



Research Article

A possible mechanism to the antidepressant-like effects of 20 (S)-protopanaxadiol based on its target protein 14-3-3 ζ

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ABSTRACT

Background: Ginsenosides and their metabolites have antidepressant-like effects, but the underlying mechanisms remain unclear. We previously identified 14-3-3 ζ as one of the target proteins of 20 (S)-protopanaxadiol (PPD), a fully deglycosylated ginsenoside metabolite.

Methods: Corticosterone (CORT) was administered repeatedly to induce the depression model, and PPD was given concurrently. The tail suspension test (TST) and the forced swimming test (FST) were used for behavioral evaluation. All mice were sacrificed. Golgi-cox staining, GSK 3β activity assay, and Western blot analysis were performed. In vitro, the kinetic binding analysis with the Biolayer Interferometry (BLI) was used to determine the molecular interactions.

Results: TST and FST both revealed that PPD reversed CORT-induced behavioral deficits. PPD also ameliorated the CORT-induced expression alterations of hippocampal Ser9 phosphorylated glycogen synthase kinase 3β (p-Ser9 GSK 3β), Ser133 phosphorylated cAMP response element-binding protein (p-Ser133 CREB), and brain-derived neurotrophic factor (BDNF). Moreover, PPD attenuated the CORT-induced increase in GSK 3β activity and decrease in dendritic spine density in the hippocampus. In vitro, 14-3-3 ζ protein specifically bound to p-Ser9 GSK 3β polypeptide. PPD promoted the binding and subsequently decreased GSK 3β activity.

Conclusion: These findings demonstrated the antidepressant-like effects of PPD on the CORT-induced mouse depression model and indicated a possible target-based mechanism. The combination of PPD with the 14-3-3 ζ protein may promote the binding of 14-3-3 ζ to p-GSK 3β (Ser9) and enhance the inhibition of Ser9 phosphorylation on GSK 3β kinase activity, thereby activating the plasticity-related CREB–BDNF signaling pathway.

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1. Introduction

Owing to their various pharmacological activities, ginsenosides are considered the principal bioactive ingredients of ginseng, which is the root of *Panax ginseng* Meyer (Araliaceae). Ginseng is known as the “king of herbs” and has been widely used for several thousand years in China, Korea, Japan, and other Asian countries. In the past

several years, many research works confirmed the antidepressant-like effects of ginsenosides and their metabolites [1–6]. Structural plasticity and neurotrophic factors are critical to the mechanism of these antidepressant-like effects. Many studies on different signal transduction pathways demonstrated that the antidepressant activity of ginsenosides may be mediated at least partially by the enhancement of the brain-derived neurotrophic factor (BDNF) expression [2,4–8]. Glycogen synthase kinase 3β (GSK 3β)–cAMP response element-binding protein (CREB) is one of the upper signaling pathways of BDNF. In a previous study, we reported that the antidepressant activity of ginseng total saponins (GTS) is induced by interfering with the signaling pathway of GSK 3β–CREB–BDNF [6].

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An increasing number of pharmacokinetic research works demonstrated that ginsenosides and their metabolites could penetrate the blood–brain barrier (BBB) or the blood–cerebrospinal fluid barrier. Through microdialysis technique and liquid chromatography–tandem mass spectrometry, Xue et al. [9] found that subcutaneously injected Rg1 existed in the brain's extracellular fluid and cerebrospinal fluid and was immediately distributed to learning- and memory-related regions in rat brain. 20 (S)-protopanaxadiol (PPD), a metabolite of ginsenosides, can also penetrate the BBB [10,11]. In our previous study, we used immunofluorescence double staining with antiginsenoside polyclonal antibody to detect ginsenosides in vascular endothelial cells, astrocytes, and neurons in brain tissues after the oral administration of high-purity ginseng total saponins [12]. These results implied that ginsenosides or their metabolites exert antidepressant effects via direct targets in brain tissues. However, studies on these targets, which need to be discovered and characterized, are few.

In recent years, we attempted to identify the immediate brain targets of ginsenosides. Through affinity chromatography, isothermal titration calorimetry, and co-crystallization, we screened several proteins that may interact with ginsenosides and identified the 14-3-3 ζ protein as one of their direct targets. Among the ginsenosides and their metabolites we have studied, PPD exhibited the highest direct molecular interaction with the 14-3-3 ζ protein [13]. The 14-3-3 proteins comprise a highly conserved protein family. They attract an increasing amount of attention as potential targets for pharmacological intervention. The family consists of seven isoforms in Mammalia and named using the Greek letters σ , ζ , β , γ , η , ϵ , and τ . By triggering or interfering with the activities of specific protein partners, they are involved in numerous physiological and pathological cellular processes [14]. Studies have identified over 500 proteins that serve as the binding partners of the 14-3-3 proteins, and this number will continue to grow with ongoing research [15].

GSK 3 β belongs to binding partners. It is one of the two isoforms of GSK 3 and may be the most active kinase in most cells, as it deals with over 100 known substrates [16]. GSK 3 β is ubiquitously expressed and particularly abundant in the brain [17]. The basal enzymatic activity of GSK 3 β depends on Tyr216 autophosphorylation; the primary way to regulate neuronal GSK 3 β activity is by controlling the inhibitory phosphorylation level of Ser9 [18]. GSK 3 β has received much attention for its role in the mechanism of mood stabilizers and the effects of psychoactive therapies, such as antidepressants, antipsychotics, and neurotrophic factors [19,20]. For example, GSK 3 β knockin mice with abnormally active GSK 3 β displayed increased susceptibility to the learned helplessness model of depression-like behavior [21]. The antidepressant activity of GSK 3 β inhibitors was also observed when they reduced the duration of immobility [22].

The neuroplasticity hypothesis on depression suggests that neurotrophic factors and structural plasticity are vital in mediating behavioral responses [23]. BDNF is key to the regulation of neuronal plasticity and plays an important role in adult neurogenesis, neuronal maturation, and synaptic plasticity [24]. Insufficient signaling by BDNF is a latent factor for depression, and antidepressant responses have been observed with the promotion of BDNF signaling [25]. The transcription of some genes, such as BDNF, is directed by activating CREB (Ser133 phosphorylation). As one of the upstream signal molecules of CREB, GSK 3 β negatively regulates the CREB–BDNF signaling pathway [26]. The antidepressant-like effects of GSK 3 β inactivation may be related to the activation of CREB–BDNF signaling pathway.

