an Animal Model of Alzheimer's Disease

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Accepted 23 April 2018

Abstract. Alzheimer's disease (AD) is a major cause of dementia in the elderly with no effective treatment. Accumulation of amyloid-β peptide (Aβ) in the brain is a pathological hallmark of AD and is believed to be a central disease-causing and disease-promoting event. In a previous study, we showed that deletion of plasminogen activator inhibitor 1 (PAI-1), a primary inhibitor of tissue type and urokinase type plasminogen activators (tPA and uPA), significantly reduced brain Aβ load in APP/PS1 mice, an animal model of familial AD. In this study, we further show that oral administration of TM5275, a small molecule inhibitor of PAI-1, for a period of 6 weeks, inhibits the activity of PAI-1 and increases the activities of tPA and uPA as well as plasmin, which is associated with a reduction of Aβ load in the hippocampus and cortex and improvement of learning/memory function in APP/PS1 mice. Protein abundance of low density lipoprotein related protein-1 (LRP-1), a multi ligand endocytotic receptor involved in transporting Aβ out of the brain, as well as plasma $A\beta_{42}$ are increased, whereas the expression and processing of full-length amyloid-β protein precursor is not affected by TM5275 treatment in APP/PS1 mice. *In vitro* studies further show that PAI-1 increases, whereas TM5275 reduces, $A\beta_{40}$ level in the culture medium of SHSY5Y-APP neuroblastoma cells. Collectively, our data suggest that TM5275 improves memory function of APP/PS1 mice, probably by reducing brain Aβ accumulation through increasing plasmin-mediated degradation and LRP-1-mediated efflux of Aβ in the brain.

Keywords: Alzheimer's disease, amyloid-β accumulation, memory, PAI-1 inhibitor

INTRODUCTION

Alzheimer's disease (AD), a neurodegenerative disease, is a major cause of dementia in the elderly. Over 5 million Americans are living with AD and the number will increase rapidly with the population aging. Despite extensive studies, there is no effective

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treatment for this devastating disease due to incomplete understanding of the etiology and pathogenesis of the disease. Deposition of amyloid-β (Aβ), senile plaques, in the brain is a pathological feature of AD and brain accumulation of Aβ peptides, soluble and aggregates, is believed to be a central disease-causing and disease-promoting event for AD [1–4]. Therefore, reducing brain Aβ loads has been a focus of many studies aimed to develop therapeutics for the treatment of AD. Although promising, no drug has been developed to cure the disease yet, partially due to side effects or failure of biologics/compounds to cross the brain-blood barrier (BBB) and partially due to our incomplete understanding of the biology associated with brain Aβ accumulation in sporadic AD [5–7].

The levels of Aβ in the brain represent a dynamic equilibrium state as a result of their biosynthesis, degradation, influx, and efflux. Mutations in amyloid precursor protein (*APP*) and presenilin1/presenilin2 (*PS1/PS2*) genes, which codes for the sub-component of γ-secretase, are responsible for brain Aβ accumulation in familial AD. The mechanism underlying brain Aβ accumulation in sporadic AD patients, who do not carry any of these mutations and show no sign of increased Aβ production, however, is unknown. Accumulating evidence suggests that decreased clearance capacity, rather than increased synthesis, may be responsible for brain Aβ accumulation in sporadic AD, although it is still debated which enzyme plays a major role in Aβ degradation [8–12]. Plasmin, a serine protease, degrades both soluble and aggregated Aβ with physiologically relevant efficiency [10, 13–16]. It has also be shown that plasmin enhances amyloid-β protein precursor (AβPP) α-cleavage [14], suggesting that plasmin may reduce the toxic build-up of Aβ by either diverting AβPP away from the β cleavage pathway or by directly degrading existing Aβ. Plasminogen deficient mice have significantly lower capacity to remove human Aβ injected into hippocampus, compared to wild type mice, further supporting the critical role of plasmin in Aβ degradation *in vivo* [17]. Plasmin is converted from the zymogen plasminogen by tissue type and urokinase type plasminogen activators (tPA and uPA), whereas PAI-1 is a primary inhibitor of tPA and uPA. Previous studies from this group and from others have shown that expression of PAI-1 increases with age in the brain of wild type and familial AD model mice as well as in AD patients [18–21]; knockout of the PAI-1 gene or inhibiting PAI-1 activity with a small-molecule PAI-1 inhibitor reduces brain Aβ burden and reversed cognitive deficits in these familial AD models [10, 20]. The results suggest that increased PAI-1 expression/activity contributes importantly to Aβ accumulation in AD and that small molecule PAI-1 inhibitors may have therapeutic potential for the treatment of AD.

This study is aimed to test the therapeutic potential of a small molecule PAI-1 inhibitor TM5275 for AD, using APP/PS1 transgenic mice. Our results show that oral administration of TM5275 reduces Aβ load in hippocampus/cortex and improves cognitive function of APP/PS1 mice. *In vitro* studies using cultured neuroblastoma cells further show that PAI-1 protein increases, whereas TM5275 reduces, the amount of $A\beta_{40}$ in cultured medium. These results further support the notion that increased PAI-1 expression/activity contributes importantly to AD pathophysiology and that small molecule PAI-1 inhibitors have therapeutic potential for AD.

