Potassium 2-(1-Hydroxypentyl)-Benzoate Improves Memory Deficits and Attenuates Amyloid and τ Pathologies in a Mouse Model of Alzheimer’s Disease

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ABSTRACT

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized by amyloid-β (Aβ) deposition and neurofibrillary tangles. DI-PHPB [potassium 2-(1-hydroxypentyl)-benzoate], 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 DI-PHPB on learning and memory in amyloid precursor protein (APP) and presenilin 1 (PS1) double-transgenic AD mouse models (APP/PS1) and the mechanisms of DI-PHPB in reducing Aβ accumulation and τ phosphorylation. Twelve-month-old APP/PS1 mice were given 30 mg/kg DI-PHPB by oral gavage for 3 months. DI-PHPB treatment significantly improved the spatial learning and memory deficits compared with the vehicle-treated APP/PS1 mice. In the meantime, DI-PHPB obviously reduced τ hyperphosphorylation at Ser199, Thr205, and Ser396 sites in APP/PS1 mice. This reduction was accompanied by APP phosphorylation reduction and protein kinase C activation. In addition, expression of cyclin-dependent kinase and glycojen synthase kinase 3β, the most important kinases involved in τ phosphorylation, was markedly decreased by DI-PHPB treatment. Phosphorylated protein kinase B and phosphoinositide 3-kinase levels of APP/PS1 mice were significantly reduced compared with levels in wild-type mice, and DI-PHPB reversed the reduction. The effects of DI-PHPB effecting a decrease in τ phosphorylation and kinase activation were further confirmed in neuroblastoma SK-N-SH cells overexpressing wild-type human APP

Introduction

Alzheimer’s disease (AD), the leading cause of dementia in older adults, is characterized by the deposition of extracellular senile plaques and intracellular neurofibrillary tangles (Selkoe, 1994). Amyloid-β peptide (Aβ), generated from amyloid precursor protein (APP) cleavage, constitutes the core of senile plaques. APP is cleaved by at least two pathways, amyloidogenic and nonamyloidogenic. In the 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 (sAPPs) 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 neurofibrillary tangles is hyperphosphorylated τ, a microtubule-associated protein that stabilizes and promotes microtubule polymerization that aggregates as paired helical filaments (Drechsel et al., 1992). At present, more than 40 serine/threonine phosphosites have been identified on paired helical filaments τ, many of which are shown specifically in AD brain tissue (Hanger et al., 2009). Phosphorylation of τ 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). Overexpression of GSK-3β and CDK-5 in transgenic mice has been reported to increase τ phosphorylation and 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 (Phiel et al., 2003; Cruz et al., 2006). GSK-3β and CDK-5 have been suggested as the drug targets for treatment of AD.

**dl-PHPB** [potassium 2-(1-hydroxypentyl)-benzoate] is a novel drug candidate for the 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 dl-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 the protein kinase C (PKC) signaling pathway (Hu et al., 2012). Recently, dl-PHPB was shown to improve learning and memory deficits, reducing oxidative stress and glia activation in the cerebral area of hypoperfused rats (Zhao et al., 2013). These results suggest that dl-PHPB might be a potential drug candidate for treatment of AD.

In the present study, we examined the effect of dl-PHPB treatment on the cognitive impairment in APP and presenilin 1 (PS1) double-transgenic AD mouse models (Trinchese et al., 2004). Moreover, we investigated the mechanisms underlying the compound on AD-related pathology, such as Aβ generation, APP processing, τ 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 (Fig. 1). APP/PS1 double-transgenic mice were obtained from The Jackson Laboratory (strain name B6C3-Tg(APPsw,PS1dE9) 85DboI; 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 central nervous system neurons (Jankowsky et al., 2001). The two transgenes cosegregate in these mice. Mice were housed in an animal room maintained at 23 ± 1°C with a 12-hour 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 distilled 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 2 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. The brain was removed; one hemibrain was snap-frozen in liquid nitrogen and stored at −80°C until analysis, and the other hemibrain was fixed in 4% paraformaldehyde for 2 hours, followed by incubation in graded sucrose at 4°C.

