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## The effects of the renin inhibitor aliskiren on neuronal toxic damage

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Received July 14, 2021, accepted August 13, 2021

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Pharmazie 76: 544-550 (2021)

doi: 10.1691/ph.2021.1729

The neurotoxicity of amyloid- $\beta$  (A $\beta$ ) and its deposition in neurons plays a critical role in the occurrence and development of Alzheimer's disease (AD). Several preclinical experiments have found that the renin inhibitor aliskiren has a wide range of physiological effects, including hindering the progression of atherosclerosis and anti-inflammatory. This study is aimed to explore the effect of aliskiren on neuronal toxic damage and the underlying mechanism. This study established an *in vitro* nerve injury model through A $\beta_{1-42}$  induction; the effects of aliskiren on the viability, inflammatory damage and apoptosis of SH-SY5Y cells were examined. For the sake of explore the underlying mechanism; SwissTargetPrediction website and molecular docking were utilized to predict the target of aliskiren. Then the impacts of the target protein overexpression were determined to verify its mediation. The results of the current study demonstrate that aliskiren has no effect on the viability of SH-SY5Y cells while A $\beta_{1-42}$  accumulation could significantly downregulate cell viability. In addition, aliskiren could alleviate neuronal inflammatory damage and apoptosis arise from A $\beta_{1-42}$  accumulation. Following confirming the high expression level of the predicted target PDE4B in damaged cells, it was found that PDE4B overexpression can reverse the influence of aliskiren on cell viability, inflammatory damage and apoptosis. In conclusion, aliskiren upregulates cell viability, reduces inflammatory damage and apoptosis induced by A $\beta$  accumulation in AD *via* inhibiting PDE4B. These findings have expanded the scope of future application of aliskiren and provided a theoretical basis.

### 1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that occurs majorly in people over 65 years old. With 65 years old as the boundary, younger patients are classified as presenile dementia (Stekete et al. 2016) while older ones as senile dementia (Teive et al. 2016). Clinically, AD is primarily characterized by comprehensive dementia containing cognitive function and behavior execution disorder, personality and behavior variation (Fonte et al. 2019). The Alzheimer's disease report pointed out that there are more than 50 million people with dementia in the world currently, and it is estimated that the number will reach 150 million by 2050 (Calsolaro et al. 2019). The pathological features of AD are dominantly senile plaques as a consequence of abnormal deposition of amyloid- $\beta$  (A $\beta$ ) and neurofibrillary tangles resulted from hyperphosphorylation of tau protein (Gallardo and Holtzman 2019). Since the first drug for the treatment of AD was launched in 1993 (de Los Ríos and Marco-Contelles 2019), plenty of pharmaceutical companies have devoted themselves to the development of new therapeutic drugs, but so far there are still very rare drugs approved for clinical treatment (Briggs et al. 2016). Specifically, there are only two types of approved drugs, including cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) antagonists (Gao et al. 2016). Although they can effectively treat the symptoms of AD, they cannot completely cure or prevent AD (Grossberg et al. 2019). Certainly, there are still abundant researchers who deepen the pathology theory of AD based on existing mechanisms, such as abnormal tau protein metabolism, A $\beta$  protein, inflammatory response, cholinergic and free radical damage, in order to develop the methods to terminate or alter the course of AD (Breijyeh and Karaman 2020).

Aliskiren is the first synthetic oral, non-peptide, direct renin inhibitor approved for marketing in 2007 (Anderson 2007). Many clinical trials have shown that aliskiren can effectively lower blood

pressure with few side effects (Luft and Weinberger 2008; Zhao et al. 2020). In animal experiments, the combined use of aliskiren and olmesartan in a diabetic rat model induced by streptozotocin (STZ) has been shown to reduce proteinuria, anti-inflammatory and downregulate the levels of circulating transforming growth factor- $\beta$ . And the combined use is better than the respective single use, accompanied by more significantly influence on lower blood pressure (Gandhi et al. 2012). Moreover, preclinical experiments have found that aliskiren has a wide range of physiological effects, not only can get a good command of high blood pressure and inhibit myocardial fibrosis (Müller et al. 2008), but more importantly, it can also hinder the progression of atherosclerosis (Seto et al. 2014) and anti-inflammatory (Aziz et al. 2020). However, whether aliskiren can play a role in neurotoxicity is still unknown. Based on this, this study established an *in vitro* nerve injury model through A $\beta$  induction, observed the effect of aliskiren on neuronal toxic damage, and explored the mechanism of aliskiren and related signal pathways, seeking to provide a theoretical basis and experimental basis for the development and clinical application of aliskiren.