MATERIALS AND METHODS

Animals and TM5275 administration

Four and half month old female APP/PS1 mice which bear two AD mutations [a mutant human presenilin 1 (DeltaE9) and a chimeric mouse/human APP] were purchased from JAXMICE. Female APP/PS1 mice were used in this study to test the therapeutic potential of TM5275 for AD as they have worse memory deficit and more brain Aβ deposition at young age than male APP/PS1 mice [22]. Mice were treated by oral gavage with 0.18 ml–0.22 ml, depending on body weight, of 5 mg/ml TM5275 dissolved in 20% DMSO/saline (vehicle) to reach final dose of 40 mg/kg of TM5275, or with vehicle alone for a period of 6 weeks. Mice were maintained on a 12-h light/dark cycle at 22°C with free access to water and food. Body weight and food intake were recorded weekly in order to monitor potential toxicity of TM5275. At the end of 6-week treatment period, learning and memory function was assessed by Morris water maze test and then mice were euthanized by intramuscular injection of a mixture of Ketamine: Xylazine. This ensures both sedation and anesthesia of the mouse within minutes. The chest is then open and blood withdrawn from heart and then transcardial perfusion was performed [20]. The brain was dissected sagittally into right and left hemispheres with the right hemisphere fixed in 10% PBS buffered formalin and the left hemisphere dissected; the hippocampus and cerebral cortex were collected

together and frozen in liquid nitrogen immediately for subsequent biochemical analyses. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

The open field and elevated plus maze tests

The open field test and then the elevated plus maze test were conducted to assess general anxiety and fear levels at UAB animal behavior core facility as we have described previously [22]. The time spent in the center, closed, or open arms (elevated plus maze) and in the center or sides (open filed test) for each mouse was recorded with a camera driven tracker system (Ethovision XT).

Water maze test

Learning and memory function was assessed by the well-established Morris water maze test at UAB animal behavior core facility after open field and elevated plus maze tests as we have described previously [22]. During day 1 through day 5 of the testing period, the mice were placed in the water next to and facing the wall successively in north (N), east (E), south (S), and west (W) positions (4 trials/day/mouse with the inter-trial interval of 2 min). All of the mice were tested at the same day in a counterbalanced order. The escape latency (from the time a mouse was placed into the water till it found the platform), swim path-length (distance), and swim speed were recorded simultaneously with a camera driven tracker system, i.e., Noldus Ethovision system (version 7.1).

Measurement of the activities of PAI-1, tPA, and uPA

PAI-1 activity in the hippocampus and cortex was determined by reverse zymography, whereas the activities of tPA and uPA assessed by zymography as we have described previously [20, 23]. For reverse zymographic analysis of PAI-1 activity, equal amounts of proteins were loaded onto 12% polyacrylamide gel containing 5 μg/ml purified human plasminogen (Molecular Innovation), 0.5 IU /ml of human uPA (672081, Milllipore), and 2 mg/ml casein. For analysis of tPA and uPA activities, 12% polyacrylamide gels containing 5 μg/ml plasminogen and 2 mg/ml casein were used. After electrophoresis at 4°C, the gels were incubated with 0.1 M glycine (pH 8.3) at 37°C for 18 h, then stained with 0.5% Coomassie brilliant blue and destained in a solution containing 45% methanol, 5% acetic acid and 50% water. To avoid the possible interference of matrix metalloproteinases (MMPs), EDTA (2 mM) was included in the glycine-NaOH buffer during the incubation period. Gels without plasminogen were also run simultaneously to ensure that the lytic bands are due to plasminogen activators. The intensities of the dark band (PAI-1 activity, due to an inhibition of casein degradation) and white lytic bands of tPA and uPA were quantified using Image J software.

Plasmin activity

Plasmin activity in the hippocampus and cortex was measured using a specific chromogenic substrate Tosyl-glycyl-prolyl-lysine-4-nitranilide-acetate (Chromzyme PL, from Roche Applied Sciences) as described before [20]. Plasmin activity was calculated using the 4-nitraniline extinction coefficient ($\varepsilon_{405nm} = 1 \times 10^4$ M^{-1} cm^{-1}) and expressed as units per protein concentration.

Immunohistochemical staining of Aβ deposits and low density lipoprotein related protein-1 (LRP-1) in the hippocampus and cortex of APP/PS1 mice

Immunohistochemical staining of Aβ deposits (plaques) was conducted using monoclonal anti-human Aβ antibody 6E10 (Covance, Emeryville, CA) as we have described previously [20, 22]. Brain Aβ plaques in the cerebral cortex and hippocampus were semi-quantified by determining the percentage of the total section positively labeled by Aβ antibody using Axiovision automatic measurement software [20, 22]. For detection of LRP-1 protein in brain microvessels, immunohistochemical staining was conducted using anti mouse LRP-1 light chain monoclonal antibody (5A6, Calbiochem).