The GSK 3 β N-terminal end is necessary for interacting with 14-3-3 ζ , and the binding of 14-3-3 proteins to GSK 3 β may inactivate GSK 3 β [27]. For instance, Chang et al. [28] reported that the 14-3-3

σ proteins regulated the proliferation of mouse embryonic stem cell by binding and sequestering phosphorylated GSK 3 β and inactivating GSK 3 β . The 14-3-3 proteins, which are newly confirmed targets of PPD in brain tissues, may play a role in regulating GSK 3 β –CREB–BDNF signaling pathway. Thus, this study aimed to determine whether the binding of PPD to 14-3-3 ζ affects the binding of 14-3-3 ζ to GSK 3 β and the activity of GSK 3 β . Moreover, this study attempted to elucidate the latent antidepressant mechanisms of PPD in view of the GSK 3 β –CREB–BDNF signaling pathway.

2. Materials and methods

2.1. *In vitro* experiments

2.1.1. Biolayer interferometry (BLI)

The binding of the 14-3-3 ζ protein to the GSK 3 β protein, the p-Ser9 GSK 3 β polypeptide, or the p-Tyr216 GSK 3 β polypeptide was measured via biolayer interferometry (BLI) by using the Octet RED96 system (ForteBio, Menlo Park, CA, USA), which is label-free and detectable in real time [29]. The detailed procedures were identical to those we described in another study [30]. Association (K_{on}) and dissociation (K_{off}) curves were fitted using a single exponential fitting model. By curve fitting, the affinity constant (K_D) was calculated ($K_D = K_{off}/K_{on}$). All experiments were repeated thrice.

The effects of PPD (purity 98%) (National Institute of Food and Drug Control, Beijing, China) on the binding of the 14-3-3 ζ protein and the p-Ser9 GSK 3 β polypeptide were detected via the competition method in BLI. Three Ni-NTA sensors were used and corresponded to the control group, PPD and PPD + GSK 3 β group, and GSK 3 β and GSK 3 β + PPD group. Data on molecular interactions were collected in real time by using the ForteBio Data Acquisition software through the balance of the sensor, immobilization of the target protein, and several related steps. Finally, the data were analyzed using the ForteBio Data Analysis software.

2.1.2. Pull-down assay

Pull-down assay was performed in accordance with the description of Wang et al. [31]. First, 200 ng purified 14-3-3 ζ was diluted to a final volume of 500 μ L with lysis buffer. Subsequently, PPD was added to the final concentration of 3.75 μ g/mL. As for the negative control, the same volume of 75% alcohol was added. The mixture was rotated at 4 °C for 4 h. Then, 20 μ L of the diluted lysate was used as input. PPD–PEGA resin was sub-packaged and washed thrice with lysis buffer. Then, it was resuspended in the diluted lysate and rotated at 4 °C for another 4 h. After the removal of the supernatant, the resin was washed thrice with lysis buffer. Western blot analysis was performed to determine whether PPD affected the binding of the 14-3-3 ζ protein to the phosphorylation site of GSK 3 β (Ser9).

2.1.3. GSK 3 β activity assay

Hippocampal tissues (500 mg) were homogenized with 2 mL ice-cold RIPA lysis buffer. Then, the homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. Approximately 20 μ L of the supernatant was collected to analyze GSK 3 β activity in accordance with the manufacturer's instructions (GMS50161.4.3 v. A; Genmed Scientifics Inc., USA). All measurements were performed in triplicate.

2.2. *In vivo* experiments

2.2.1. Experimental animals

Male C57BL/6 N mice (18–22 g) were obtained from the Laboratory Animal Center of Nanjing Medical University (Nanjing,

China) and maintained in an SPF laboratory animal center in Nanjing University of Chinese Medicine. The mice were housed at five mice per cage with free water and food under controlled environmental conditions (room temperature of $22 \pm 2^\circ\text{C}$ and 12 h light/12 h dark cycle). The mice were allowed to habituate for 1 week. All animal experiments were strictly conducted in accordance with the experimental animal guidelines of Laboratory Animal Research Institute and were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine. Efforts were exerted to minimize animal suffering and reduce the number of mice used in the experiment.

2.2.2. Experimental design

Chronic corticosterone (CORT)-induced mouse depression model was prepared, and the mice were synchronously treated with fluoxetine (FLU) or different doses of PPD in 4 weeks (Fig. 1). The mice's depression-like behavior was evaluated through forced swimming test (FST) and the tail suspension test (TST). After the last dose of CORT, all the animals were sacrificed for subsequent Western blot analysis, GSK 3 β enzyme activity assay, and Golgi staining. The expressions of neuroplasticity-related proteins and the GSK 3 β –CREB–BDNF signaling pathway were determined via Western blot. Spine density in the hippocampus was observed via Golgi staining.

2.2.3. Grouping and drug treatments in mice

Sixty male mice were divided randomly into six matched groups (N = 10/group), as follows: (a) control group; (b) CORT group (20 mg/kg); (c) CORT + 10 mg/kg FLU (CORT + FLU) group; (d) CORT + 50 mg/kg PPD (CORT + PPDH) group; (e) CORT + 25 mg/kg PPD (CORT + PPDM) group; and (f) CORT + 12.5 mg/kg PPD (CORT + PPDL) group.

