Potassium 2-(1-hydroxypentyl)-benzoate improves memory deficits and attenuates amyloid and tau pathologies in a mouse model of Alzheimer’s disease

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Running title: PHPB attenuates Aβ and tau pathologies in APP/PS1 mice

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Abbreviations:

AD, Alzheimer’s disease; Aβ, amyloid-β; dl-PHPB, Potassium 2-(1-hydroxypentyl)-benzoate;
NFT, neurofibrillary tangles; APP, amyloid precursor protein; PS1, presenilin 1; PKC, protein kinase C; GSK-3β, glycogen synthase kinase 3β; CDK-5, cyclin-dependent kinase; PI3K,
Phosphoinositide 3 kinase; SK-N-SH APPwt, neuroblastoma SK-N-SH cells over-expressing WT human APP695; SAMP8, senescence-accelerated mouse prone 8; PKB, protein kinase B; ADAM, A member of the disintegrin and metalloprotease; IHC, Immunohistochemistry.

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ABSTRACT

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized by amyloid-β (Aβ) deposition and neurofibrillary tangles (NFT). Potassium 2-(1-hydroxypentyl)-benzoate (dl-PHPB), has been shown to have neuroprotective effects on cerebral ischemic, vascular dementia and Aβ-induced animal models by inhibiting oxidative injury, neuronal apoptosis and glial activation. The aim of the present study was to examine the effect of dl-PHPB on learning and memory in amyloid precursor protein (APP) and presenilin 1 (PS1) double-transgenic AD mouse model (APP/PS1) and the mechanisms of dl-PHPB in reducing Aβ accumulation and tau phosphorylation. Twelve-month old APP/PS1 mice were given 30 mg/kg dl-PHPB by oral gavage for 3 months. Dl-PHPB treatment significantly improved the spatial learning and memory deficits compared to the vehicle-treated APP/PS1 mice. In the meantime, dl-PHPB obviously reduced tau hyperphosphorylation at Ser199, Thr205 and Ser396 sites and slightly resition and in APP/PS1 mice. This was accompanied by APP phosphorylation reduction and protein kinase C (PKC) activation. In addition, the expressions of cyclin-dependent kinase (CDK-5) and glycogen synthase kinase 3β (GSK-3β), the most important kinases involved in tau phosphorylation, were markedly decreased by dl-PHPB treatment. Phosphorylated Akt and phosphoinositide 3 kinase (PI3K) levels of APP/PS1 mice were significantly reduced compared to wild-type mice, and dl-PHPB reversed the reduction. The effects of dl-PHPB on decreasing tau phosphorylation and kinases activations were further confirmed in neuroblastoma SK-N-SH cells over-expressing WT human APP695 (SK-N-SH APPwt). These data raised the possibility that dl-PHPB might be a promising multi-target neuronal protective.
agent for the treatment of AD.
Introduction

Alzheimer’s disease (AD), the leading cause of dementia in the elderly, is characterized by the extracellular senile plaques (SP) deposition and intracellular neurofibrillary tangles (NFT) (Selkoe, 1994). β-amyloid peptide (Aβ), generated from amyloid precursor protein (APP) cleavage, compose the core of SP. APP is cleaved by at least two pathways, amyloidogenic and non-amyloidogenic. In amyloidogenic pathway, APP is cleaved by β-secretase and γ-secretase (Haass et al, 1992). In the alternative pathway, APP is cleaved by α-secretase within the sequence of the Aβ peptide and a secreted form of APP fragment (αAPPs) is released into the extracellular media thereby precluding the formation of Aβ (Esch et al, 1990). APP phosphorylation at its C-terminal Thr668 facilitates its processing (Cruz et al, 2006). The main component of NFT is hyperphosphorylated tau that aggregates as paired helical filaments (PHFs). Tau is a microtubule-associated protein that stabilizes and promotes microtubule polymerization (Drechsel et al, 1992). At present, more than 40 serine/threonine phospho-sites have been identified on PHF tau, many of which are shown specifically in AD brain tissue (Hanger et al, 2009). Phosphorylation of tau is balanced by the activity of multiple kinases and phosphatases. Glycogen synthase kinase 3 (GSK-3) and cyclin-dependent kinase (CDK-5) have been identified as the main kinases for pathogenesis (Wen et al, 2008). The levels and enzyme activities of GSK-3β and CDK-5 have been shown to be increased in AD brains (Pei et al, 1998). Overexpressions of GSK-3β and CDK-5 in transgenic mice have been reported to increase tau phosphorylation and to induce cognitive impairment (Lucas et al, 2001; Cruz et al, 2003). In addition, CDK-5 and GSK-3β have been shown to promote Aβ synthesis, with inhibition of GSK-3 reported to decrease Aβ levels...
(Cruz et al, 2006; Phiel et al, 2003). GSK-3β and CDK-5 have been suggested as the drug targets for treatment of AD.

Potassium 2-(1-hydroxypentyl)-benzoate (d/-PHPB) is a novel drug candidate for treatment of cerebral ischemia. It was approved by the Food and Drug Administration of China for clinical trial of ischemic stroke in 2009. At present, the phase II clinical trial has been started. Previous studies showed that d/-PHPB improved the neurobehavioral deficits and reduced infarct volume in the cerebral ischemic animal model (Zhang et al, 2006). It might protect neurons against H2O2-induced apoptosis by modulating PKC signaling pathway (Hu et al, 2012). Recently, d/-PHPB was shown to improve the learning and memory deficits, reducing oxidative stress and glia activation in the cerebral hypoperfused rats (Zhao et al, 2013). These results suggested that d/-PHPB might be a potential drug candidate for treatment of AD.