ELISA analyses of $A\beta_{40}$ and $A\beta_{42}$

For the measurement of soluble and insoluble Aβ in the brain, the hippocampus and cortex were processed as we have described previously [20, 22, 24]. Briefly, cortex and hippocampus tissues were homogenized in an Aβ extraction buffer containing 20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 1% Triton X-100, 2% sodium dodecyl sulfate soluble (SDS), and protease inhibitor cocktail and centrifuged at 100,000×g for 1 h. Supernatants were collected (SDS soluble)

and stored at –80°C until analysis. Pellets were dissolved in 70% formic acid (FA) with gentle shaking at room temperature for 1 h, and then centrifuged at 100,000×g for 1 h. The supernatants were collected (SDS insoluble/FA soluble) and neutralized/diluted 20 times with a neutralizing buffer containing 1 M Tris, 0.5M Na_2HPO_4, and 0.05% NaN_3, before used for ELISA. The amounts of SDS soluble and insoluble $A\beta_{42}$ and $A\beta_{40}$ in hippocampus and cortex as well as the amounts of $A\beta_{42}$ and $A\beta_{40}$ in the plasma were quantified using the ELISA kits from Covance (Emeryville, CA).

Western analysis

Mouse hippocampus and cortex were homogenized in a tissue extraction buffer containing 2% SDS and protease inhibitor cocktail. Western analyses of full-length AβPP, alpha/beta C-terminal fragments (α-/β-CTFs), and LRP-1 were performed on 4–20% gradient gels (Invitrogen). Full-length AβPP protein was detected with 6E10 antibody; α- and β-CTFs determined using rabbit polyclonal antibody CT695 against the C terminus of AβPP (Invitrogen) [20]; LRP-1 protein was detected using anti mouse LRP-1 light chain monoclonal antibody (5A6) as we have described previously [24]. Semi-quantification of the band intensities was performed using Image J software and normalized by β-actin band.

SHSY5Y-APP cell culture and treatment

Neuroblastoma cells, which overexpress human APP695 mutant protein SHSY5Y-APP (kindly provided by Dr. Chris Miller at Kings College, London), were cultured in DMEM medium supplemented with 1% penicillin/streptomycin, 10% FBS (heating), 500 μg/ml G418 antibiotic for 48 h. After washing with serum-free DMEM medium to remove FBS, cells were cultured in the medium containing 1% endothelial cell growth stimulant (ECGS) plus 8 μg/ml plasminogen and 0.16 μg/ml uPA (Calbiochem, CN: 672081) plus or minus 1.0 μg/ml of active human PAI-1 (PAI-A, Molecular Innovations) with or without TM5275 (25 μM) for 24 h. The culture medium was collected and concentrated; the amounts of $A\beta_{40}$ and $A\beta_{42}$ were determined by ELISA.

Statistical analysis

The results presented are means and standard deviations of 6-8 mice. Memory function data were analyzed by the two-way analysis of variance (ANOVA) (time and treatment). *Post hoc* analyses were conducted with Tukey's test. Statistical analyses of other parameters were conducted by one-way ANOVA and the statistical significance was determined by Fisher LSD test wherein $p < 0.05$ is considered statistically significant.

RESULTS

TM5275 administration causes no obvious toxicity

Four and half month old female APP/PS1 mice were gavaged daily with either vehicle or 40 mg/kg of TM5275 for 6 weeks. The results show that there is no significant difference in body weights between TM5275 and vehicle treated mice (data not shown), suggesting that TM5275, under the treatment conditions, does not cause obvious toxicity to mice. The results further support the notion that TM5275 is relatively safe when administrated to animals as shown in the previous studies from this and from others groups [25, 26].

Oral administration of TM5275 inhibits PAI-1 activity and increases the activities of tPA/uPA and plasmin in the hippocampus and cerebral cortex of APP/PS1 mice

PAI-1 is a physiological inhibitor of tPA and uPA. To confirm that oral administration of TM5275 inhibits PAI-1 activity in mouse brain, reverse zymography, which reveals PAI-1 activity, and zymography, which measures tPA and uPA activities, were conducted as we have described previously [20, 24]. The results show that treatment with TM5275 significantly reduces PAI-1 activity and increases the activities of uPA and tPA (Fig. 1A, B) as well as plasmin (Fig. 1C) in the hippocampus and cortex of APP/PS1 mice. These results suggest that TM5275 can get into the brain and is effective in blocking PAI-1 activity *in vivo*.