CORT was suspended in physiological saline containing 0.1% dimethyl sulfoxide and 0.1% Tween-80 at the concentration of 30 mg/mL. The suspension was intraperitoneally injected (20 mg/kg, TCI C0388) once a day during the light phase for 4 weeks to induce the depression model [32]. Plasma CORT levels were persistently elevated, and the peaks occurred within 4 h of the intraperitoneal injection [33]. The control group was injected with the vehicle. FLU (Ark Pharm) was dissolved in physiological saline (NaCl, 0.9%) at a concentration of 10 mg/kg. PPD (purity 98%) (National Institute of Food and Drug Control, Beijing, China) was dissolved in 0.9% saline, to which 1% sodium carboxymethylcellulose was added. For 4 weeks, FLU or PPD was administered daily to respective groups by gastric gavage, whereas the control and CORT

groups received distilled water. By following the clinical routine usage of ginseng and PPD, we employed the oral route of PPD. Owing to the relatively low bioavailability of the drug when administered by oral gavage, we administered higher PPD doses (12.5, 25, and 50 mg/kg/day) by gavage in contrast to the approach used in previous studies (intraperitoneal injection) [4,5].

2.2.4. Behavioral tests

Behavioral tests included FST and TST. They were performed in JLBHV-FSG-4 sound insulation boxes with a DigBehav animal behavior video analysis system (Shanghai Jiliang Software Technology Co., Ltd., Shanghai, China). The system can record and analyze animal movements automatically to provide total immobility time. FST and TST were conducted on D26 and D27, respectively. Depression-like behavior was inferred from the increase in immobility time.

FST was performed in accordance with the method described by Porsolt et al. [34]. The mice were individually placed in 10 cm-deep water at constant temperature ($25 \pm 1^\circ\text{C}$) in a 2 L cylindrical beaker and allowed to swim freely for 5 min. The duration of immobility time was recorded during the last 4 min. TST was conducted in accordance with the method outline by Steru et al. [35]. At 24 h after FST, TST was performed. Mice was suspended individually on the edge of a shelf at 58 cm above the bottom by using an adhesive tape placed 1 cm from the tip of the tail. These mice were hung for 6 min, and the immobility time was recorded during the last 4 min.

2.2.5. Western blot analysis

Mouse hippocampal tissues were homogenized with a cocktail of protease/phosphatase inhibitors in ice-cold PIPA lysis buffer. A ball mill instrument was used to thoroughly break up tissues. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C , and supernatants were collected. The concentration of total proteins was valued using a BCA protein assay kit (CWBIOTECH, CW0014S). Equal amounts of protein (40 μg) were loaded on SDS-PAGE gels for electrophoretic separation and then transferred onto PVDF membranes. The PVDF membranes were blocked in 5% BSA for 1.5 h and then incubated overnight at 4°C with different primary antibodies. The primary antibodies were as follows: rabbit polyclonal anti-GSK 3 β (1:1000, Affinity Biosciences, AF5016), rabbit polyclonal anti-Phospho-GSK 3 β (Ser9) (1:1000, Affinity Biosciences, AF2016), rabbit polyclonal anti-CREB (1:1000, Affinity Biosciences, AF6188), rabbit polyclonal anti-Phospho-CREB (Ser133) (1:1000, Affinity Biosciences, AF3189), rabbit monoclonal anti-BDNF (1:1000, Abcam, ab108319), and mouse monoclonal anti- β -actin (1:5000,

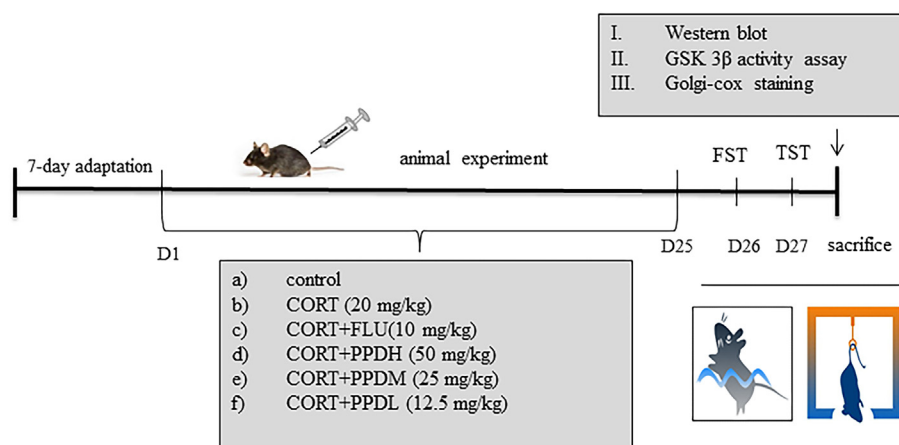


Fig. 1. Timeline of the in vivo experimental procedure.

Proteintech, 66009-1-Ig). The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000, Proteintech) and goat anti-rabbit IgG (1:5000, Proteintech). All values indicating protein levels were normalized against the amount of β -actin obtained from the same sample. All experiments were performed at least thrice.

2.2.6. Golgi–Cox staining

Golgi–Cox staining was performed in accordance with the manufacturer’s protocol (Golgi–Cox OptimStain™ PreKit; Histo-biotech Corp. Kingsport, TN, USA). Whole brains were quickly rinsed in ddH₂O and soaked in impregnated silver solution for 2 weeks at room temperature (RT) in the dark. The brain tissues were then transferred to a tissue protection solution, maintained in the dark at 4 °C for 72 h, and coronally sectioned at 120 μ m on a cryostat. These sections were mounted in the tissue protection solution on adhesive microscope slides and air-dried at RT in the dark. Dendritic spine density was assayed for quantification by counting the spine number along the same length of dendritic segments in hippocampal CA1 regions.

2.3. Statistical analysis

Data were expressed as mean \pm standard error of mean (S.E.M) for the indicated number of experiments. Statistical Package for Social Sciences (SPSS) software (version 10.1) was used for statistical analysis, and statistical significance was determined by one-way ANOVA. In case of significant interactions, Tukey’s honest significant difference test of multiple comparisons was performed.

A significance level was set at $p \leq 0.05$ for all statistical comparisons.