In the present study, we examined the effect of d/-PHPB treatment on the cognitive impairment in APP and presenilin 1 (PS1) double-transgenic AD mouse model (Trinchese et al, 2004). Moreover, we investigated the mechanisms underlying the compound on AD related pathology, such as Aβ generation, APP processing, tau hyperphosphorylation and the signaling pathway.
Materials and Methods

Animals and treatment

dl-PHPB (purity >98%) was synthesized by the Department of Medical Synthetic Chemistry, Institute of Materia Medica and dissolved in distilled water at a concentration of 30 mg/ml. APP/PS1 double-transgenic mice used were got from The Jackson Laboratory (strain name, B6C3-Tg(APPswe,PSEN1dE9)85Dbo/J; stock number 004462). These mice express a chimeric mouse/human APP containing the K595N/M596L Swedish mutations and a mutant human PS1 carrying the exon 9-deleted variant under the control of mouse prion promoter elements, directing transgene expression predominantly to CNS neurons (Jankowsky et al, 2001). The two transgenes cosegregate in these mice. Mice were group-housed in an animal room maintained at 23±1°C with a 12h light/dark cycle and free access to water and food. All experiments were approved and performed in accordance with the institutional guidelines of the Experimental Animal Center of the Chinese Academy of Medical Science, Beijing, China. Male APP/PS1 transgenic mice and wild-type littermates were randomly divided into three groups: treated APP/PS1 mice, untreated APP/PS1 mice and untreated wild-type mice. Treated groups received dl-PHPB by oral gavage 5 days per week at a dose of 30 mg/kg body weight. Untreated groups received distill water alone as a vehicle control. Treatment was started when the mice were 12 months old and was continued for 12 weeks. The body weight of each mouse was recorded every two weeks. After behavioral testing was completed, the mice were sacrificed by CO₂ inhalation and blood was collected by cardiac puncture followed by transcardial perfusion with 20-30 ml phosphate buffered saline (PBS). The brain was
removed. One hemi-brain was snap frozen in liquid nitrogen and stored at -80°C until analysis, and the other hemi-brain was fixed in 4% paraformaldehyde for 2h followed by incubation in graded sucrose at 4°C.

**Step-down passive avoidance test**

The effect of *dl*-PHPB on memory impairments in mice was studied using a step-down passive avoidance test according to the previously reported method (Takashima et al, 1989), but with a minor modification. The experimental compartment was a (15 cm × 15 cm × 30 cm) wooden box. The floor of the box consisted of parallel stainless steel bars. A wooden insulating platform (4 cm × 4 cm × 4 cm) was placed on the center of the grid floor. The experiment lasted three days. At the first day, mice were accustomed to the experimental box for 5 min. The acquisition trials were performed at the second day. An electric footshock (36 V) was delivered to the paws of the animal through the grid floor. The mice had to jump onto the platform to avoid the foot shock. The process would last 3 min until the mice were removed from the compartment. 24 hours after training, the retention was performed. The mouse was placed on the platform and the latency to jump down from the platform and error times were recorded as the measures of the retention. Error time means that how many times the mice jumped down the platform. A maximum retention latency of 3 minutes was given to mice that did not jump down from the platform.

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed using the ELITE ABC method (Vector
Laboratories, Burlingame, CA, USA) as previously described (Peng et al, 2010). Ten micron sagittal cryosections of mouse brain were mounted on glass slides. Aβ plaques, microglia and astrocytes were recognized by the primary antibodies, 6E10 (Covance, Princeton, NJ, USA), anti-CD45 (Serotec Raleigh, NC, USA) and anti-GFAP (DAKO Carpinteria, CA, USA). Secondary biotinylated antibodies (anti-mouse, anti-rat and anti-rabbit) were obtained from Vector laboratories (Burlingame, CA, USA). Thioflavin S staining for fibrillar Aβ was performed by incubating slides in a 1% aqueous solution of Thioflavin S for 10 min followed by rinsing in 80% and 95% ethanol, and then distilled water. To quantify immunoreactivity and Thioflavin S staining, acquisition of images was performed in a single session using a Nikon camera mounted on a Nikon Eclipse 80i microscope (Nikon, Japan). Image analysis was performed using Image-Pro Plus 5.1 (Media Cybernetics, Bethesda, MD, USA). The threshold of detection was held constant during analysis. For all treatment groups, the percent area occupied by Aβ and Thio S in the cortex and hippocampal area were calculated for 3 equidistant sections per mouse.

**Determination of Aβ levels**

Aβ levels were measured in the brain homogenates (TBS, TBS-T and Guanidine-soluble fractions). Aβ-specific sandwich ELISAs were performed by using human Aβ42 immunoassay kit (Invitrogen, Carlsbad, CA, USA). The absorbance was recorded at a 450 nm wavelength using an uQuant microplate spectrophotometer (Bio-Tek Instruments. Inc. Rockville, MD, USA).
Cell culture

SK-N-SH/SK-N-SH APP695 human neuroblastoma cells were cultured using Dulbecco’s modified Eagle’s medium (DMEM) culture, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin. In addition, SK-N-SH APP695 cells were supplemented with 200 μg/ml G418. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. Prior to treatment, cells were maintained in serum-free media overnight. After incubation with the dl-PHPB for 24h, cells were collected, and total protein was extracted from the cells using lysis buffer containing 150 mM NaCl, 10 mM Tris, 1%NP-40, 1 mM EGTA, 1 mM Na3VO4, 10 mM NaF, 10% Glycerol and Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN, USA). Protein was determined by the Bradford method.

