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Short Communication

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Leptin signaling plays a critical role in the geniposide-induced decrease of tau phosphorylation

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Abstract

We have previously demonstrated that geniposide attenuates the production of $A\beta_{1-42}$ both *in vitro* and *in vivo* via enhancing leptin receptor signaling. But the role played by geniposide in the phosphorylation of tau and its underlying molecular mechanisms remain unclear. In this study, we investigated the effect of geniposide on the phosphorylation of tau and the role of leptin signaling in this process. Our data suggested that, accompanied by the up-regulation of leptin receptor expression, geniposide significantly decreased the phosphorylation of tau in rat primary cultured cortical neurons and in APP/PS1 transgenic mice, and this geniposide-induced decrease of tau phosphorylation could be prevented by leptin antagonist (LA). Furthermore, LA also prevented the phosphorylation of Akt at Ser-473 site and GSK-3β at Ser-9 site induced by geniposide. All these results indicate that geniposide may regulate tau phosphorylation through leptin signaling, and geniposide may be a promising therapeutic compound for the treatment of Alzheimer's disease in the future.

Key words: Alzheimer's disease, geniposide, GSK-3β, leptin, tau

Introduction

With improvement of living conditions, life expectancy is increasing steadily and as a consequence more individuals are suffering from age-related disorders such as Alzheimer's disease (AD). The prevalence of AD is predicted to rise rapidly in the coming decades, which is likely to pose a huge burden on health care services in the future [1]. Although it is widely accepted that senile plaques (SP) composed of an extra-cellular core of the insoluble form of β-amyloid (Aβ) are associated with AD, intracellular neurofibrillary tangles (NFTs) formed by the hyperphosphorylation of tau protein also play an essential role in the neurodegeneration and cognitive decline of AD [2].

In recent years, there has been significant progress in the studies of tau hyperphosphorylation, and a large number of protein kinases and protein phosphatases have been identified to be involved in the regulation of tau phosphorylation [3]. Glycogen synthase kinase-3β (GSK-3β) (also known as tau protein kinase [4]) is a proline-directed serine/threonine kinase which has been identified as one of the major candidates found in brain tissues from AD [5]. Abnormal activation of GSK-3β will lead to tau phosphorylation. However, the signal transduction pathways involved in the abnormal activation of GSK-3β have not been completely clarified.

It has been demonstrated that leptin can inactivate GSK-3β through phosphorylation of Ser-9, leading to the reduction of tau phosphorylation [6]. Most interestingly, leptin-deficient mice have distinctively different synaptic profiles from wild-type mice, as revealed by electron microscopy and electrophysiology [7]. And in AD patients, the level of circulating leptin is inversely proportional to the severity of cognitive decline. These observations suggest that leptin supplementation therapy may be beneficial for AD patients [8].

Our previous work demonstrated that geniposide could decrease the production of $A\beta_{1-42}$ in primary cultured cortical neurons and in APP/PS1 transgenic mice [9]. Geniposide could also attenuate the level of $A\beta_{1-42}$ and tau phosphorylation in streptozotocin (STZ)-induced diabetic rats [10,11]. But the role of leptin signaling on

geniposide-regulating tau phosphorylation remains to be explored. In this study, we tried to investigate the effect of geniposide on the phosphorylation of tau *in vitro* and *in vivo*, and to clarify its molecular mechanisms.

Materials and Methods

Animal experiment

APP_{swe}/PS_{1dE9} double transgenic AD mice (C57BL/6 background) were obtained from Model Animal Research Center of Nanjing University (Nanjing, China). These mice express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9). All experiments and protocols described in the present study were performed in accordance with the principles and guidelines of the Chinese Council Animal Care and also approved by the Institutional Animal Care and Use Committee at Chongqing University of Technology (Chongqing, China).