3. Results

3.1. The 14-3-3 ζ protein specifically bound to the p-Ser9 GSK 3 β polypeptide

The binding affinity of the 14-3-3 ζ and GSK 3 β proteins and that of the p-Ser9 GSK 3 β (Gly3-pSer9-Glu12, GRPRTTSFAE) and p-Tyr216 GSK 3 β polypeptides (Glu211-pTyr216-Arg220, EPNV-SYICSR) were determined via BLI. As shown in Fig. 2A, B, and 2C, the 14-3-3 ζ protein had no immediate interaction with non-phosphorylated GSK 3 β protein or p-Tyr216 GSK 3 β polypeptide but was specifically bound to the p-Ser9 GSK 3 β polypeptide. The affinity (K_D) of the 14-3-3 ζ protein for the p-Ser9 GSK 3 β polypeptide was $4.68 \pm 3.20E-5$ M. The K_{on} and K_{off} ratios of the 14-3-3 ζ protein to the p-Ser9 GSK3 β polypeptide were $5.79E2$ 1/Ms and $1.85E-2$ 1/s, respectively (Fig. 2D).

3.2. PPD promoted the binding of the 14-3-3 ζ protein to the p-Ser9 GSK 3 β polypeptide

The effects of PPD on the binding of the 14-3-3 ζ protein and the p-Ser9 GSK 3 β polypeptide were detected by using the competition method. As shown in Fig. 3A and B, the 14-3-3 ζ protein combined with the mixture of PPD and the p-Ser9 GSK 3 β polypeptide after being bound to PPD showed a significantly higher response (0.9654 nm) than direct interaction with the p-Ser9 GSK 3 β polypeptide (0.074 nm).

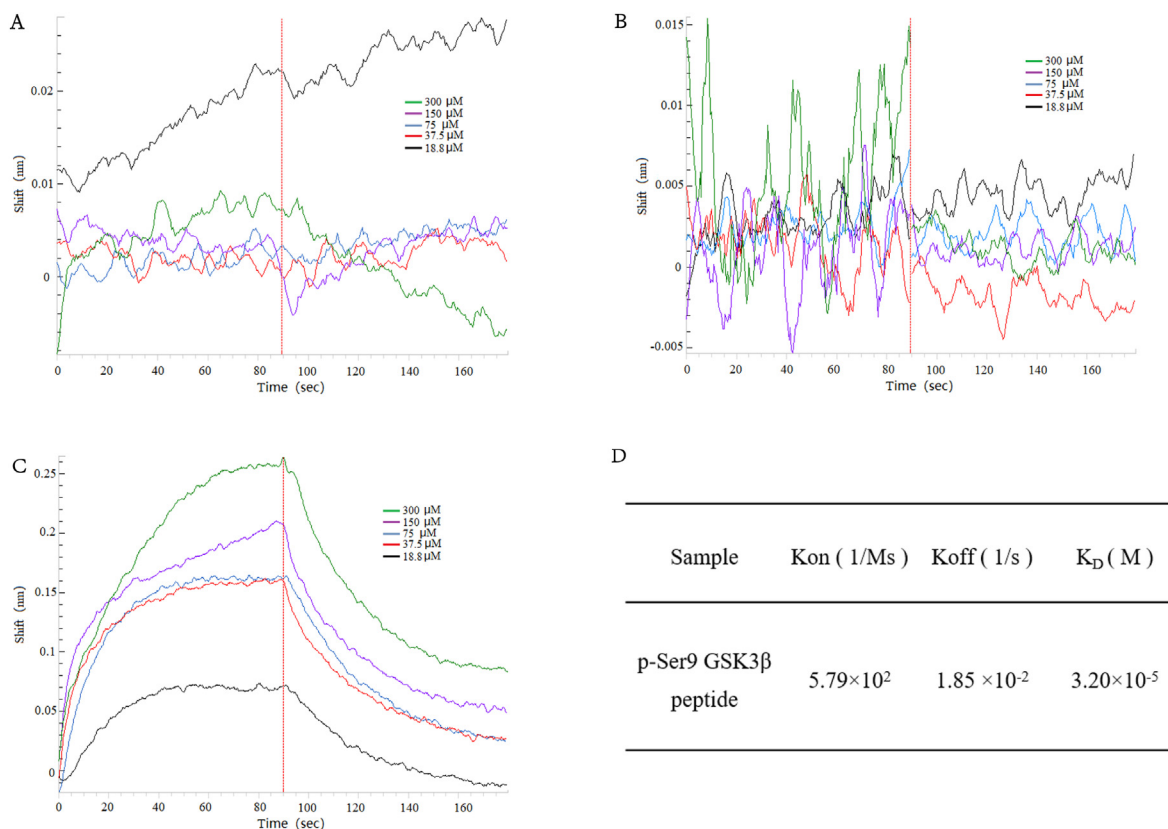


Fig. 2. The 14-3-3 ζ protein specifically bound to the p-Ser9 GSK 3 β polypeptide. (A) BLI detection images of the 14-3-3 ζ protein and the GSK 3 β protein. (B) The 14-3-3 ζ protein and the Tyr216 phosphorylated GSK 3 β polypeptide, and (C) the 14-3-3 ζ protein and the Ser9 phosphorylated GSK 3 β polypeptide. (D) Affinity constants of the 14-3-3 ζ protein and the Ser9 phosphorylated GSK 3 β polypeptide. The results represent no less than three experiments. Data are expressed as mean \pm S.E.M. (n = 3).