Western Blot Analysis

Standard western blotting analysis was performed. The samples of cortex and hippocampus were homogenized in the lysis buffer containing: 150 mM NaCl, 20 mM Tris–HCl (pH 7.6), 1 mM EDTA, 5 mM DTT, 1% Triton-X100, 10 mM NaF, 10 mM Na4O7P2, 1 mM Na3VO4 with a protease inhibitor cocktail. 40 μg protein per lane were run on polyacrylamide gel, transferred on to a PVDF membrane, blocked with 5% milk solution (nonfat dry milk in TBST) for 2 h, and subsequently incubated with primary antibodies diluted in blocking solution overnight. The primary antibodies used in the study are summarized in Table 1. After washing with TBST for 5 times, the membrane was incubated with secondary antibodies (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG) at room temperature for 1
h. The signals were detected using an enhanced chemiluminescence (ECL) kit, scanned using an LAS4000 Fujifilm imaging system (Fujifilm, Tokyo Japan), and analyzed by densitometric evaluation using the Quantity-One software (Bio-Rad, Hercules, CA, USA). The values were normalized to β-actin intensity levels.

**Statistical analysis**

All the data were expressed as mean ± the standard error of the means (SEM). One-way analysis of variance (ANOVA) using SPSS ver. 10.0 software was used for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.
Results

*Dl*-PHPB treatment improved the memory deficits in APP/PS1-Tg AD mice

During *d/-PHPB treatment of three months, we had been monitoring the general health of mice and did not find any abnormal changes. In addition, the body weights of mice were not significant different between *d/-PHPB treated and vehicle control mice (data not shown). Together, these data indicate that long-term treatment of *d/-PHPB had no significant toxicity in mice.

It has been widely reported that APP/PS1 AD transgenic mouse model develop memory impairment with increasing age (Trinchese et al, 2004). In this study, we evaluated whether *d/-PHPB contributed to attenuate Aβ-dependent behavioral deficits at the APP/PS1-Tg AD mice of 15 months old by step-down passive avoidance test. In the acquisition trial, all mice were able to actively jump onto the platform after footshock, and the latency time was not different across groups (data not shown). In the memory retention trial, the wild type mice spent 123.9±23.9 s on the platform before they jumped down it first time. The latency of APP/PS1-Tg AD mice treated with the vehicle control were 20±8.4 s (Fig.2A). In addition, the number of errors of APP-PS1-Tg mice was more than that of wild type mice (3.2±0.7 vs. 0.9±0.4) (Fig.2B). There was significant difference between the groups in the latency and number of errors (p<0.001 and p<0.01), indicating that APP-PS1-Tg mice had obvious memory deficits. However *d/-PHPB treatment extended the latency to 138.4±20.5 s and lowered the number of errors to 0.5±0.2. The therapeutic of *d/-PHPB markedly reversed the reduction of the latency (p<0.001) and the increase of number of errors in the transgenic mice.
(p<0.001). The above data suggested that *dl*-PHPB were likely to enhance the memory retention in APP/PS1-Tg AD mice.

In addition, we observed that there was not cognitive enhancement in the wild type mice treated *dl*-PHPB (Supplement Figure 1), indicating that *dl*-PHPB was not a cognitive enhancer.

**Dl-PHPB treatment reduced Aβ plaque deposition in APP/PS1-Tg AD mice**

APP/PS1 mice begin to develop Aβ deposition at 6 months of age (Reiserer et al, 2007). At 12 months old, they exhibit moderate levels of Aβ depositions (Peng et al, 2012). To determine whether the improvement of *dl*-PHPB on the memory deficits correlated with changes in Aβ levels in the brain, all mice were sacrificed following behavioral testing. The brains were removed for biochemical and immunohistochemical analyses. Total Aβ plaque load, including diffuse and compacted, fibrillar plaques, was detected by Aβ immunolabeling with a general Aβ monoclonal antibody 6E10, and fibrillar amyloid deposits by Thioflavin S staining. The results showed that long-term oral administration of *dl*-PHPB did not reduce total Aβ plaque burden in the cortex and hippocampus. However *dl*-PHPB significantly reduced Thio-S-positive plaque deposition in the hippocampus, indicating *dl*-PHPB might inhibit Aβ aggregation or promote Aβ plaque degradation (Fig.3A-B).

Then, we analyzed cerebral Aβ levels by ELISA and found that *dl*-PHPB had no significant effect on lowering Aβ levels of cortex (Fig.3C) and hippocampus (Fig.3D) in TBS-soluble, TBST-soluble and Guanidine-soluble brain homogenates. We deduced that


dl-PHPB had slightly Aβ-lowering effect in APP/PS1 mice, which might be not the main mechanism of dl-PHPB in cognitive improvement.

**dl-PHPB attenuated glial activation in APP/PS1-Tg AD mice**

Activated astrocytes and microglia are associated with Aβ plaque deposition in the brains of AD patients and transgenic AD mouse models (Itagaki et al., 1989; Matsuoka et al., 2001). Thus, we investigated the ability of dl-PHPB treatment to suppress astrocyte and microglial reactivity in the current study. The results showed that dl-PHPB treatment did not significantly reduced CD45 and GFAP immunoreactivity in APP/PS1-Tg mice (Fig. 4). It indicated that dl-PHPB did not appear to involve Abeta accumulation or inflammation (e.g., gliosis).

**Dl-PHPB directed APP processing towards the non-amyloidogenic pathway in APP/PS1-Tg AD mice**

We found that dl-PHPB treatment improved memory deficits and lowered Aβ plaque deposition in the brain of APP/PS1-Tg mice. To elucidate the possible mechanism, we investigated the effect of dl-PHPB on APP processing by Western-blot measurement. We chose the polyclonal antibody, R1736 (a gift from Dr. D. Selkoe), which was raised in rabbits against residues 595-611 of APP695 and labels αAPPs as a 98 kD band and full-length APP at ~110 kD. Dl-PHPB treatment increase the release of αAPPs by 23% in the cortex and had no effect in the hippocampus (Fig. 5A and 5B), indicating dl-PHPB might lightly regulate APP processing towards the non-amyloidogenic pathway. In addition, we determined the
effect of *dl*-PHPB on full-length APP levels by using the C-terminal APP polyclonal antibody, C8 (a gift from Dr. D. Selkoe). *Dl*-PHPB treatment had no effect on APP steady-state levels (Fig. 5A).