Oral administration of TM5275 improves memory of APP/PS1 mice

Morris water maze tests were conducted to assess learning and memory function after TM5275 treatment and the results were analyzed with the two-way ANOVA followed by Turkey's test. The results show that, compared to vehicle treated control, treatment

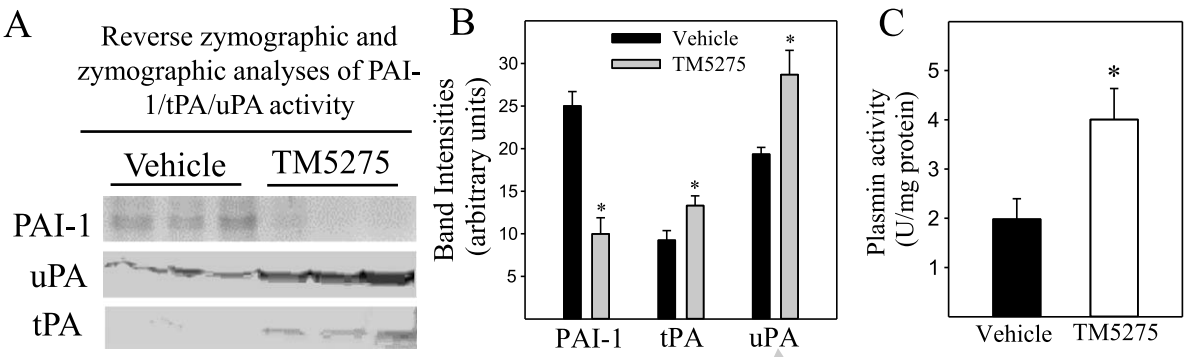

Fig. 1. Effect of oral administration of TM5275 on the activities of PAI-1, tPA, and uPA and plasmin in the hippocampus and cortex of APP/PS1 mice. A) Reverse zymographic and zymographic analyses of the activities of PAI-1, tPA, and uPA. B) The band intensities were semi-quantified using Image J software. C) Plasmin activity was determined using a specific chromogenic substrate as described in the Materials and Methods section. *Significantly different from corresponding vehicle treated mice ($p < 0.05$, $n = 5$–6).

with TM5275 significantly decreases the escape latencies in APP/PS1 mice by day 3, day 4 and day 5 (Fig. 2A). There is no significant difference in swimming distances (Fig. 2B) or swimming speeds (Fig. 2C) between vehicle and TM5275 treated mice in all other time points except day 4, in which time the swimming distance is significantly decreased in TM5275 versus vehicle treated group (Fig. 2B). The open field and elevated plus maze test results show that there is no significant difference between vehicle and TM5275 treated APP/PS1 mice in their anxiety level (Fig. 2D, E). Together, the results suggest that the decrease in the escape latencies in TM5275 treated APP/PS1 mice results from improved learning/memory function rather than changes in the motor activity or anxiety level.

Oral administration of TM5275 reduces Aβ burden in the cortex and hippocampus of APP/PS1mice

To elucidate whether improvement of cognitive function in TM5275 treated APP/PS1 mice was associated with a decrease in brain Aβ burden, we measured SDS soluble and insoluble $A\beta_{42}$ and $A\beta_{40}$ as well as Aβ plaques in the cortex and hippocampus by ELISA and immunohistochemistry staining techniques, respectively. The results show that inhibition of PAI-1 activity with TM5275 significantly reduces the amounts of Aβ plaques (Fig. 3A, B) as well as soluble and insoluble $A\beta_{40}$ (Fig. 3C) and $A\beta_{42}$ (Fig. 3D) in the hippocampus and cerebral cortex of APP/PS1 mice. These data suggest that TM5275 improves memory of APP/PS1 mice probably by reducing brain Aβ burden.

Oral administration of TM5275 has no significant effect on the steady state levels of full length AβPP, alpha-C-terminal fragment, or beta-C-terminal fragment

To elucidate the mechanism whereby TM5275 treatment reduces brain Aβ burden in APP/PS1 mice, the amounts of full-length AβPP (FL-AβPP), alpha C-terminal fragment (α-CTF), and beta C-terminal fragment (β-CTF) were determined by western analyses. The results show that there is no significant difference in the amounts of FL-AβPP (≈100 kDa), α-CTF, or β-CTF between APP/PS1 mice treated with vehicle or TM5275 (Fig. 4A, B). The results suggest that TM5275 reduces Aβ burden in the hippocampus and cerebral cortex of APP/PS1 mice not by decreasing AβPP synthesis or Aβ production.

Oral administration of TM5275 increases the protein abundance of LRP-1 at the BBB and plasma concentration of $A\beta_{42}$ in APP/PS1 mice

Low-density lipoprotein receptor related protein 1 (LRP-1), a multi-function endocytic receptor expressed on endothelium of the BBB, plays a major role in transporting Aβ out of the brain (efflux). In addition to APP/Aβ, LRP-1 also binds to a diverse array of structurally and functionally unrelated ligands, including PAI-1. By forming a complex with LRP-1 through the uPA-uPA receptor, PAI-1 triggers internalization of LRP-1 protein [27, 28]. To determine whether administration of TM5275 alters brain LRP-1 protein level, immunohistochemical staining and Westerns were conducted. The results show that oral administration of TM5275 signifi-