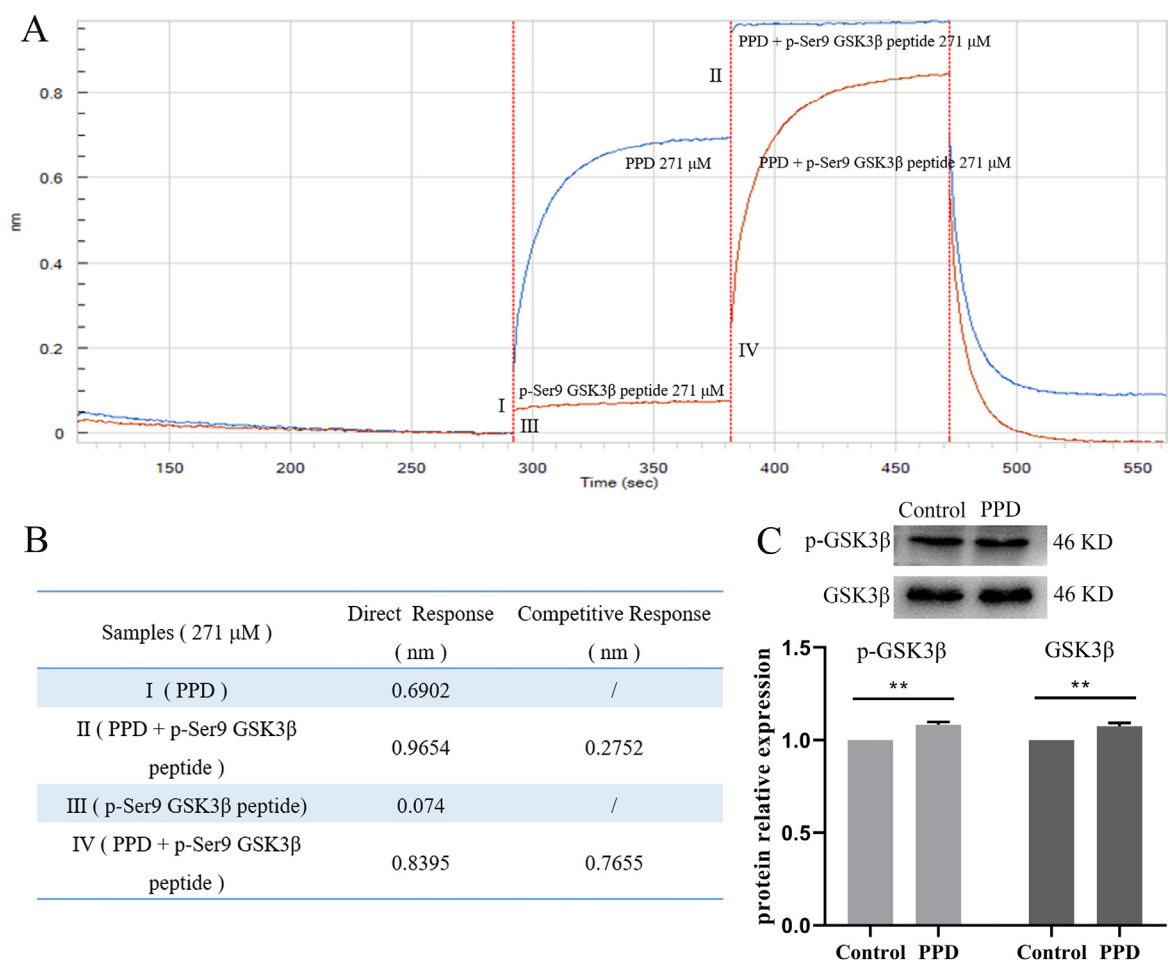


Fig. 3. PPD boosted the binding of the 14-3-3 ζ protein and the p-Ser9 GSK 3β polypeptide. (A) Image of competitive BLI method. (B) Response data from the competitive BLI method. (C) Western blot analysis showed that more p-Ser9 GSK 3β was pulled down in the PPD group. ***p* < 0.01 vs. the control group.

The effects of PPD on the binding of the 14-3-3 ζ protein to the GSK 3β p-Ser9 site were detected through the pull-down technique combined with Western blot. As shown in Fig. 3C, significantly more p-Ser9 GSK 3β and GSK 3β were pulled down in the PPD group than that in the control group (*p* < 0.01), and the relative expression levels of p-Ser9 GSK 3β and GSK 3β in the PPD group were similar.

3.3. PPD inhibited GSK 3β activity by regulating Ser9 phosphorylation

The effects of PPD at three concentrations on GSK 3β activity in mouse brain tissues in vitro were determined. As shown in Fig. 4A, PPD significantly decreased GSK 3β activity, especially in the PPDm group (*p* < 0.01), indicating that the binding of PPD to the 14-3-3 ζ protein enhanced the inhibitory effect of GSK 3β Ser9 phosphorylation on GSK 3β activity.

The quantitative determination of kinase activity in the hippocampal tissues of the CORT-induced depression model was performed to determine whether PPD had the same effect on GSK 3β activity in vivo. As shown in Fig. 4B, the GSK 3β activity in the CORT group significantly increased in comparison with that in the control group (*p* < 0.05). Compared with the activity of GSK 3β in the CORT

group, that in the groups treated with FLU or different doses of PPD significantly decreased (all *p* < 0.05).

3.4. PPD ameliorated depression-like behavior and promoted the protein levels of p-Ser9 GSK 3β, p-Ser133 CREB, and BDNF in the CORT-induced depression model

Immobility times significantly differed among the groups in FST (Fig. 5A) and TST (Fig. 5B). Repeated CORT injections significantly increased the immobility time during FST and TST compared with the control group (*p* < 0.05). Compared with the CORT group, synchronous treatment with FLU (*p* < 0.05 in both FST and TST), PPDH (*p* < 0.05 in both FST and TST), PPDm (*p* < 0.05 in both FST and TST), or PPDl (*p* < 0.05 in FST) reversed the immobility time.

As shown in Fig. 5E, the ratio of p-Ser9 GSK 3β and GSK 3β statistically differed among groups (*p* < 0.05). The ratio significantly decreased in the CORT group compared with the control group (*p* < 0.05), and it increased in the FLU (*p* < 0.05), PPDH (*p* < 0.05), PPDm (*p* < 0.05), and PPDl (*p* < 0.05) groups compared with the CORT group.

Post hoc comparison results showed that the p-Ser133 CREB/CREB ratio was lower in the CORT group than in the control group (*p* < 0.05) and higher in groups treated with FLU or different doses of PPD than in the CORT group (all *p* < 0.05).