ADAM family enzymes catalyze the shedding of the ectodomain of APPs (Allinson et al., 2003). We examined ADAM 10 and ADAM17 which related to the pathogenesis of Alzheimer's disease (Buxbaum et al., 1998). *Dl*-PHPB treatment increased ADAM10 and ADAM17 levels to 24% and 11% in the cortex, and slightly downregulated ADAM10 and ADAM17 activities in the hippocampus. But the difference did not reach significance (Fig. 5A and 5C).

APP phosphorylation at C-terminal Thr668 plays an important role in its processing (Lee et al., 2003) and neurodegeneration (Chang et al., 2006). Thus, we determined APP phosphorylation in the brain tissues by Western blotting using an antibody against phospho-Thr668 of APP. The result showed that a robust elevation of phosphorylated APP was detected in the vehicle-treated APP/PS1 mice. In contrast, *dl*-PHPB significantly reduced the APP phosphorylation in the *dl*-PHPB-treated APP/PS1 mice (Fig. 5A). Quantitative analysis showed a 28% decrease in the cortex and 23% decrease in the hippocampus in the *dl*-PHPB-treated APP/PS1 mice compared with the vehicle-treated APP/PS1 mice (Fig. 5D).

It is well known that protein kinase C (PKC) is involved in the regulation of APP processing (Nitsch et al., 1992; Peng et al., 2007). PKC agonist phorbol esters have been shown to increase αAPPs release and decrease Aβ levels (Chen and Fernandez, 2004). PKCα was assessed by Western blot as shown in Figure 5A. Quantitative analysis of Western blot bands indicated a significant 23% increase in PKCα expression (p<0.05) and a 57% increase.
(p<0.05) in the hippocampus in dl-PHPB-treated APP/PS1 mice compared to vehicle control mice (Fig. 5E), indicating that dl-PHPB might enhance PKCα signaling thereby directing APP processing towards non-amyloidogenic pathway.

**Dl-PHPB treatment inhibited tau hyperphosphorylation in APP/PS1-Tg AD mice and APP-transfected SK-N-SH cells**

It has been demonstrated that the tau hyperphosphorylation appeared in the APP/PS1 mouse brain after the onset of Aβ deposition (Kurt et al, 2003). Tau protein can be phosphorylated in at least 38 serine/threonine sites in AD (Hanger et al, 1998). In an Aβ-intracereventriculally infused rat model and senescence-accelerated mouse prone 8 (SAMP8) animal model, we have found that dl-PHPB reduced tau abnormal hyperphosphorylation (data unpublished). In the present study, we examined tau hyperphosphorylation by western blot using antibodies against different phosphorylation sites on tau, including Ser199, Thr205, Ser396 and Ser404. As shown in Figure 6A, a markedly increase of tau phosphorylation at sites of Ser 199, Thr205 and Ser396 was observed in both cortex and hippocampus of the vehicle-treated APP/PS1 mice. In contrast, except for a slight decrease in the phosphorylation at Ser404 site, dl-PHPB treatment strongly reduced tau phosphorylation in Thr205 and Ser396 sites in both cortex and hippocampus. Quantitative analysis exhibited the decrease of tau phosphorylation at Thr205 were 30% and 47% in the cortex and hippocampus, respectively after dl-PHPB treatment (Fig. 6C). Dl-PHPB reduced tau phosphorylation at Ser396 site by 28% in the cortex and by 52% in the hippocampus relative to vehicle-treated APP/PS1 mice (Fig. 6D). In addition, a robust decrease about 57% in the tau phosphorylation at Ser199 was showed in the
hippocampus rather than in the cortex of the dl-PHPB-treated mice (Fig. 6B).

Our previous study has shown that tau phosphorylations at Ser199, Thr205 and Ser396 sites APP were significantly increased in Human neuroblastoma SK-N-SH cells overexpressing wild-type APP695 (SK-N-SH APPwt) (Peng et al, 2012). After a 24 hr treatment of the cells with dl-PHPB, the tau phosphorylation levels at Thr205 and Ser396 sites were dose-dependently decreased (Fig. 7A). Particularly treated with 10 μM dl-PHPB, tau phosphorylations at Thr205 and Ser396 sites were reduced by 42% (p<0.05) and 24%, (p<0.05) respectively (Fig. 7C and D). However, dl-PHPB treatment had no effect on tau phosphorylation at Ser199 site. The in vitro and in vivo data jointly confirmed that dl-PHPB could reverse tau phosphorylation.

*Dl*-PHPB attenuated the expressions of phosphorylated CDK-5 and GSK-3β in APP/PS1-Tg AD mice and APP-transfected SK-N-SH cells