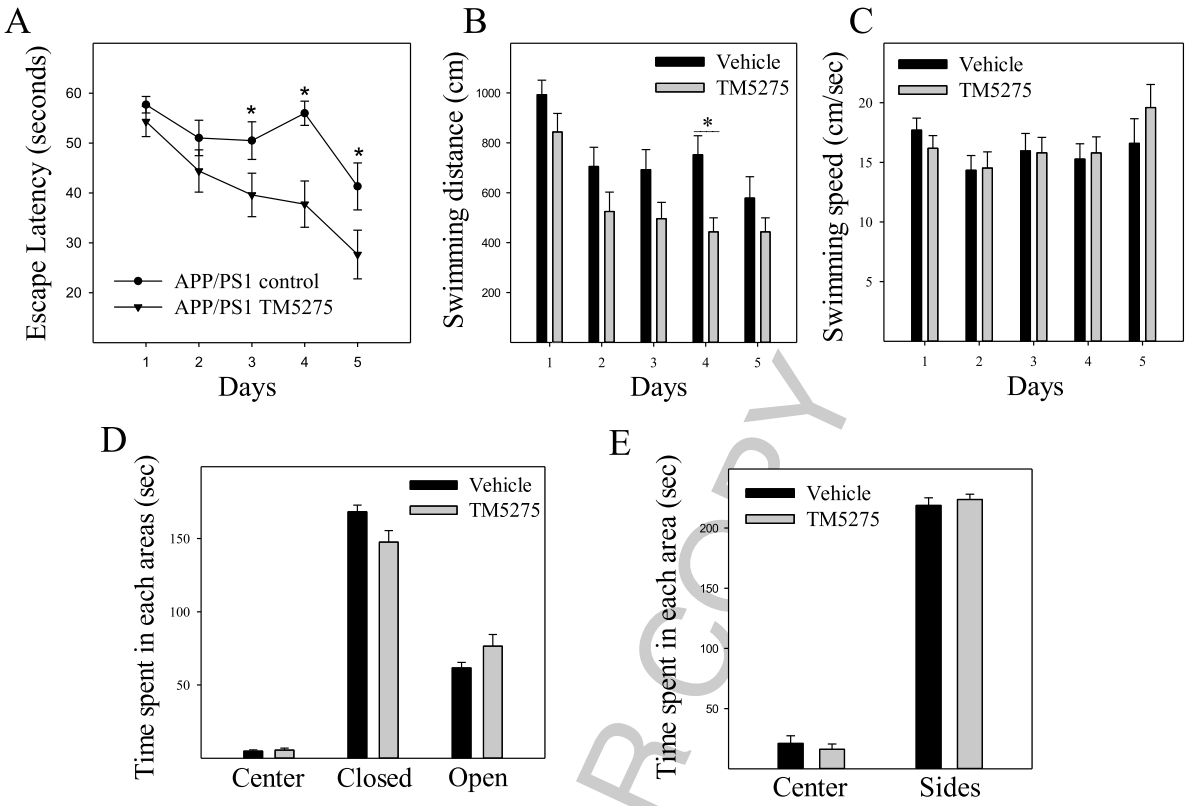

Fig. 2. Effect of TM5275 on learning and memory function of APP/PS1 mice. Learning and memory function was assessed by a well-established Morris water maze test as described in the Materials and Methods section. A) Time-dependent changes in the escape latencies of control (vehicle) and TM5275 treated APP/PS1 mice; B) Swimming distances; C) Swimming speeds. Elevated plus maze (D) and open field test (E) were also conducted to evaluate the fear and anxiety levels of mice. *Significantly different from vehicle treated controls ($p < 0.05$, $n = 6–7$).

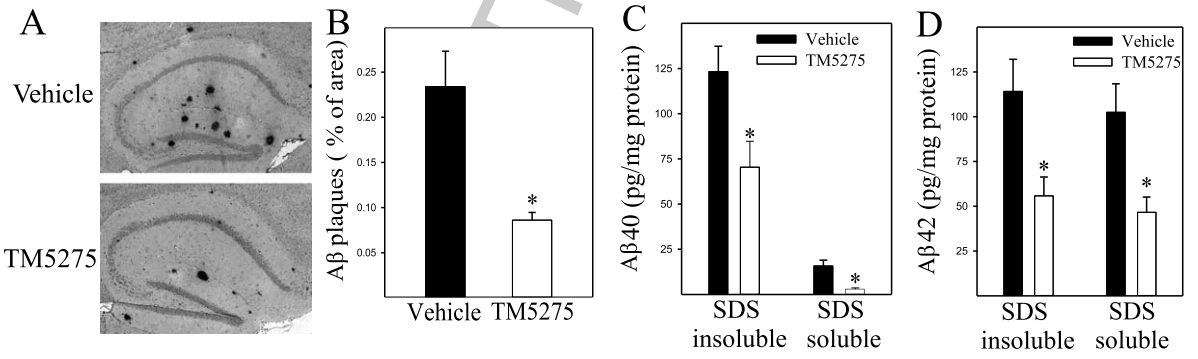

Fig. 3. Effect of TM5275 on Aβ loads in the hippocampus and cortex of APP/PS1 mice. A) Aβ deposits/plagues were revealed by immunohistochemical staining, quantified by the histomorphometry system, and expressed as percentage of total area of the cortex and hippocampus (B). The amounts of SDS soluble and SDS insoluble $A\beta_{40}$ (B) and $A\beta_{42}$ (C) in the cortex and hippocampus of APP/PS1 mice were determined by ELISA and calculated based on the total protein content. *Significantly different from vehicle treated controls ($p < 0.05$, $n = 6–8$).