Geniposide, bought from Sichuan Dicotyledonous Bio-tech Co, Ltd (purity is over 99.5%, UR20060421; Chengdu, China), was administered with intragastric administration (once a day) for 4 weeks. As a positive control, liraglutide (Lir; Novo Nordisk, Bagsvaerd, Denmark), a human GLP-1 analog, was administered with intraperitoneal injection (i.p.) at 100 μg/kg once a day.

Primary cortical neuronal culture and treatment

Rat primary cortical neurons were prepared from fetus cortex at embryonic day 19–20 as described before [12,13]. Briefly, the cortexes were dissected in cold PBS. Tissues were collected and washed with PBS, and 0.25% (V/V) trypsin was added for digestion at 37°C for 15 min. The digestion was stopped by addition of fetal bovine serum (FBS, Neurobasal; Gibco, Carlsbad, USA) at a final concentration of 10% (V/V). Cells were collected by centrifugation at 1000 rpm for 10 min to remove PBS, and were then suspended in neurobasal medium (Gibco) supplemented with 10% FBS.

Cells were plated onto poly-D-lysine (10 μg/ml) pre-coated 24-well plates and were cultured at 37°C in 95% humidified atmosphere with 5% CO_2 until use. The initial medium (Neurobasal containing 10% FBS) was removed on day 2 and replaced by fresh medium (Neurobasal containing 2% B27) without serum. After seven days, the cells were incubated with indicated dose of geniposide for 1 h, the cell lysates were used to determine the level of phosphorylated tau protein. To determine the effect of geniposide on the phosphorylation of GSK-3β at ser-9 site and Akt ser-473 site, cells were incubated with 10 μM geniposide for the indicated time in the presence or absence of leptin antagonist (LA, leptin antagonist triple mutant rat recombinant; Prospec, Rehovot, Israel), and the phosphorylation of Akt at ser-473 site and GSK-3β at ser-9 site were measured by western blot analysis.

Western blot analysis

Hemibrain samples were taken from mice and snap-frozen for biochemical assays. For western blot analysis, each sample was homogenized in 10 volumes of modified RIPA buffer (Biyotime, Shanghai, China) containing 150 mM NaCl, 50 mM Tris. HCl (pH 8.0), 1 mM EDTA, 1% igepal, 0.5% sodium deoxycholate, 0.1% SDS and protease/phosphatase inhibitor cocktail (Calbiochem, La Jolla, USA) and centrifuged at 10,000 *g* for 10 min to remove the insoluble material. Protein concentrations were determined by a BCA protein assay kit (Biyotime), and 10–20 μg protein was resolved on 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking, membranes were probed with anti-leptin receptor (OBR), anti-Akt, anti-GSK-3β, anti-β-Actin, anti-phos-Akt473 and anti-phos-GSK-3β antibodies (1:2000–3000; Santa Cruz Biotechnology, Santa Cruz, USA), anti-tau and anti-phos-tau at ser-396 site (Immunoway, Newark, USA) or horseradish peroxidase labeled anti-GAPDH antibody (1:10,000, Genetimes Technology Inc., Shanghai, China). After extensive wash, membranes were then incubated with horseradish peroxidase-conjugated secondary IgG. Immunoblot signals were visualized by an ECL chemiluminescence substrate reagent kit (Millipore, Billerica, USA) and were quantified by densitometric scanning and image analysis using the software of Quanty One (Bio-Rad, Hercules, USA).

Statistical analysis

Data are presented as mean ± SD from at least three independent experiments. Analysis of variance was carried out using Origin software, with $P < 0.05$ considered statistically significant, as assessed by student's *t*-test with corrections for multiple comparisons to a single group (Dunnett's *t*-test) and between multiple groups (Bonferroni and Tukey's tests).