Tau phosphorylation and dephosphorylation is in homeostasis in normal condition, and dependent on the activities of protein kinases and phosphatases. The abnormal tau hyperphosphorylation could be induced by upregulating tau kinase and downregulating tau phosphatases in AD brain. To further elucidate the mechanisms of *dl*-PHPB to inhibit tau hyperphosphorylation in APP/PS1 mice, we assessed the expressions of GSK-3β and CDK-5, two major kinases implicated in tau hyperphosphorylation at abundant sites. GSK-3β is activated through the phosphorylation at Tyr216 and inhibited by phosphorylating Ser9. Active GSK-3β contributes to phosphorylate tau and facilitates tangle formation (Wang et al, 1998; Cohen and Frame, 2001). Whereas, CDK-5 activation requires the phosphorylation at
Ser159 site (Sharma et al, 1999). Furthermore, GSK-3β and CDK-5 are the most important kinases responsible for APP phosphorylation. Thus, we detected the levels of phosphorylated GSK-3β and CDK-5 by using specific antibodies. The results showed that CDK-5 phosphorylation at Ser159 was significantly increased in the cortex (p<0.05) and hippocampus (p<0.05) in vehicle-treated APP/PS1 transgenic mice compared to the wild-type control mice. Dl-PHPB treatment exhibited a robust decrease in the phosphorylation of CDK-5 by 35% in the cortex and 37% in the hippocampus compared to the vehicle-treated APP/PS1 mice (Fig. 8A and 8B). In addition, we did not find a difference of total CDK-5 levels among groups (Fig. 8A and 8C). The data indicated that dl-PHPB treatment might reduce CDK-5 activity. Western blot analysis with an antibody against the phosphorylated GSK-β (Ser9) showed that dl-PHPB had no effect on the phosphorylation of GSK-3β at Ser9 in the cortex and hippocampus of APP/PS1 mice (Fig. 8A). However, western blot analysis of GSK-3α/β (Tyr279/216) showed a markedly enhancement (~79%) of GSK-3β phosphorylation at Tyr216 in the hippocampus of vehicle-treated APP/PS1 mice compared to wild-type controls (p<0.01). In contrast, dl-PHPB treatment robustly reduced the level of GSK-3β phosphorylation at Tyr216 in the hippocampus of APP/PS1 mice (~46%) compare to vehicle-treated APP/PS1 mice (p<0.01) (Fig. 8A and 8D). Although a slight increase in the phosphorylation of GSK-3α at Tyr279 was observed in the brain of the vehicle control-treated APP/PS1 mice relative to the wild-type mice, no significant difference was shown between the dl-PHPB and vehicle-treated APP/PS1 mice (Fig. 8A). We did not observed a significant difference in the total GSK-3β levels among the groups (Fig. 8A and 8E). The above results suggested that dl-PHPB long-term treatment has an inhibitory effect
on GSK-3β activity. In the meantime, we determined the PP2A activity, the most important phosphatase in the wild-type and APP/PS1 mice. The Western blot results showed that there were not a statistical difference in PP2A activities among the groups (Fig. 8A), indicating that dl-PHPB mainly inhibited tau hyperphosphorylation by regulating protein kinase in APP/PS1 mice.

Meantime, we also examined the GSK-3β and CDK-5 activities in SK-N-SH APPwt cells. The results were consistent with the in vivo data. The level of CDK-5 phosphorylation at Ser159 site was down-regulated by 23% after 10 μM dl-PHPB treatment, whereas the expression of total CDK-5 was not altered (Fig. 9A-C). In addition, western blot analysis showed that 1 μM dl-PHPB incubation markedly increased the phosphorylation of GSK-3β (Ser9) by 71% compared to control group (Fig. 9A and D). Moreover, western blot analysis exhibited that dl-PHPB robustly decreased GSK-3α (Tyr279) and GSK-3β (Tyr216) levels. Quantitative data indicated that dl-PHPB at the concentration of 10 μM reduced GSK-3α/β (Tyr279/216) by 49% (p<0.05) and 51% (p<0.01), respectively (Fig. 9A and E-F), respectively. No obvious change in the expression of total GSK-3β was observed after dl-PHPB treatment (Fig. 9A and G). These results indicated that dl-PHPB reduced tau hyperphosphorylation by regulating protein kinases CDK-5 and GSK-3β activities.

**Dl-PHPB up-regulated PI3K and Akt signaling pathway in APP/PS1-Tg AD mice**

Akt/protein kinase B (PKB) is a serine-threonine protein kinase in the downstream of phosphoinositide 3 kinase (PI3K). PI3K-Akt signaling pathway is necessary and sufficient for neuronal survival (Dudek et al, 1997). Activation of PI3K and Akt may be implicated in the
early signaling events involved in downregulating the level of phosphorylated Tau through GSK3β (Baki et al., 2004). A recent study also suggests that GSK-3β phosphorylation can be associated with phosphorylation of Akt at Ser473 residue (Sutton and Rushlow, 2011). Thus, we further investigated the effect of \textit{dl}-PHPB on PI3K-Akt signaling pathway by Western blot assay. The results showed that phosphorylated Akt levels were significantly decreased by 29% and 32% in the cortex and hippocampus of APP/PS1 mice compared to wild-type mice. Long-term treatment of \textit{dl}-PHPB inhibited the reduction of phosphorylated Akt activity in the cortex compared to vehicle-treated APP/PS1 mice (p<0.05). We did not found a significant difference in the total Akt levels among the groups (Fig. 10A-F). Moreover, PI3K levels were significantly reduced in the cortex (p<0.01) and hippocampus (p<0.01) of APP/PS1 mice compared to wild-type mice. In contrast, the reduction of PI3K level of cortex was reversed by \textit{dl}-PHPB treatment (p<0.05). The above results suggested that \textit{dl}-PHPB long-term treatment has an up-regulatory effect on PI3K-Akt signaling pathway (Fig. 10A, G-H).

**Discussion**

As a promising candidate for the treatment of stroke, although \textit{dl}-PHPB has been demonstrated to contribute to improve the learning and memory deficits in the hypoperfused rats, an usually used vascular dementia model, the actual therapeutic role of \textit{dl}-PHPB in AD pathology and dementia has not yet been ascertained. The present study firstly discloses a therapeutic effect of \textit{dl}-PHPB in APP/PS1 double transgenic AD mouse model. \textit{Dl}-PHPB treatment by oral gavage for three months significantly reversed memory impairment in APP/PS1 mice. Step-down passive avoidance task is a fear-aggravated test used to evaluate
learning and memory in rodent models of CNS disorder. One of the most common animal
tests in memory research is the inhibition to imitate activities or learned habits. The term
“passive avoidance” is usually employed to describe experiments in which the animal learns
to avoid a noxious event by suppressing a particular behavior. Step-down passive avoidance
test is also hippocampus-dependent. The CA1 region of the hippocampus is essential for
memory formation of one-trial passive avoidance (Izquierdo and Medina, 1997).