**Step-Down Passive Avoidance Test.** The effect of dl-PHPB on memory impairment in mice was studied using a step-down passive avoidance test according to the previously reported method (Takushima and Itoh, 1989) but with a minor modification. The experimental compartment was a (15 × 15 × 30-cm) wooden box. The floor of the box consisted of parallel stainless steel bars. A wooden insulating platform (4 × 4 × 4-cm) was placed on the center of the grid floor. The experiment lasted 3 days. On the first day, mice were introduced to the experimental box for 5 minutes. The acquisition trials were performed on the second day. An electric foot shock (36 V) was delivered to the paws of the animals through the grid floor. The mice had to jump onto the platform to avoid the foot shock. The process lasted 3 minutes until the mice were removed from the compartment. Twenty-four hours after training, 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 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 was performed using the ELITE ABC method (Vector Laboratories, Burlingame, CA) as previously described (Peng et al., 2010). Ten-micron sagittalcryosections of mouse brain were mounted on glass slides. Aβ plaques, microglia, and astrocytes were recognized by the primary antibodies 6E10 (Covance, Princeton, NJ), anti-CD45 (Serotec Raleigh, NC), and anti–glial fibrillary acidic protein (DAKO, Carpinteria, CA). Secondary biotinylated antibodies (anti-mouse, anti-rat, and anti-rabbit) were obtained from Vector Laboratories. Thioflavin-S staining for fibrillar Aβ was performed by incubating the slides in a 1% aqueous solution of Thioflavin-S for 10 minutes, 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, Tokyo, Japan). Image analysis was performed using Image-Pro Plus 5.1 (Media Cybernetics, Bethesda, MD). The threshold of detection was held constant during analysis. For all treatment groups, the percentage of the area occupied by Aβ and Thioflavin-S in the cortex and hippocampal area was calculated for three equidistant sections per mouse.

**Determination of Aβ Levels.** Aβ levels were measured in the brain homogenates (Tris-buffered saline (TBS), TBS-Tween 20 (TBST), and guanidine-soluble fractions). Aβ-specific sandwich enzyme-linked immunosorbent assays were performed by using human Aβ42 immunnoassay kit (Innogenetix, Carlsbad, CA). The absorbance was recorded at a 450-nm wavelength using an uQuant microplate spectrophotometer (BioTek Instruments, Inc., Rockville, MD).

**Cell Culture.** SK-N-SH/SHSK-N-SH APP695 human neuroblastoma cells were cultured using Dulbecco’s modified Eagle’s medium culture, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 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. Before treatment, cells were maintained in serum-free media overnight. After incubation with the dl-PHPB for 24 hours, 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 EDTA, 1 mM Na3VO4, 10 mM NaF, 10% glycerol and Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN). 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 the following: 150 mM NaCl,
20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 5 mM DTT, 1% Triton X-100, 10 mM NaF, 10 mM Na$_4$O$_2$P$_2$, 1 mM Na$_3$VO$_4$ with a protease inhibitor cocktail; 40 μg of protein per lane was run on polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, blocked with 5% milk solution (nonfat dry milk in TBST) for 2 hours, 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 five times, the membrane was incubated with secondary antibodies (horseradish peroxidase–conjugated anti-rabbit IgG) at room temperature for 1 hour. The signals were analyzed by densitometric evaluation using the Quantity-One software (Bio-Rad, Hercules, CA). The values were normalized to β-actin intensity levels.

**Statistical Analysis.** All data were expressed as mean ± the S.E.M. One-way analysis of variance using SPSS version 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 dl-PHPB treatment for 3 months, we monitored the general health of the mice and did not find any abnormal changes. In addition, the body weights of the mice were not significantly different in dl-PHPB–treated and vehicle control mice (data not shown). Together, these data indicate that long-term treatment of dl-PHPB had no significant toxicity in mice.

It has been widely reported that APP/PS1 AD transgenic mice develop memory impairment with increasing age (Trinchese et al., 2004). In this study, we evaluated whether dl-PHPB contributed to attenuation of Aβ-dependent behavioral deficits at the APP/PS1-Tg AD mice 15 months of age by the step-down passive avoidance test. In the acquisition trial, all mice were able to jump actively onto the platform after foot shock, and the latency time was not different across the groups (data not shown). In the memory retention trial, the wild-type mice spent 123.9 ± 23.9 seconds on the platform before they jumped down it the first time. The latency of APP/PS1-Tg AD mice treated with the vehicle control was 20 ± 8.4 seconds (Fig. 2A). In addition, the number of errors of APP/PS1-Tg mice was greater than that of wild-type mice (3.2 ± 0.7 versus 0.9 ± 0.4) (Fig. 2B). There was a 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, dl-PHPB treatment extended the latency to 138.4 ± 20.5 seconds and lowered the number of errors to 0.5 ± 0.2. The therapeutic effect of dl-PHPB markedly reversed the reduction of the latency ($P < 0.001$) and the increase in the number of errors in the transgenic mice ($P < 0.001$). These data suggested that dl-PHPB was likely to enhance memory retention in APP/PS1-Tg AD mice.