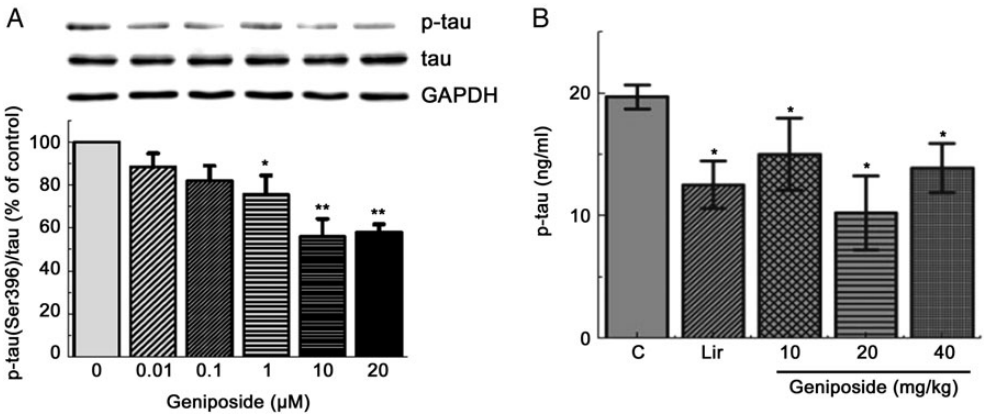

Figure 1. Effects of geniposide on the phosphorylation of tau *in vitro* and *in vivo* (A) After rat primary cortical neurons were incubated with 0, 0.01, 0.1, 1 10 or 20 μM geniposide for 60 min, the cell lysates were collected to determine the level of phosphorylated tau by western blot analysis. Data are shown as mean ± SD from three independent experiments, $*P < 0.05$, $**P < 0.01$ vs. control. (B) To determine the effect of geniposide on the phosphorylation of tau in APP/PS1 transegnic mice, the mice were treated with 10, 20 and 40 mg/kg geniposide (i.g. once daily) for 4 weeks, the level of phosphorylated tau in the brain tissues were determined with ELISA according to the manufacturer's protocol. Data are mean ± SD ($n = 6$–10 mice per group). Liraglutide (100 μg/kg; i.p. once daily), the human GLP-1 analog, was used as positive control (Lir) in this experiment. $*P < 0.05$ vs. control (C).

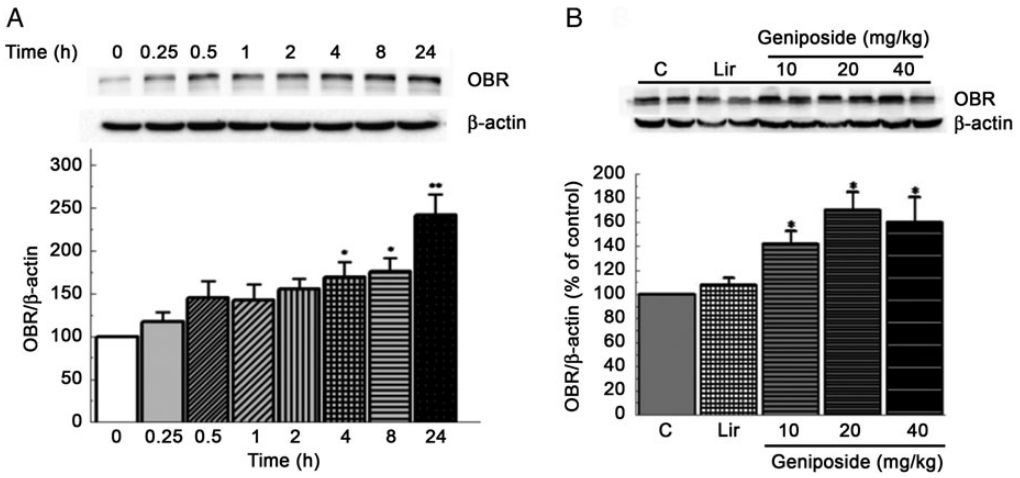

Figure 2. Effects of geniposide on the expression of leptin receptor *in vitro* and *in vivo* (A) After rat primary cortical neurons were incubated with 10 μM geniposide for 0, 0.25, 0.5, 1, 2, 4, 8 and 24 h, the cell lysates were collected to determine the expression level of OBR by western blot analysis. Data are shown as mean ± SD from three independent experiments, $^{*}P<0.05$, $^{**}P<0.01$ vs. control (0 h). (B) To explore the influence of geniposide on the phosphorylation of tau in APP/PS1 transegnic mice, the mice were treated with 10, 20 and 40 mg/kg geniposide (i.g.) for 4 weeks, the level of phosphorylated tau in the brain tissues were determined with ELISA. Data are mean ± SD ($n=6$–10 mice per group). Liraglutide (100 μg/kg) was used as positive control (Lir) in this experiment. $^{*}P<0.05$ vs. control (C).