cantly increases the abundance of LRP-1 protein at the BBB of APP/PS1 mice (Fig. 5A-C). Associated with the increased abundance of LRP-1 protein at the BBB, plasma level of $A\beta_{42}$ is significantly increased in TM5275 treated mice, compared to vehicle treated control APP/PS1 mice (Fig. 5D). These results suggest that increased PAI-1 expression in AD may lead to decreased Aβ efflux through internalizing/reducing LRP-1 expression at the BBB. TM5275 treatment reduces brain Aβ load at least in part by

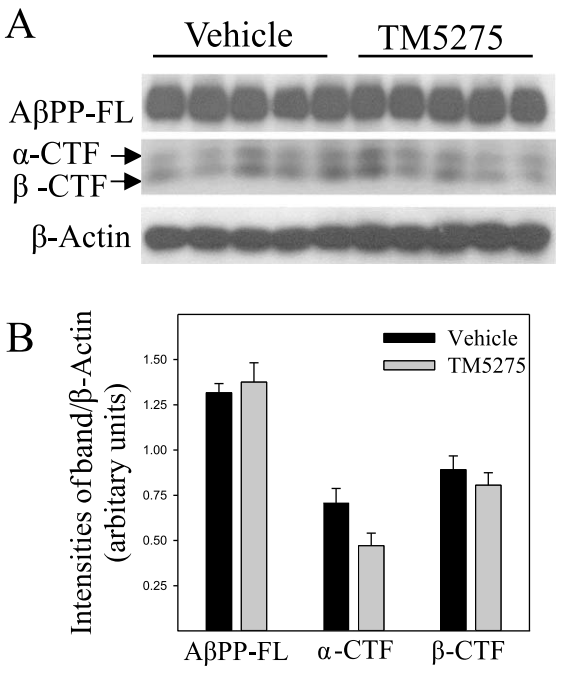

Fig. 4. Effect of TM5275 on the amounts of full-length AβPP, α-CTF, and β-CTF in the hippocampus and cortex of APP/PS1 mice. Full-length AβPP (FL-AβPP) and α/β-CTF in hippocampus and cortex homogenate of APP/PS1 mice were determined by Western analyses using 4–20% gradient SDS-PAGE and antibodies to human Aβ (6E10) and C terminus of human AβPP (CT695). The intensities of the bands were semi-quantified by densitometric scanning and normalized by β-actin. *Significantly different from vehicle treated mice ($p < 0.05$, $n = 5$–6).

promoting Aβ efflux through inhibiting PAI-1 and thereby LRP-1 internalization/degradation.

TM5275 reduces Aβ level in the culture medium of SHSY5Y-APP neuroblastoma cells

To determine whether TM5275 promotes Aβ degradation, SHSY5Y-APP neuroblastoma cells, which have been stably transfected with APP695,

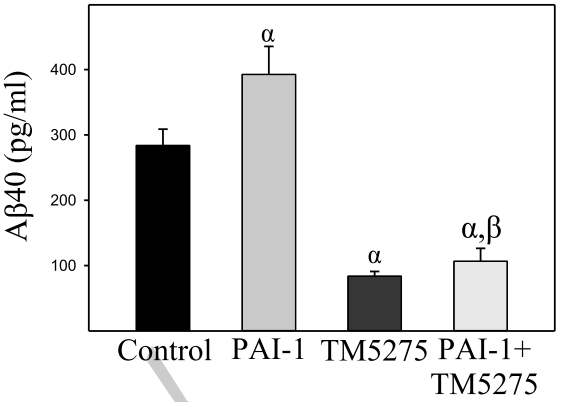

Fig. 6. Effect of TM5275 on Aβ concentration in the culture medium of SHSY5Y-APP cells. The levels of $A\beta_{40}$ in the culture medium of SHSY5Y-APP cells were determined by ELISA as described in the Materials and Methods section. α, Significantly different from vehicle treated control; β, significantly different from PAI-1 alone treated group ($p < 0.05$, $n = 3$).

were cultured in serum-free medium containing plasminogen and uPA plus or minus active human PAI-1 with or without TM5275 for 24 h. The concentrations of $A\beta_{40}$ in the medium were determined by ELISA. The results show that incubation of SHSY5Y-APP cells with active PAI-1 protein significantly increases the level of $A\beta_{40}$ in the cultured medium (Fig. 6). Treatment of SHSY5Y-APP cells, with TM5275 with or without PAI-1 protein, on the other hand, dramatically reduces $A\beta_{40}$ level in the culture medium (Fig. 6). The results suggest that TM5275 treatment decreases $A\beta_{40}$ burden in the hippocampus and cerebral cortex at least in part through inhibiting PAI-1 activity and thereby promoting Aβ degradation.