### 2. Investigations and results

#### 2.1. Aliskiren upregulates cell viability and reduces inflammatory damage of A $\beta$ -induced SY5Y cells

The viability of SH-SY5Y cell lines treated with different concentrations of aliskiren was determined using a CCK8 assay. The highest experimental concentration of aliskiren had no significant effect on cell viability (Fig. 1A). Cells that had not undergone any treatment were deemed as the control group. The results of the CCK8 assay demonstrated that the viability of the cells treated by A $\beta$  had been significantly reduced, but the decline of that in

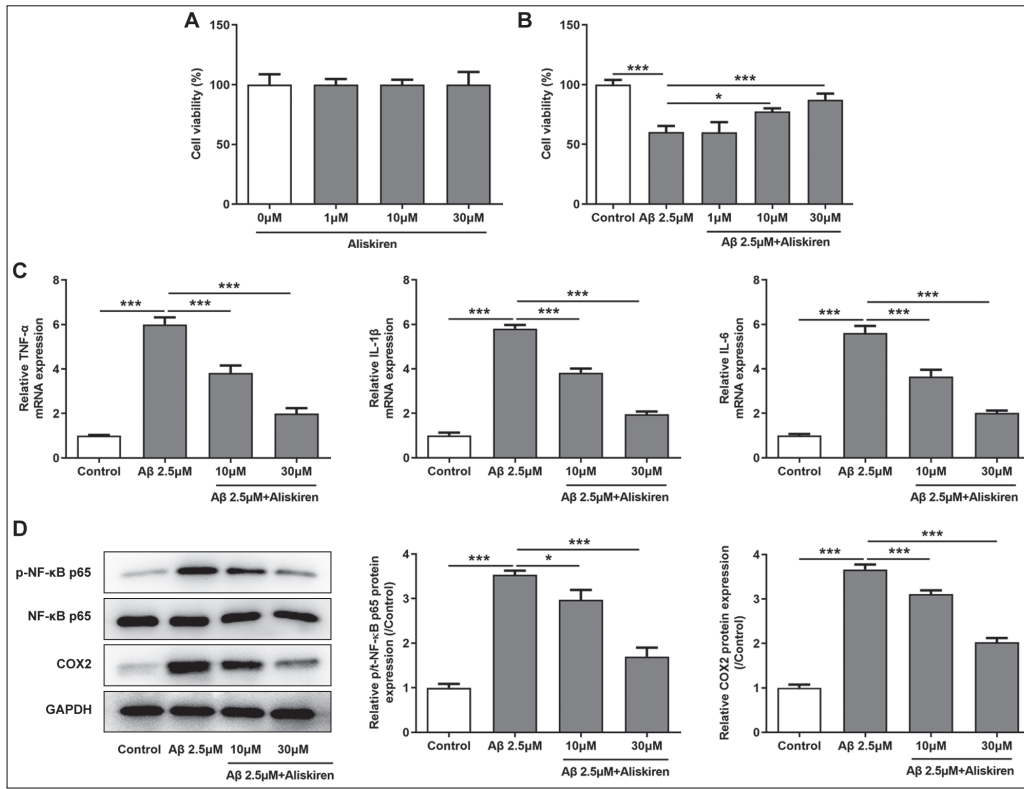


Fig. 1: Aliskiren upregulates cell viability and reduces inflammatory damage of Aβ-induced SY5Y cells. (A) The viability of SH-SY5Y cell lines treated with different concentrations of aliskiren was determined using a CCK8 assay. (B) The viability of SH-SY5Y cell lines treated with Aβ and aliskiren was determined using a CCK8 assay. (C) The expression levels of TNF-α, IL-1β and IL-6 were examined using RT-qPCR. (D) The expression levels of p-NF-κB p65, NF-κB p65, Cox2 were examined using western blotting. \*P<0.05, \*\*\*P<0.001.