It is well known that Aβ plaque deposition and tau hyperphosphorylation are closely
associated with cognitive impairments in an AD mouse model (Chen et al, 2000;
Schindowski et al, 2006). The present study indicated that the beneficial effect of dl-PHPB on
cognitive improvement was possibly attributable to reduce Aβ plaque deposition and inhibit
tau abnormal hyperphosphorylation.

*dl*-PHPB significantly reduced Thioflavin S-positive compact Aβ plaque accumulation
might be due to directing APP processing towards a non-amyloidogenic pathway, because
dl-PHPB-treated APP/PS1 mice showed the enhancement of αAPPs release and the reduction
of APP phosphorylation at Thr668 site compared to the vehicle-treated APP/PS1 mice.
Alpha-APPs has been shown to be beneficial for memory function, and possesses
neuroprotective and neurotrophic properties (Mattson, 1997). It is likely that αAPPs derived
from *dl*-PHPB-mediated APP processing may serve as a neuroprotective agent and contribute
to the long-term benefit of *dl*-PHPB on memory in APP/PS1 mice. In addition, our previous
study had demonstrated that 3-n-butylphthalide (NBP) isoform could regulate APP
processing and lowered Aβ generation in AD mouse models (Peng, 2010, Peng 2012). It has
been demonstrated that Thr668 phosphorylated APP was significantly increased in AD brains.
Thr668 phosphorylation might regulate APP metabolism and facilitate the BACE1 cleavage of APP to increase Aβ generation (Lee et al, 2003). Thr668 in the cytoplasmic domain of APP was phosphorylated by a number of protein kinases, such as GSK-3β, CDK-5 and CDC-2 (Aplin et al, 1996; Iijima et al, 2000). GSK-3β and CDK-5 are major kinases of tau protein phosphorylation, and our study also confirmed that dl-PHPB obviously inhibited GSK-3β and CDK-5 activities in vivo and in vitro. Thus, the regulation of Thr668 phosphorylation of APP might play an important role in dl-PHPB-mediated APP processing.

PKC messenger pathways have been well known to be involved in regulating the non-amyloidogenic processing of APP by changing α-secretase activities or APP trafficking by protein phosphorylation (Hung et al, 1993; Koo, 1997), rather than phosphorylating APP (Hung and Selkoe, 1994). A member of the disintegrin and metalloprotease (ADAM) families has been put forward as candidate α-secretases (Buxbaum et al, 1998). ADAM10 and ADAM17 are considered likely candidates for α-secretase APP cleavage (Lammich et al, 1999; Nunan and Small, 2000). In our study, the long-term treatment of dl-PHPB significantly upregulated PKCα levels in the brains of APP/PS1 mice compared to the vehicle-treated group. Consistent with this, ADAM17 protein level was significantly elevated and ADAM10 level was modest increased in dl-PHPB-treated APP/PS1 mice compared to vehicle-treated group. Taken together, dl-PHPB might regulate APP processing toward to nonamyloidogenic pathway by PKC-dependent and PKC-independent pathway.

Microglia has a complex role in Aβ deposition and the cognitive impairment that accompanies this process. At the early stage of Aβ plaque development, it appears that microglia inhibit Aβ deposition; thus, the clearance of Aβ can be by activating microglial. In
contrast, in the late stage of disease, mice harboring existing \( \text{A} \beta \) plaques, inhibiting microglial activation had little effect on \( \text{A} \beta \) load (Seabrook, et al, 2006). In this study, L-NBP did not show strong efficiency on the microglia activation, thus, we deduced that cognitive improvement might be not attribute to \( \text{A} \beta \) plaque reduction.

Tau is abundant in neurons and works as microtubule-stabilizer within cells. The elevated tau phosphorylation enhances susceptibility to toxic stimuli and neurodegeneration and also results in increased production of \( \text{A} \beta \) (Sotiropoulos et al, 2008.). The affinity of hyperphosphorylated tau for microtubules is reduced, and then, microtubule network is broken and result in axonal transport deficits which ultimately induce neuritic atrophy and cell death. Hyperphosphorylated tau leads to memory deficits and loss of functional synapses in a transgenic mouse model (Schindowski et al, 2006). At present, about 40 serine-threonine tau phosphorylation sites have been identified (Hanger et al, 2009). Another finding of the present study is \( dl \)-PHPB-induced markedly inhibition of tau hyperphosphorylation in APP/PS1 transgenic mice. We detected the effect of \( dl \)-PHPB on tau hyperphosphorylation at several specific phosphorylated sites, such as Ser199, Thr205, Ser396 and Ser404, which represented different stage of phosphorylation. The results showed that \( dl \)-PHPB significantly inhibited tau hyperphosphorylation at Ser199, Thr205 and Ser396 sites, and also moderately reduced tau phosphorylation at Ser404 site in APP/PS1 mice, suggesting \( dl \)-PHPB could inhibit tau abnormal hyperphosphorylation at different stage. This effect was confirmed by treatment of SK-N-SH APP cells with \( dl \)-PHPB. Thus, the beneficial effect of \( dl \)-PHPB on memory deficits in APP/PS1 transgenic mice is mainly attributable to inhibiting tau phosphorylation. CDK-5 and GSK-3\( \beta \) are known as the most important kinases in regulating
tau hyperphosphorylation that are associated with AD (Lovestone and Reynolds, 1997). We found that $dl$-PHPB significantly inhibited CDK-5 and GSK-3β activities in APP/PS1 mice and in SK-N-SH APP cells. In addition, $dl$-PHPB also decreased GSK-3α expression in SK-N-SH APP cells. GSK-3α mainly involved in APP processing and Aβ generation (Phiel et al, 2003). Tau abnormal hyperphosphorylation may result from an imbalance between tau kinases and protein phosphatases. Our study showed that $dl$-PHPB had no effect on PP2A, one of the most important phosphatases affecting tau hyperphosphorylation in AD brain, indicating that $dl$-PHPB mainly inhibited tau phosphorylation by regulating kinases. At the beginning of project, we detected the tau hyperphosphorylation at 12 month-old mice, and found that only the phosphorylation of Ser396 site was markedly increased in the APP/PS1 mice compared to the wild-type mice. The tau phosphorylation at the other specific phosphorylated sites, such as Ser199, Thr205, and Ser404 had not been shown to enhance robustly. The result implied that $dl$-PHPB might have therapeutic and prevention dual function in reducing abnormal tau hyperphosphorylation. In addition, inhibiting tau hyperphosphorylation may be the main mechanism of L-NBP on improving cognition rather than reducing Aβ plaque deposition.