DISCUSSION

Accumulation of Aβ in the brain, a pathological feature of AD, is considered to be a central

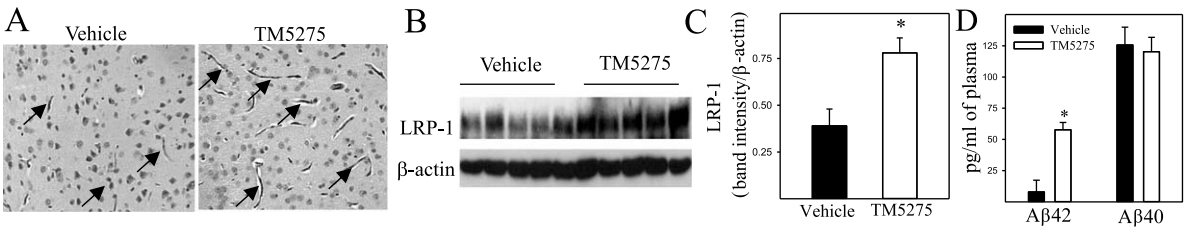

Fig. 5. Effect of TM5275 on the protein abundance of LRP-1 at the BBB of the hippocampus and cortex as well as plasma Aβ levels in APP/PS1 mice. A) Immunohistochemical staining of LRP-1 at the BBB; B) Western blotting pictures of LRP-1 protein in hippocampus and cortex homogenate; C) Semiquantified western data and the band intensities normalized by β-actin. D) Plasma $A\beta_{40}$ and $A\beta_{42}$ concentrations were measured by ELISA. *Significantly different from vehicle treated mice ($p < 0.05$, $n = 5$).

disease-causing and disease-promotion event [1–4]. Although it has been well-documented that increased production of Aβ, due to *APP* and *PS1/PS2* gene mutations, is attributed to brain Aβ accumulation in familial AD, the mechanism underlying brain Aβ accumulation in sporadic AD, which account >95% of AD cases, remains unclear. PAI-1 is a primary inhibitor of tPA and uPA, which convert plasminogen into plasmin, a serine protease playing a critical role in Aβ degradation [13–17]. Previous studies from this laboratory and from others have shown that PAI-1 expression is increased in the brain of APP/PS1 mice and in AD [19–21]; knockout of the PAI-1 gene or inhibiting PAI-1 activity with a small-molecule PAI-1 inhibitor reduced brain Aβ burden and reversed cognitive deficits in these familial AD models [10, 20]. In this study, we further show that oral administration of a small molecule PAI-1 inhibitor TM5275 increases the activities of tPA, uPA, and plasmin as well as the expression of LRP-1 at the BBB, a salvage receptor involved in Aβ transport out of the brain, and plasma $A\beta_{42}$ level. This is associated with a reduction of Aβ load in the hippocampus/cortex and an improvement of learning/memory function of APP/PS1 mice. Together, the data suggest that increased PAI-1 expression contributes importantly to brain Aβ accumulation and memory decline and that TM5275 may have therapeutic potential for the treatment of AD in part through reducing brain Aβ accumulation.

The mechanism whereby TM5275 decreases brain Aβ load is unclear. As PAI-1 is a primary inhibitor of tPA and uPA and thereby plasminogen activation and as plasmin plays a critical role in the degradation of Aβ, it is hypothesized that TM5275 reduces brain Aβ load by inhibiting PAI-1 activity and thereby stimulating plasmin-mediated Aβ degradation. This hypothesis is supported by the following data: 1) Administration of TM5275 to APP/PS1 mice leads to inhibition of PAI-1 activity, increases in the activities of tPA, uPA, and plasmin, and a decline in Aβ load in the hippocampus and cortex; 2) Treatment of SHSY5Y-APP cells, which overexpress and secrete full-length of AβPP protein [29, 30], with active human PAI-1 increases, whereas treatment with TM5275 reduces, $A\beta_{40}$ in the cultured medium; and 3) Treatment of APP/PS1 mice with TM5275 has no significant effect on the amounts of full-length AβPP, α-CTF, or β-CTF in the hippocampus/cortex, suggesting that TM5275 does not affect AβPP synthesis or Aβ production.