In addition, we observed no cognitive enhancement in the wild-type mice treated with dl-PHPB (Supplemental Fig. 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 the age of 12 months, 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 after 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 the total Aβ plaque burden in the

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GFAP, glial fibrillary acidic protein; IHC, immunohistochemistry; P-Akt, phospho-protein kinase B; WB, Western blot.
cortex and hippocampus. However, dl-PHPB significantly reduced Thioflavin-S-positive plaque deposition in the hippocampus, indicating that dl-PHPB might inhibit Aβ aggregation or promote Aβ plaque degradation (Fig. 3, A and B).

Then we analyzed cerebral Aβ levels by enzyme-linked immunosorbent assay and found that dl-PHPBs had no significant effect on lowering Aβ levels of the 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 reduce CD45 and glial fibrillary acidic protein immunoreactivity in APP/PS1-Tg mice (Fig. 4), indicating that dl-PHPB did not appear to be involved in Aβ accumulation or inflammation (e.g., in gliosis).

**DL-PHPB Directed APP Processing toward 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 brains 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 aAPPs as a 98-kDa band and full-length APP at ~110 kDa. DL-PHPB treatment increased the release of aAPPs by 23% in the cortex and had no effect in the hippocampus (Fig. 5, A and B), indicating that dl-PHPB might lightly regulate APP processing toward the nonamyloidogenic 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).

Disintegrin and metalloprotease family (ADAM) 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 AD (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. 5, A and C).

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 a 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 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 the release of aAPPs and decrease Aβ levels (Chen and Fernandez, 2004). PKCα was assessed by Western blot (Fig. 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 with vehicle control mice (Fig. 5E), indicating that dl-PHPB might enhance PKCα signaling, thereby directing APP processing toward a nonamyloidogenic pathway.

**DL-PHPB Treatment Inhibited τ Hyperphosphorylation in APP/PS1-Tg AD Mice and APP-Transfected SK-N-SH Cells.** It has been demonstrated that the τ hyperphosphorylation appeared in the APP/PS1 mouse brain after the onset of Aβ deposition (Kurt et al., 2003). In AD, τ protein can be phosphorylated in at least 38 serine/threonine sites (Hanger et al., 1998). In an Aβ-intracerebroventricularly infused rat model and senescence-accelerated mouse prone 8 animal model, we found that dl-PHPB reduced τ abnormal hyperphosphorylation (data not shown). In the present study, we examined τ hyperphosphorylation by Western blot using antibodies against different phosphorylation sites on τ, including Ser199, Thr205, Ser396, and Ser404. As shown in Fig. 6A, a marked increase of τ phosphorylation at sites of Ser199, Thr205, and Ser396 was...
observed in both the cortex and hippocampus of the vehicle-treated APP/PS1 mice. In contrast, except for a slight decrease in the phosphorylation at the Ser404 site, *dl*-PHPB treatment strongly reduced τ phosphorylation in Thr205 and Ser396 sites in both the cortex and hippocampus. Quantitative analysis exhibited decreases in τ phosphorylation of Thr205 of 30 and 47% in the cortex and hippocampus, respectively, after *dl*-PHPB treatment (Fig. 6C). *dl*-PHPB reduced τ phosphorylation at the Ser396 site by 28% in the cortex and by 52% in the hippocampus in vehicle-treated APP/PS1 mice (Fig. 6D). In addition, a robust decrease of about 57% in τ phosphorylation at Ser199 was shown in the hippocampus rather than in the cortex of the *dl*-PHPB–treated mice (Fig. 6B).

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

**Dl-PHPB Attenuated the Expression of Phosphorylated CDK-5 and GSK-3β in APP/PS1-Tg AD Mice and APP-Transfected SK-N-SH Cells.** Both τ phosphorylation and dephosphorylation are in homeostasis under normal conditions and are dependent on the activities of protein kinases and phosphatases. Abnormal τ hyperphosphorylation could be induced by upregulating τ kinase and downregulating τ phosphatases in the brain affected by AD. To elucidate further the mechanisms of dl-PHPB to inhibit τ hyperphosphorylation in APP/PS1 mice, we assessed the expressions of GSK-3β and CDK-5, two major kinases implicated in τ hyperphosphorylation at abundant sites. GSK-3β is activated through the phosphorylation at Tyr216 and inhibited by phosphorylating Ser9. Active GSK-3β contributes to phosphorylate τ and facilitates tangle formation (Wang et al., 1998; Cohen and Frame, 2001), whereas CDK-5 activation requires phosphorylation at the 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 with 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 with vehicle-treated APP/PS1 mice (Fig. 8, A and B). In addition, we did not find a difference in total CDK-5 levels among the groups (Fig. 8, A and C). The data indicated that dl-PHPB treatment might reduce CDK-5 activity.