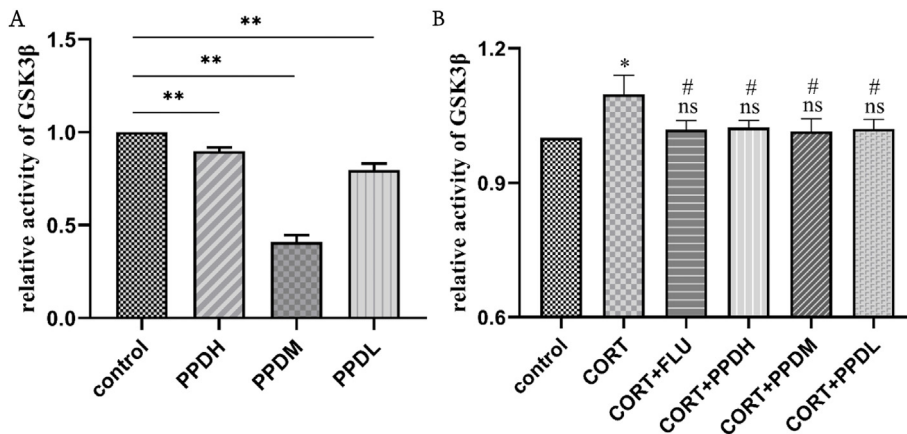


Fig. 4. Effects of PPD on GSK3β activity. (A) Effects of high, medium, and low doses of PPD (PPDH, PPDM, and PPDL) on GSK3β kinase activity in mouse brain tissues in vitro. (B) Effects of fluoxetine (FLU) and different doses of PPD on GSK 3β activity in hippocampal tissues of the corticosterone (CORT)-induced depression model. ***p* < 0.01 vs. the control group, **p* < 0.05 vs. the control group, ^{ns}no statistical significance vs. the control group, #*p* < 0.05 vs. the CORT group.

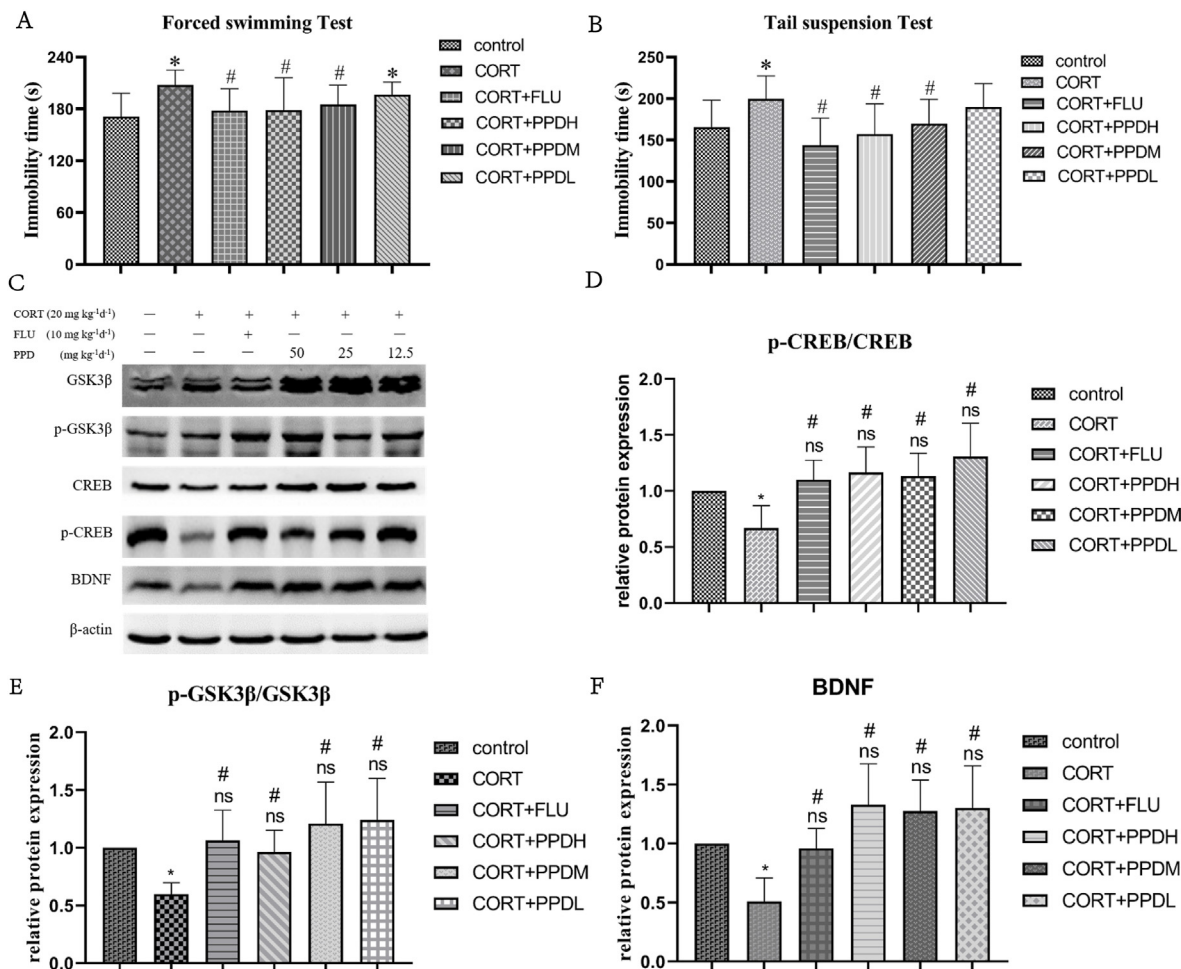


Fig. 5. Effects of fluoxetine (FLU) or PPD on the immobility time in the forced swimming test (FST) (A) and tail suspension test (TST) (B) in the corticosterone (CORT)-induced depression model. Representative Western blot images of GSK 3β, p-Ser9 GSK 3β, CREB, p-Ser133 CREB, BDNF, and β-actin expression levels (C). Western blot analysis showing relative protein levels of p-Ser133 CREB (D), p-Ser9 GSK 3β (E), and BDNF (F). **p* < 0.05 vs. the control group, ^{ns}no statistical significance vs. the control group, #*p* < 0.05 vs. the CORT group.

The hippocampal protein level of BDNF in the CORT group was statistically lower than that in the control group (*p* < 0.05), whereas daily treatment with FLU (*p* < 0.05), PPDH (*p* < 0.05), PPDM

(*p* < 0.05), or PPDL (*p* < 0.05) during chronic CORT injections reversed the aforementioned detrimental effects.