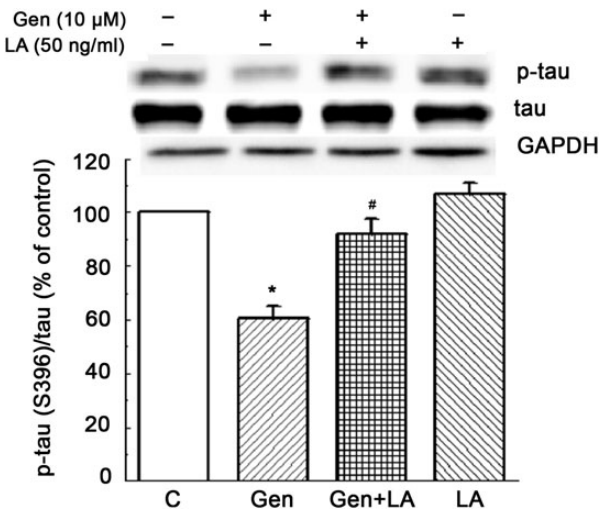

Figure 3. Effect of LA on the phosphorylation of tau in primary cultured cortical neurons To determine the influence of LA on geniposide regulating the phosphorylation of tau protein, rat primary cortical neurons were pre-incubated with 50 ng/ml LA for 30 min before addition of geniposide. After the cells were treated with 10 μM geniposide for 60 min, the cells lysates were used to determine the phosphorylation of tau protein by western blot assay. Data are shown as mean ± SD from three independent experiments. $^{*}P<0.05$, vs. control (C) and $^{\#}P<0.05$ vs. the group treated with geniposide alone (Gen).

Results

Effects of geniposide on the phosphorylation of tau *in vitro* and *in vivo*

As shown in **Fig. 1A**, geniposide decreased the phosphorylation of tau directly in a dose-dependent manner in rat primary cultured cortical neurons. Treatment with 20 μM geniposide for 1 h could decrease the level of phosphorylated tau by 40%. To confirm the effect of geniposide on the phosphorylation of tau *in vivo*, we treated the APP/PS1 transgenic mice with geniposide (intragastric administration, once daily) at indicated dosages for 4 weeks. The results demonstrated that geniposide also attenuated the level of phosphorylated tau protein in the brain of APP/PS1 transgenic mice (**Fig. 1B**).

Effect of geniposide on the protein of leptin receptor *in vitro* and *in vivo*

It was speculated that a high concentration of leptin receptor (OBR) in the hippocampus (center for cognition and memory) underscores the possibility of a multifaceted role for leptin [8]. We therefore determined the influence of geniposide on the expression of OBR. Our results indicated that geniposide could induce the expression of OBR in primary cultured cortical neurons; and treatment with 10 μM geniposide for 24 h increased the expression level of OBR by 2.2-folds (**Fig. 2A**). Additionally, the influence of geniposide on the protein level of OBR *in vivo* was also determined. **Figure 2B** showed that geniposide also significantly enhanced the expression of OBR in APP/PS1 transgenic mice.

Effect of leptin signaling on geniposide-regulated tau phosporylation

To determine the role of leptin signaling in geniposide-regulated tau phosphorylation, we tested the influence of leptin antagonist (LA) on the phosphorylation of tau in primary cultured cortical neurons. Our data showed that inhibition of geniposide on the phosphorylation of tau was prevented by the pre-incubation with 50 nM LA (**Fig. 3**).