Western blot analysis with an antibody against the phosphorylated GSK-3β (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 marked enhancement (~79%) of GSK-3β phosphorylation at Tyr216 in the hippocampus of vehicle-treated APP/PS1 mice compared with 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%) compared with vehicle-treated APP/PS1 mice (P < 0.01) (Fig. 8, A and D). Although a slight increase in phosphorylation of GSK-3α at Tyr279 was observed in the brains of the vehicle control-treated APP/PS1 mice (P < 0.05), respectively (Fig. 7, C and D). However, dl-PHPB treatment had no effect on τ phosphorylation at the Ser199 site. The in vitro and in vivo data jointly confirmed that dl-PHPB could reverse τ phosphorylation.

**Fig. 4.** Dl-PHPB treatment had no effect on glial activation in the hippocampus of APP/PS1 mice. (A) Representative images of CD45-positive microglia and glial fibrillary acidic protein (GFAP)-positive astrocytes in the hippocampus of APP/PS1 mice. (B) Quantitative image analysis of CD45- and GFAP-immunoreactivity. Values represent group mean ± S.E.M.; n = 6 mice per group. ROI, region of interest.
mice relative to the wild-type mice, no significant difference was found between the dl-PHPB- and vehicle-treated APP/PS1 mice (Fig. 8A). We did not observe a significant difference in the total GSK-3β levels among the groups (Fig. 8A and E). These results suggest that long-term dl-PHPB treatment has an inhibitory effect on GSK-3β activity. In the meantime, we determined the
protein phosphatase 2 (PP2A) activity; PP2A is the most important phosphatase in the wild-type and APP/PS1 mice. The Western blot results showed no statistical differences in PP2A activities among the groups (Fig. 8A), indicating that dl-PHPB mainly inhibited \(\tau\) hyperphosphorylation by regulating protein kinase in APP/PS1 mice.

**Fig. 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 parts of the brain. (B–E) Homogenates of wild-type or APP/PS1 mice treated with vehicle or dl-PHPB, respectively. Quantitative analysis of phosphorylated \(\tau\) at Ser199 (B), Thr205 (C), Ser396 (D), and Ser404 (E). Quantified results were normalized to \(\beta\)-actin expression. Values were expressed as percentages compared with vehicle-treated wild-type mice (set to 100%) and represented as group mean ± S.E.M.; \(n = 9–13\) 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.
We also examined the GSK-3\(\beta\) 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 the Ser159 site was downregulated by 23% after 10 \(\mu\)M dl-PHPB treatment, whereas the expression of total CDK-5 was not altered (Fig. 9, A–C). In addition, Western blot analysis showed that 1 \(\mu\)M dl-PHPB incubation markedly increased the phosphorylation of GSK-3\(\beta\) (Ser9) by 71% compared with the control group (Fig. 9, A and D). Moreover, Western blot analysis exhibited that dl-PHPB robustly decreased GSK-3\(\alpha\) (Tyr279) and GSK-3\(\beta\) (Tyr216) levels. Quantitative data indicated that dl-PHPB at the concentration of 10 \(\mu\)M reduced GSK-3\(\alpha/\beta\) (Tyr279/216) by 49% \((P < 0.05)\) and 51% \((P < 0.01)\), respectively (Fig. 9, A, E, and F). No obvious change in the expression of total GSK-3\(\beta\) was observed after dl-PHPB treatment (Fig. 9, A and G). These results indicate that dl-PHPB reduced \(\tau\) hyperphosphorylation by regulating protein kinases CDK-5 and GSK-3\(\beta\) activities.

**DL-PHPB Upregulated Phosphoinositide 3-Kinase and Protein Kinase B Signaling Pathway in APP/PS1-Tg AD Mice.** Protein kinase B (Akt) is a serine-threonine protein kinase in the downstream of phosphoinositide 3-kinase (PI3K). The 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 GSK3\(\beta\) (Baki et al., 2004). A recent study also suggests that GSK-3\(\beta\) phosphorylation can be associated with phosphorylation of Akt at Ser473 residue (Sutton and Rushlow, 2012). Thus, we further investigated the effect of 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, respectively, of APP/PS1 mice compared with levels in wild-type mice. Long-term treatment of dl-PHPB inhibited the reduction of phosphorylated Akt activity in the cortex compared with vehicle-treated APP/PS1 mice \((P < 0.05)\). We did not find a significant difference in the total Akt levels among the groups (Fig. 10, A–F). Moreover, PI3K levels were significantly reduced in the cortex \((P < 0.01)\) and hippocampus \((P < 0.01)\) of APP/PS1 mice compared with wild-type mice. In contrast, the reduction of PI3K level of cortex was reversed by dl-PHPB treatment \((P < 0.05)\). These results suggest that long-term treatment with dl-PHPB has an upregulatory effect on the PI3K-Akt signaling pathway (Fig. 10, A, G, and H).