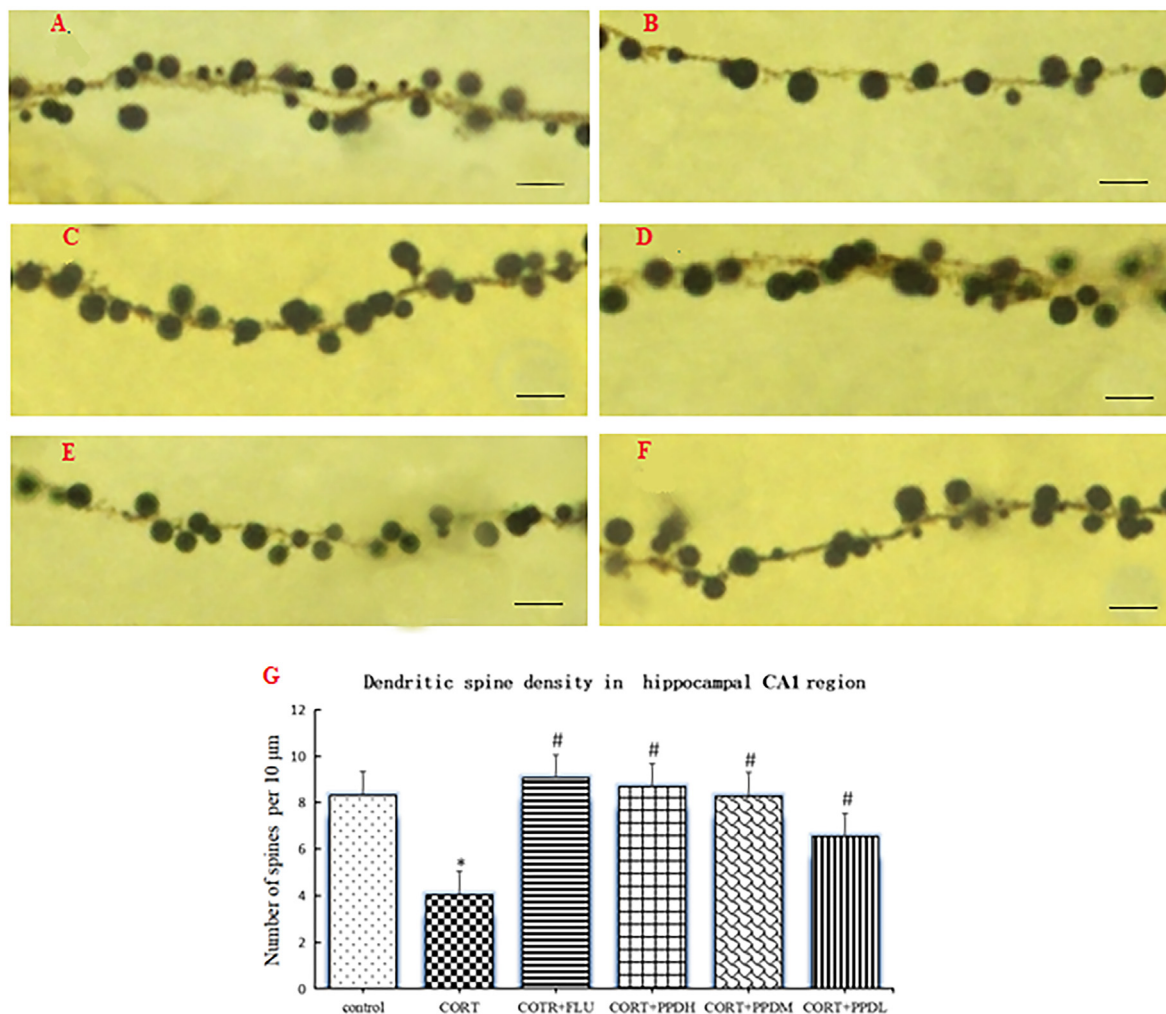


Fig. 6. Dendritic spine density in hippocampal CA1 region differed among treatment groups. A–F: control, CORT, CORT + FLU, CORT + PPDH, CORT + PPDM, and CORT + PPDL. * $p < 0.05$ vs. the CORT group, # $p < 0.05$ vs. the control group.

3.5. PPD attenuated the decrease in the dendritic spine density in the CORT-induced depression model

The dendritic spine density in the CA1 region of the hippocampi was analyzed via Golgi–Cox staining. Fig. 6 shows that repeated CORT injections induced a significant decrease in the number of dendritic spines compared with the control group ($p < 0.05$). Compared with the CORT group, spine density was significantly reversed by simultaneous treatment with FLU ($p < 0.05$), PPDH ($p < 0.05$), PPDM ($p < 0.05$), or PPDL ($p < 0.05$).

4. Discussion

Depression is a mood disorder characterized by a constant sense of hopelessness and despair. Although the underlying pathogenesis and etiology of depression remain unclear, BDNF, a crucial regulatory component in neuronal plasticity, is postulated to play an important role in depression [36]. BDNF serves as a transducer that links the antidepressant drug and neuroplastic changes, resulting in the improvement of depressive symptoms [37]. In the present study, we evaluated the antidepressant-like effects of PPD by observing alterations in depression-like behavior, hippocampal neuronal structural plasticity (dendritic spine density), and BDNF expression. Compared with the widely used chronic mild stress

(CMS) model, the CORT-induced model has no obvious difference in depression-like behaviors and in the levels of hippocampal depression-related proteins; the CORT-induced model has a simpler operation and a shorter modeling cycle than the former [38–40]. Owing to its convenience and reliability, the CORT-induced model has been increasingly accepted by the academic community. This model was adopted in the present study.

The present study demonstrated the antidepressant-like effects of PPD on the CORT-induced model. Similar to the classical antidepressant FLU, PPD can reverse the increased immobility time in the behavior tests, activate the suppressed hippocampal CREB–BDNF signaling pathway, and ameliorate the decrease in dendritic spine density. These results were consistent with the findings of other studies on ginsenosides in which the CMS model was used. For instance, the neuroprotective and ameliorating effects of ginsenoside Rg1 on depression-like behavior were mediated, at least in part, by activating the CREB–BDNF system in CMS rat model [7,41]. Zhang et al. [42] found that ginsenoside Rg3 had an apparent antidepressant effect on both the NMDA-treated cell model and the CMS mouse model. Moreover, this effect was mediated by the CREB–BDNF signaling pathway.