It has been reported that the inhibition of the PI3K-Akt pathway could promote AD pathological changes, and the activation of the PI3K-Akt pathway was effective against AD-like lesions (Baki et al, 2004). Akt-dependent phosphorylation, and inactivation of GSK3β, which can attenuate tau hyperphosphorylation. These are consistent with our experimental results. In APP/PS1 transgenic mice, the expressions of p-Akt and PI3K were obviously lower than that in wild-type mice. In contrast, $dl$-PHPB significantly activated
PI3K-Akt pathway. Akt can downregulate the activity of GSK3β by phosphorylating it at the residue Ser9 (Cross et al, 1995). In this study, we found that \textit{dl}-PHPB activated the phosphorylation of GSK3β at Ser 9 site in SK-N-SH APP cells. It implied that PI3K-Akt pathway might be involved in \textit{dl}-PHPB-regulated GSK3β activation. But more detailed studies are needed to determine whether the PI3K-Akt signaling pathway can be a target of \textit{dl}-PHPB treatment.

In conclusion, our data demonstrate that \textit{dl}-PHPB was able to inhibit tau abnormal hyperphosphorylation and improve cognitive impairment in an APP/PS1 transgenic AD mouse model. Inhibitory effects of CDK-5 and GSK-3β signaling pathway might be underlying mechanism. PI3K/Akt might involve in the effect of \textit{dl}-PHPB. Together with the previous findings that \textit{dl}-PHPB attenuated learning and memory deficits in the animal models of vascular-dementia and Aβ-intracerebroventricular infusion, \textit{dl}-PHPB appears to be promising as a new therapeutic agent of Alzheimer’s disease.
Authorship Contributions

Participated in research design: Peng, and Wang.

Conducted experiments: Peng, Hu, Xu, Rong, Li, Li, and Wang,

Contributed new reagents: Yang

Performed data analysis: Peng, and Xu

Wrote or contributed to the writing of the manuscript: Peng, and Wang
References


Takashima A and Itoh S (1989) Effect of V-9-M, a peptide fragment derived from


Footnotes

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Figure Legends

Figure 1. Chemical structure of \textit{dl}-PHPB.

Figure 2. Long-term \textit{dl}-PHPB treatment improved spatial learning and memory deficits in APP/PS1 mice. The latency (A) and error times (B) were assessed in the step-down passive avoidance test. \textit{Dl}-PHPB treatment significantly attenuated the learning and memory deficits in the compared to vehicle-treated APP/PS1 mice. Values represent group mean ±SEM. n=9-13 mice per group. ##p<0.01, ###p<0.001 versus vehicle-treated wild-type group. ***p<0.001 versus vehicle-treated APP/PS1 group.

Figure 3. The effect of \textit{Dl}-PHPB on Aβ depositions in APP/PS1 mice. \textit{A}, Representative image of total Aβ plaque staining (top) and Thioflavin S positive compact plaques staining (bottom) in hippocampus in vehicle-treated wild-type mice (left), vehicle-treated APP/PS1 mice (middle) and \textit{dl}-PHPB-treated APP/PS1 mice (right). \textit{B}, Statistical analysis showed \textit{dl}-PHPB significantly reduced Thio-S-positive plaque deposition in the hippocampus. \textit{C}, Aβ_{42} levels of cortical brain homogenates. \textit{D}, Aβ_{42} levels of hippocampal brain homogenates. Values represent group mean ±SEM. n=9-13 mice per group. ***p<0.05 versus vehicle-treated APP/PS1 group.

Figure 4. \textit{Dl}-PHPB treatment had no effect on glial activation in the hippocampus of APP/PS1 mice. \textit{A}, Representative images of CD45-positive microglia and GFAP-positive astrocytes in the hippocampus of APP/PS1 mice. \textit{B}, Quantitative image analysis of CD45- and GFAP-immunoreactivity. Values represent group mean ±SEM. n=6 mice per group.
Figure 5. *Dl*-PHPB regulated APP processing and phosphorylation. **A**, Representative Western blots of αAPPs, APP, ADAM10, ADAM17, phosphorylated APP (Thr668) and PKCα in cortical and hippocampal lysates of APP/PS1 mice treated with vehicle and *dl*-PHPB, respectively. (B-E) Quantitative analysis of αAPPs (B), ADAM17 (C), phosphorylated APP (D), and PKCα (E) from APP/PS1 mice treated with vehicle or *dl*-PHPB. Quantified results were normalized to β-actin expression. Values were expressed as percentages compared to vehicle-treated APP/PS1 mice (set to 100%), and represented as group mean ±SEM. n=9-13 mice per group. *p<0.05 versus vehicle-treated APP/PS1 group.