**Discussion**

As a promising candidate for the treatment of stroke, although dl-PHPB has been demonstrated to contribute to improvement in learning and memory deficits in hypoperfused rats, an often-used vascular dementia model, the actual therapeutic role of dl-PHPBs in AD pathology and dementia has not yet been ascertained. The present study first discloses a therapeutic effect of dl-PHPB in the APP/PS1 double transgenic AD mouse model. DL-PHPB treatment by oral gavage for 3 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 central nervous system disorders. One of the most common animal tests in memory research is inhibition of imitating activities or learned habits. The term passive avoidance is usually used 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 τ 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 τ abnormal hyperphosphorylation.
Dl-PHPB significantly reduced Thioflavin-S–positive compact Aβ plaque accumulation, which might be due to directing APP processing toward a nonamyloidogenic pathway because dl-PHPB–treated APP/PS1 mice showed enhancement of aAPPs release and reduction of APP phosphorylation at the Thr668 site compared with vehicle-treated APP/PS1 mice. aAPPs have been shown to be beneficial for memory function and to possess neuroprotective and neurotrophic properties (Mattson, 1997). It is likely that aAPPs derived from dl-PHPB–mediated APP processing may serve as neuroprotective agents and contribute to the long-term benefit of dl-PHPB on memory in APP/PS1 mice. In addition, our previous study demonstrated that 3-n-butylphthalide (NBP) isoform could regulate APP processing and lower Aβ generation in AD mouse models (Peng et al., 2010, 2012). It has been demonstrated that Thr668-phosphorylated APP was significantly increased in AD brains. Thr668 phosphorylation might regulate APP metabolism and facilitate the β-secretase 1 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 τ 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 nonamyloidogenic processing of APP by changing α-secretase activities or APP trafficking by protein phosphorylation (Hung et al., 1993; Koo, 1997) rather

**Fig. 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 1 μM or without dl-PHPB (control) for 24 hours. (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 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 and F), and total GSK-3β (G). Results are shown as the mean ± S.E.M. and represent six independent experiments. *P < 0.05; **P < 0.01 versus control group.
Fig. 10. Effect of dl-NBP on PI3K/Akt signaling pathway in APP/PS1 mice. (A and 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 dl-HPHB, 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 with vehicle-treated wild-type mice (set to 100%) and represented as group mean ± S.E.M.; 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.
than by phosphorylating APP (Hung and Selkoe, 1994). A member of the 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; Nunn 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 with the vehicle-treated group. Consistent with this finding, ADAM17 protein level was significantly elevated, and ADAM10 level was modestly increased in dl-PHPB-treated APP/PS1 mice compared with the vehicle-treated group. These results together indicate that dl-PHPB might regulate APP processing toward to nonamyloidogenic pathway by PKC-dependent and -independent pathways.

Microglia has a complex role in Aβ deposition and in 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 microglia. In contrast, in the late stage of disease, mice harboring existing Aβ plaques, inhibiting microglial activation, had little effect on Aβ load (Seabrook et al., 2006). In this study, L-NBP did not show strong efficiency on microglia activation; thus, we deduced that cognitive improvement might be not attributable to Aβ plaque reduction.

In neurons, τ is abundant and works as microtubule stabilizer within cells. Elevated τ phosphorylation enhances susceptibility to toxic stimuli and neurodegeneration and also results in increased production of Aβ (Schindowski et al., 2006). At present, about 40 serine-threonine kinase and protein phosphatases. Our previous findings that dl-PHPB attenuated learning and memory deficits in the animal models of vascular dementia and Aβ-intracerebroventricular infusion, dl-PHPB appears to be promising as a new therapeutic agent for Alzheimer's disease.

Authorship Contributions

Participated in research design: Peng, Wang.
Conducted experiments: Peng, Xu, Rong, J. Li, P. Li, Wang.
Contributed new reagents or analytic tools: Yang.
Performed data analysis: Peng, Xu.
Wrote or contributed to the writing of the manuscript: Peng, Wang.

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Supplement Data

Article title: 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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Figure legend:

Supplement Figure 1: Long-term dl-PHPB treatment had no cognition enhancement in wild type mice. The latency (A) and error times (B) were assessed in the step-down passive avoidance test. Values represent group mean ±SEM. n=9-13 mice per group.
Supplement Figure