Effect of leptin signaling on geniposide-regulated GSK-3β and Akt phosporylation

Much evidence has suggested that GSK-3β is physically complexed with tau in the brain and phosphorylates tau *in vitro* and *in vivo* [14,15], and plays an important role on the phosphorylation of tau through the activation of phosphatidylinositol 3-kinase (PI3K) [16,17]. To clarify the role of leptin signaling on the activation of Akt and GSK-3β, we determined the effects of LA on the phosphorylation of Akt at ser-473 site and GSK-3β at ser-9 site in rat primary cultured cortical neurons. The results demonstrated that geniposide

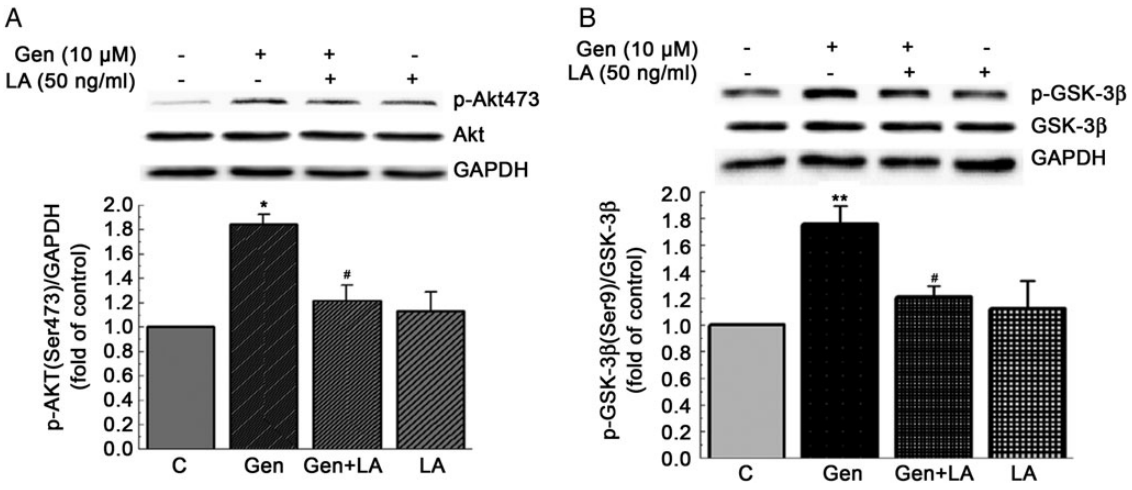

Figure 4. Effect of LA on the phosphorylation of Akt at ser-473 and GSK-3β in primary cultured cortical neurons Cells were pre-incubated with 50 ng/ml LA for 30 min, and then treated with 10 μM geniposide for 60 min. After that, the phosphorylation of Akt473 (A) and GSK-3β (B) were determined by western blot assay. Data are shown as mean ± SD from three independent experiments. *P < 0.05, **P < 0.01 vs. control (C) and #P < 0.05 vs. the group treated with geniposide alone (Gen).

induced the phosphorylation of Akt and GSK-3β, and pre-incubation with 50 nM LA prevented the role of geniposide on the phosphorylation of Akt at ser473 and GSK-3β (**Fig. 4A,B**). Together with the result of LA on geniposide-regulated tau phosphorylation (**Fig. 3**), the present data indicated that geniposide enhanced the leptin signaling to regulate phosphorylation of Akt and GSK-3β, which might play a critical role on geniposide-regulated tau phosphorylation.

Discussion

Alzheimer's disease (AD) is one of the most common cases of dementia, and formation of NFT from hyperphosphorylated tau is one of the typical hallmark lesions of AD. Although the pathogenesis of this disease is not yet fully understood, it is reported that NFTs are structures present in the neuronal cell body, and GSK-3β plays an critical role in the phosphorylation of tau [5,14]. GSK-3β is physically complexed with tau and phosphorylates tau *in vitro* and *in vivo* [15,18]. In the present study, our data suggested that geniposide decreased the level of phosphorylated tau in rat primary cortical neurons and in APP/PS1 transgenic mice via enhancing leptin signaling to regulate the phosphorylation of Akt at ser-473 site and GSK-3β at ser-9 site, indicating that geniposide might be useful for the treatment of AD.