Our results on GSK 3 β , one of the negative upstream signal molecules of the CREB–BDNF signaling pathway, showed that PPD substantially ameliorated the decrease in Ser9 p-GSK 3 β /GSK 3 β

ratio in the CORT-induced depression model, similar to a previously report's results on the total saponins of ginseng [6]. As a multi-tasking kinase that is abundantly expressed throughout the brain, GSK 3 β is positioned at various signaling crossroads [43]. Lin et al. [44] found that PPD stimulates the Ser9 phosphorylation of GSK 3 β in vitro, resulting in the activation of the Wnt/GSK 3 β / β -catenin pathway and the promotion of the proliferation and differentiation of neural stem cells. Although Lin et al. [44] and the present study differed in experimental designs, the same result was obtained, i.e., PPD improved Ser9 phosphorylation of GSK 3 β . The increase in the levels of Ser9 p-GSK 3 β , p-CREB, and BDNF in the hippocampus was the underlying antidepressant mechanism of Jieyu chufan, a well-known traditional Chinese medicine used to treat depression [45]. PPD administration improved the ratios of Ser9 p-GSK 3 β /GSK 3 β and p-CREB/CREB, as well as the total protein levels of GSK 3 β and CREB, in the CORT-induced depression model. Yu et al. [7] reported similar effects of ginsenoside Rg1 on p-CREB/CREB and CREB levels in the chronic unpredictable mild stress rat model. These observations might be attributed to the multiple target nature of ginsenosides and their metabolites.

Given that GSK 3 β phosphorylation at Ser9 inhibits its kinase activity, the increased Ser9 phosphorylation of GSK 3 β is an indirect index of the decrease in GSK 3 β activity [46]. However, correctly estimating kinase activity by using Ser9 phosphorylation level is difficult [17]. Therefore, we performed a GSK 3 β kinase activity assay. The results definitively demonstrated that PPD depressed GSK 3 β kinase activity in vitro and ameliorated the increase in GSK 3 β kinase activity induced by CORT in vivo. To our limited knowledge, the present study directly confirmed the effects of PPD on GSK 3 β kinase activity for the first time. The results implied that the neuroprotective properties of PPD may be at least partially mediated by the inhibition of GSK 3 β activity.

However, evidence indicating the direct interaction of PPD with GSK 3 β is lacking. According to our previous report, GSK 3 β is not among the five candidate target proteins of ginsenosides in brain tissues. Among these potential targets, the 14-3-3 ζ protein has been recently confirmed via several techniques [11]. The present study explored whether the effects of PPD on GSK 3 β are mediated by the 14-3-3 ζ protein by investigating the direct interaction of the 14-3-3 ζ protein with GSK 3 β via BLI. The 14-3-3 ζ protein had no intermolecular interaction with unphosphorylated GSK 3 β protein or Tyr216 phosphorylated GSK 3 β peptide but selectively interacted with Ser9 phosphorylated GSK 3 β peptide. This finding was supported by earlier research reports. Mwangi et al. [47] found that the glial cell line-derived neurotrophic factor enhances the phosphorylation of GSK 3 β at its N-terminal serine-9 residue and demonstrated the specificity of the interaction between p-GSK 3 β (Ser9) and 14-3-3 protein by co-immunoprecipitation combined with Western blot analysis. Chang et al. [26] observed that the interaction of 14-3-3 σ with GSK 3 β is reduced when S9A mutated but not T309A, thereby indicating that 14-3-3 σ preferentially binds to p-GSK 3 β (Ser9).

After confirming the direct binding interaction between 14-3-3 ζ and p-GSK 3 β (Ser9), we further studied the role of PPD in the interaction. Results of competitive BLI and pull-down assay indicated that PPD promoted the binding of the 14-3-3 ζ protein to p-GSK 3 β (Ser9). The results of pull-down assay also showed that PPD increased GSK 3 β levels, which were higher than those of p-Ser9 GSK 3 β in the same groups. The 14-3-3 ζ protein specifically bound to p-Ser9 GSK 3 β , and p-Ser9 GSK 3 β and GSK 3 β had similar relative expression levels. Thus, the PPD-induced increase in GSK 3 β level may be due to the increase in p-Ser9 GSK 3 β level. In general, antibodies against nonphosphorylated sites have a higher affinity than phosphorylation site-specific antibodies, which may explain the higher levels of GSK 3 β than p-Ser9 GSK 3 β .

Given that PPD exerted inhibitory effects on GSK 3 β activity, the coupling of the 14-3-3 ζ protein to p-GSK 3 β (Ser9) likely inhibited GSK 3 β kinase activity. PPD combined with its target protein 14-3-3 ζ promoted the binding of the 14-3-3 ζ protein to p-GSK 3 β (Ser9), thereby enhancing the inhibitory effect of S9 phosphorylation on GSK 3 β . The present study established the antidepressant-like effects of PPD in view of the hippocampal GSK 3 β –CREB–BDNF signaling pathway and the consequential alteration in neuronal structural plasticity. Moreover, it clarified how PPD inhibited GSK 3 β activity. As far as our knowledge, these findings are the first to elucidate the possible antidepressant mechanism of PPD on the basis of its direct target protein in brain tissues.

5. Conclusion

The combination of PPD with its target protein 14-3-3 ζ promoted the binding of the 14-3-3 ζ protein to p-GSK 3 β (Ser9), thereby enhancing the inhibitory effect of Ser9 phosphorylation on GSK 3 β kinase activity. PPD exerted antidepressant-like activities through its function on the GSK 3 β –CREB–BDNF signaling pathway and hippocampal structural plasticity.

Author contributions

Yunan Zhao and Lin Chen designed the project. Ruimei Li performed Western blot assay and GSK 3 β activity assay. Feiyan Chen performed intermolecular interaction detections. Hantao Zhang and Shuyi Xu performed model building and drug treatments. Zhu Zhu performed behavioral tests. Yao Cheng performed Golgi-cox staining. Lin Chen, Ruimei Li, and Feiyan Chen wrote the manuscript. All authors discussed and commented on the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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