In addition to inhibition of tPA and uPA activities, PAI-1 has many other functions, including binding to uPA-uPA receptor (uPAR) and thereby modulating cell attachment and signaling. LRP-1 is a multi-function endocytic receptor expressed on endothelium of the BBB and plays a major role in transporting Aβ out of the brain [31–33]. It has been reported that the expression of LRP-1 at the BBB is decreased in AD and in a pre-AD stage referred to as amnestic mild cognitive impairment, associated with a decline in Aβ efflux and an increase in brain Aβ burden [31, 34, 35]. LRP-1 expression is also decreased with increased age in the brain of rodents [36]. The mechanism underlying the decrease in LRP-1 expression during aging and in AD, however, is unclear. In addition to AβPP/Aβ, LRP-1 also binds to a diverse array of structurally and functionally unrelated ligands, including PAI-1. By forming a complex with LRP-1 through the uPA-uPAR, PAI-1 triggers internalization of LRP-1 protein [27, 28]. Interestingly, our previous studies showed that suppression of PAI-1 expression with tert-butylhydroquinone was associated with an increase in the abundance of LRP-1 at the BBB and a decrease in brain Aβ load in APP/PS1 mice [24]. In this study, we show that treatment of APP/PS1 mice with TM5275 also increases LRP-1 protein abundance at the BBB in hippocampus/cortex. Association with the increase in the expression of LRP-1 at the BBB, TM5275 treatment increases plasma level of $A\beta_{42}$, but not $A\beta_{40}$. The mechanism underlying differential effects of TM5275 administration on plasma $A\beta_{40}$ and $A\beta_{42}$ level is unclear at moment. Together, the data suggest that PAI-1 may promote brain Aβ accumulation through reducing LRP-1 expression at the BBB and thus the efflux of Aβ from the brain. TM5275 treatment reduces brain Aβ load probably in part through blocking the interaction between PAI-1, uPA/uPAR, and LRP-1 and thus preventing LRP-1 from internalization and degradation, leading to an increase in Aβ efflux from the brain.

Besides involved in Aβ clearance, plasminogen system also plays a role in memory formation. Long-term potentiation (LTP) at hippocampal synapses correlates with learning and memory. Tissue type plasminogen activator (tPA) is a major type of plasminogen activator expressed in the brain and plays an important role in hippocampal LTP [37–39]. tPA activity is decreased with age and in AD patients [40–43] and tPA heterozygous knockout mice have significantly decreased synaptic and memory function, compared to tPA wild type Tg2576 mice [44].

It has also been reported that systemic chronic administration of recombinant tPA improves the cognitive function in Tg256 mice [11]. Together, these data suggest that decreased tPA activity may contribute to LTP impairment in AD. Whether TM5275 treatment improves memory of APP/PS1 mice through inhibiting PAI-1 and thus increasing tPA activity, which promotes LTP, remains to be determined. It should also be pointed out that a recent study shows that inhibition of PAI-1 with another PAI-1 inhibitor, PAI-039, reverted Aβ-mediated tau hyperphosphorylation and inhibition of brain-derived neurotrophic factor (BDNF) maturation in cultured neurons [45]. Administration of PAI-039 to 14-month-old Tg2576 mice restored the level of matured BDNF and improved cognitive function but had no significant effect on brain Aβ load [45]. The authors concluded that upregulation of PAI-1 leads to insufficient neurotrophic support and thus neurodegeneration in AD [45]. The mechanism underlying the different effects of PAI-039 and TM5275 on brain Aβ load is unclear at moment and is probably related to the differences in mouse models (Tg2576 versus APP/PS1 mice), age of the mice (14 months versus 4.5 months), the chemical properties and doses used in these two studies. Whether TM5275 administration improves learning and memory function of APP/PS1 mice through promoting BDNF maturation remains to be determined.

We want stress that PAI-1, as a multiple functional molecule, is involved in the pathogenesis of many diseases besides AD, including cardiovascular diseases, diabetics, fibrosis, and cancer. In general, increased PAI-1 expression/activity is believed to contribute to the pathogenesis of these diseases. Therefore, treatment of AD with PAI-1 inhibitor may have beneficial effect for the treatment of these co-current diseases. However, it should be mentioned that emerging evidence suggests that there is an inverse relationship between AD and cancer [46–50], although some studies do not support this notion [51–53]. More well-controlled prospective studies are needed to conclude the negative relation between AD and cancer and to identify the factors responsible for this relationship. If it is true that there is a negative relationship between AD and cancer, treatment of AD patients with TM5275 may post increasing risk of developing cancer to the patients.

In summary, we demonstrate in this study that oral administration of a small-molecule PAI-1 inhibitor TM5275 significantly decreases brain Aβ burden and improves memory of APP/PS1 mice at a dose which induces no obvious toxicity. We also show that oral administration of TM5275 inhibits PAI-1 activity and increases the activity of tPA, uPA, and plasmin in the hippocampus and cortex, suggesting that TM5275 can pass the BBB efficiently. These data provide first line of evidence that TM5275 is a relatively safe and potent anti-amyloidosis agent with promising therapeutic potential for the treatment of AD.

ACKNOWLEDGMENTS

The work was supported by the grants from National Institute of Health and National Institute of Aging to Rui-Ming Liu (R01 HL088141 and R21 AG046701). This work was also supported in part by P30 NS47466 (UAB animal behavior core facility). The authors would also like to thank Drs. Namasivayam Ambalavanan and Brian A. Halloran for their technical assistance.

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/18-0241r2).

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