Our previous studies suggested that geniposide isolated from *Gardenia jasminoide* was a novel agonist for glucagon-like peptide 1(GLP-1) receptor. It induced the neuronal differentiation of PC12 cells via the MAP kinase pathway by the activation of GLP-1 receptor [19]. Pretreatment with geniposide also prevented neurons from oxidative stress via PI3K signaling pathway in PC12 cells and primary cultured cortical neurons [20,21]. Furthermore, with the activation of GLP-1 receptor, geniposide also up-regulated insulin-degrading enzyme expression to antagonize the cytoxicity of Aβ in primary cultured cortical neurons [13]. A recent study revealed that a single injection of geniposide to the lateral ventricle prevented STZ-induced spatial learning deficit by ~40% and reduced tau phosphorylation by ~30%, demonstrated by Morris water maze test and quantitative immunohistochemical analysis, respectively [10]. But the mechanisms through which geniposide regulates tau phosphorylation are still unknown.

A number of studies have demonstrated that leptin treatment reduces Aβ production and tau phosphorylation in neuronal cultures [22]. Furthermore, leptin treatment reversed these effects by decreasing the expression of BACE1 and the activity of GSK-3β [2,23]. These studies support the scenario where leptin inhibits Aβ production and tau phosphorylation. Currently, leptin therapy may provide promising hopes for the treatment of both the metabolic syndrome and AD. In this study, geniposide was shown to induce the expression level of leptin receptor and decrease the phosphorylation of tau in rat primary cultured cortical neurons as well as in APP/PS1 transgenic mice. Our data further revealed that geniposide is able to enhance the phosphorylation of Akt at ser-473 site and GSK-3β at ser-9 site, which leads to the decrease of its activity, and thus prevents the phosphorylation of tau protein. Furthermore, our data also indicated that LA suppresses the effect of geniposide on the phosphorylation of tau, Akt and GSK-3β in rat primary cortical neurons. All these results suggest that leptin signaling may play a critical role in the geniposide-regulated tau phosphorylation.

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References

1. Harvey J. Leptin: the missing link in Alzheimer disease? *Clin Chem* 2010, 56: 696–697.
2. Greco SJ, Sarkar S, Casadesus G, Zhu X, Smith MA, Ashford JW, Johnston JM, *et al*. Leptin inhibits glycogen synthase kinase-3beta to prevent tau phosphorylation in neuronal cells. *Neurosci Lett* 2009, 455: 191–194.
3. Lee VM, Goedert M, Trojanowski JQ. Neurodegenerative tauopathies. *Ann Rev Neurosci* 2001, 24: 1121–1159.