Figure 6. *Dl*-NBP treatment decreased tau hyperphosphorylation in APP/PS1 mice. **A**, Representative Western blots of phosphorylated tau at Ser199, Thr205, Ser396 and Ser404 in the cortical and hippocampal brain homogenates of wild-type or APP/PS1 mice treated with vehicle or *dl*-PHPB, respectively. **B-E**, Quantitative analysis of phosphorylated tau at Ser199 (B), Thr205 (C), Ser396 (D) and Ser404 (E). Quantified results were normalized to β-actin expression. Values were expressed as percentages compared to vehicle-treated wild-type mice (set to 100%), and represented as group mean ±SEM. n=9-13 mice per group. *p<0.05, **p<0.01 and ***p<0.001 versus vehicle-treated wild-type group. *p<0.05, **p<0.01 versus vehicle-treated APP/PS1 group.

Figure 7. *Dl*-NBP decreased tau phosphorylation levels in cultured neuroblastoma SK-N-SH APPwt cells. Cells were incubated with *dl*-PHPB at 0.1, 1µM and 10µM or without *dl*-PHPB (control) for 24 hr. **A**, Representative Western blots of phosphorylated tau
at Ser199, Thr205, and Ser396, B-D. Quantitative analysis of the Western blot was expressed as a percentage of phosphorylated tau at Ser199 (B), Thr205 (C), and Ser396 (D). Results are shown as the mean ± SEM and represent six independent experiments. *p<0.05 versus control group.

Figure 8. Dl-NBP treatment inhibited CDK-5 and GSK-3β activities in APP/PS1 mice. A, Representative Western blots of phosphorylated CDK-5 (Ser159), total CDK-5, phosphorylated GSK-3β (Ser9), phosphorylated GSK-3α, β (Tyr216), total GSK-3β and PP2A in the cortical and hippocampal brain homogenates of wild-type or APP/PS1 mice treated with vehicle or dl-PHPB, respectively. B-E, phosphorylated CDK-5 (Ser159) (B), total CDK-5 (C), phosphorylated GSK-3β (Tyr216) (D) and total GSK-3β (E). Quantified results were normalized to β-actin expression. Values were expressed as percentages compared to vehicle-treated wild-type mice (set to 100%), and represented as group mean ±SEM. n=9-13 mice per group. *p<0.05, **p<0.01 versus vehicle-treated wild-type group. *p<0.05, **p<0.01 versus vehicle-treated APP/PS1 group.

Figure 9. Dl-NBP decreased CDK-5 and GSK-3β activities in cultured neuroblastoma SK-N-SH APPwt cells. Cells were incubated with dl-PHPB at 0.1 and 1µM or without dl-PHPB (control) for 24 hr. A, Representative Western blots of phosphorylated CDK-5 (Ser159), total CDK-5, phosphorylated GSK-3β (Ser9), phosphorylated GSK-3α, β (Tyr216) and total GSK-3β in SK-N-SH APPwt cells. B-G, Quantitative analysis of the Western blot was expressed as a percentage of phosphorylated CDK-5 (Ser159) (B), total CDK-5 (C), phosphorylated GSK-3β
(Ser9) \( (D) \), phosphorylated GSK-3α, β (Tyr216) \( (E-F) \) and total GSK-3β \( (G) \). Results are shown as the mean ± SEM and represent six independent experiments.

\* \( p < 0.05 \), \** \( p < 0.01 \) versus control group.

Figure 10. Effect of \textit{dl}-NBP on PI3K/Akt signaling pathway in APP/PS1 mice. \textbf{A-B}, Representative Western blots of phosphorylated Akt, total Akt and PI3K in the cortical and hippocampal brain homogenates of wild-type or APP/PS1 mice treated with vehicle or \textit{dl}-PHPB, respectively. Quantitative analysis of the Western blot was expressed as a percentage of phosphorylated Akt \( (C) \), total Akt \( (D) \) and PI3K \( (E) \) in the cortex and hippocampus. Quantified results were normalized to β-actin expression. Values were expressed as percentages compared to vehicle-treated wild-type mice (set to 100%), and represented as group mean ±SEM. \( N=9-13 \) mice per group. \# \( p < 0.05 \), \## \( p < 0.01 \) versus vehicle-treated wild-type group. \* \( p < 0.05 \) versus vehicle-treated APP/PS1 group.
Table 1. Primary antibodies used in this study

<table>
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<th>Application</th>
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IHC, immunohistochemistry; WB, Western blot.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

(A) Western blots of cortical and hippocampal tissues from hAPPs Swiss Tg and Tg PHPB mice. The blots show the expression levels of α-APPs, APP, ADAM10, ADAM17, P-APP (T668), PKCα, and β-actin.

(B) Graphs showing the relative intensity of α-APPs in cortical and hippocampal tissues from Tg and Tg PHPB mice.

(C) Graphs showing the relative intensity of ADAM17 in cortical and hippocampal tissues from Tg and Tg PHPB mice.

(D) Graphs showing the relative intensity of P-APP (T668) in cortical and hippocampal tissues from Tg and Tg PHPB mice.

(E) Graphs showing the relative intensity of PKCα in cortical and hippocampal tissues from Tg and Tg PHPB mice.
Figure 6

A

Cortex

Hippocampus

β-actin

B

WT Tg Tg PHPB

WT Tg Tg PHPB

C

WT Tg Tg PHPB

WT Tg Tg PHPB

D

WT Tg Tg PHPB

WT Tg Tg PHPB

E

WT Tg Tg PHPB

WT Tg Tg PHPB

P-Tau (S199)
P-Tau (T205)
P-Tau (S396)
P-Tau (S404)

Relative intensity of P-Tau (S199) (% of WT)
Figure 7
Figure 8
Figure 9
Figure 10