4. Mandelkow EM, Drewes G, Biernat J, Gustke N, Van Lint J, Vandenheede JR, Mandelkow E. Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. *FEBS Lett* 1992, 314: 315–321.
5. Llorens-Martin M, Jurado J, Hernandez F, Avila J. GSK-3beta, a pivotal kinase in Alzheimer disease. *Front Mol Neurosci* 2014, 7: 46.
6. Greco SJ, Sarkar S, Johnston JM, Tezapsidis N. Leptin regulates tau phosphorylation and amyloid through AMPK in neuronal cells. *Biochem Biophys Res Commun* 2009, 380: 98–104.
7. Pinto S, Roseberry AG, Liu H, Diano S, Shanabrough M, Cai X, Friedman JM, *et al*. Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science* 2004, 304: 110–115.
8. Tezapsidis N, Johnston JM, Smith MA, Ashford JW, Casadesus G, Robakis NK, Wolozin B, *et al*. Leptin: a novel therapeutic strategy for Alzheimer's disease. *J Alzheimers Dis* 2009, 16: 731–740.
9. Zhang Y, Yin F, Liu J, Liu Z, Guo L, Xia Z, Zidichouski J. Geniposide attenuates insulin-deficiency-induced acceleration of beta-amyloidosis in an APP/PS1 transgenic model of Alzheimer's disease. *Neurochem Int* 2015, 89: 7–16.
10. Gao C, Liu Y, Jiang Y, Ding J, Li L. Geniposide ameliorates learning memory deficits, reduces tau phosphorylation and decreases apoptosis via GSK3beta pathway in streptozotocin-induced Alzheimer rat model. *Brain Pathol* 2014, 24: 261–269.
11. Liu J, Zhang Y, Deng X, Yin F. Geniposide decreases the level of Abeta1–42 in the hippocampus of streptozotocin-induced diabetic rats. *Acta Biochim Biophys Sin* 2013, 45: 787–791.
12. Negishi T, Ishii Y, Kyuwa S, Kuroda Y, Yoshikawa Y. Inhibition of staurosporine-induced neuronal cell death by bisphenol A and nonylphenol in primary cultured rat hippocampal and cortical neurons. *Neurosci Lett* 2003, 353: 99–102.
13. Yin F, Zhang Y, Guo L, Kong S, Liu J. Geniposide regulates insulin-degrading enzyme expression to inhibit the cytotoxicity of Abeta(1)(-)(4)(2) in cortical neurons. *CNS Neurol Disord Drug Targets* 2012, 11: 1045–1051.
14. Takashima A. GSK-3 is essential in the pathogenesis of Alzheimer's disease. *J Alzheimers Dis* 2006, 9: 309–317.
15. Wagner U, Utton M, Gallo JM, Miller CC. Cellular phosphorylation of tau by GSK-3 beta influences tau binding to microtubules and microtubule organisation. *J Cell Sci* 1996, 109: 1537–1543.
16. Li X, Lu F, Tian Q, Yang Y, Wang Q, Wang JZ. Activation of glycogen synthase kinase-3 induces Alzheimer-like tau hyperphosphorylation in rat hippocampus slices in culture. *J Neural Transm* 2006, 113: 93–102.
17. Xu GG, Deng YQ, Liu SJ, Li HL, Wang JZ. Prolonged Alzheimer-like tau hyperphosphorylation induced by simultaneous inhibition of phosphoinositol-3 kinase and protein kinase C in N2a cells. *Acta Biochim Biophys Sin* 2005, 37: 349–354.
18. Rankin CA, Sun Q, Gamblin TC. Tau phosphorylation by GSK-3beta promotes tangle-like filament morphology. *Mol Neurodegener* 2007, 2: 12.
19. Liu J, Zheng X, Yin F, Hu Y, Guo L, Deng X, Chen G, *et al*. Neurotrophic property of geniposide for inducing the neuronal differentiation of PC12 cells. *Int J Dev Neurosci* 2006, 24: 419–424.
20. Liu J, Yin F, Zheng X, Jing J, Hu Y. Geniposide, a novel agonist for GLP-1 receptor, prevents PC12 cells from oxidative damage via MAP kinase pathway. *Neurochem Int* 2007, 51: 361–369.
21. Liu JH, Yin F, Guo LX, Deng XH, Hu YH. Neuroprotection of geniposide against hydrogen peroxide induced PC12 cells injury: involvement of PI3 kinase signal pathway. *Acta Pharmacol Sin* 2009, 30: 159–165.
22. Greco SJ, Bryan KJ, Sarkar S, Zhu X, Smith MA, Ashford JW, Johnston JM, *et al*. Leptin reduces pathology and improves memory in a transgenic mouse model of Alzheimer's disease. *J Alzheimers Dis* 2010, 19: 1155–1167.
23. Greco SJ, Hamzelou A, Johnston JM, Smith MA, Ashford JW, Tezapsidis N. Leptin boosts cellular metabolism by activating AMPK and the sirtuins to reduce tau phosphorylation and beta-amyloid in neurons. *Biochem Biophys Res Commun* 2011, 414: 170–174.