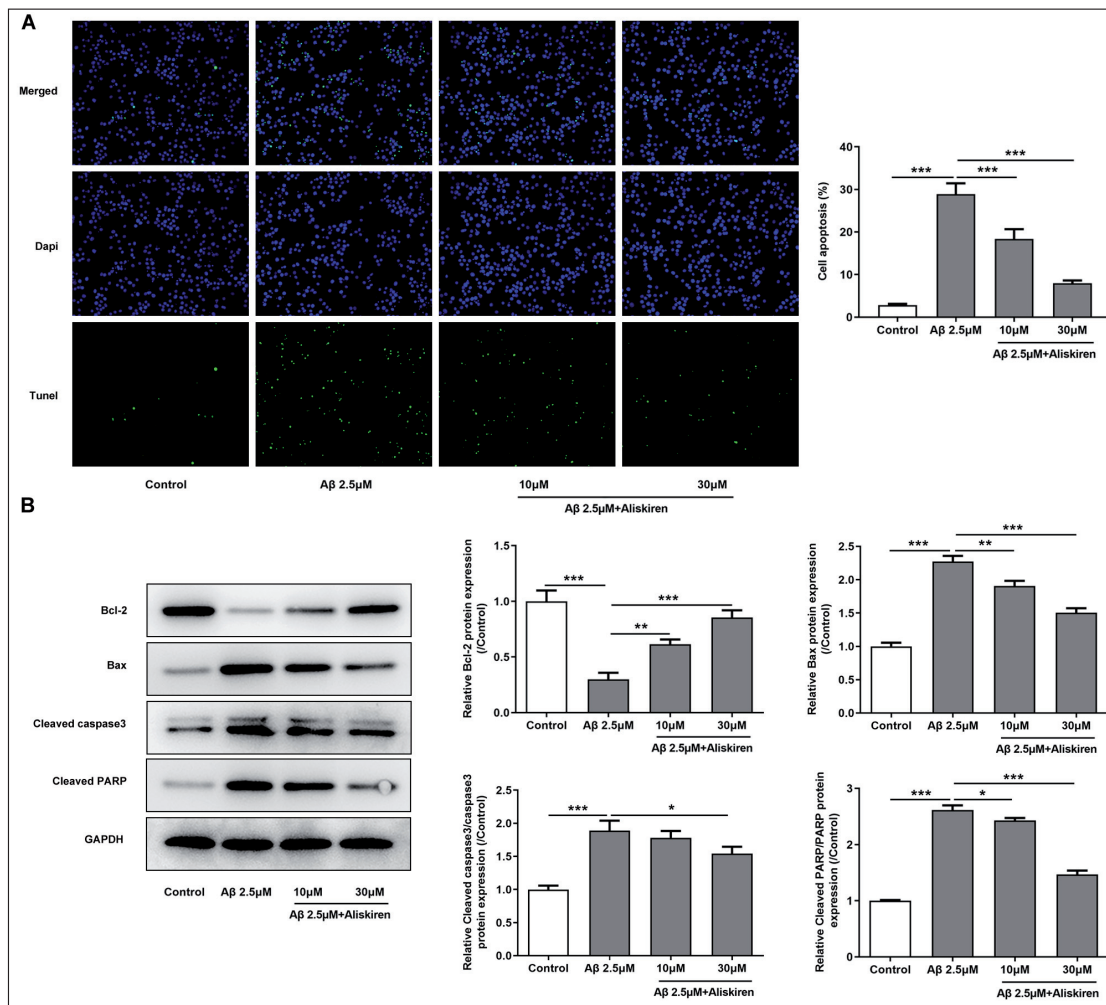


Fig. 2: Aliskiren reduces the apoptosis of Aβ-induced SY5Y cells. (A) The apoptosis levels of cells were determined using TUNEL assay (B) The expression levels of protein relevant to apoptosis were determined using western blotting. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

cells pretreated with aliskiren was alleviated in a concentration-dependent manner (Fig. 1B). The expression levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were examined using RT-qPCR. Since aliskiren at a concentration of 1  $\mu\text{mol/L}$  had no obvious protective effect on cell activity, as a result, only aliskiren at concentrations of 10 and 30  $\mu\text{mol/L}$  were applied for confirming the effect of aliskiren on cell inflammatory damage. The results of RT-qPCR proved that aliskiren can significantly inhibit the mRNA expression of inflammatory factors (Fig. 1C). In addition, the expression levels of p-NF-kB p65, NF-kB p65, Cox2 were examined using western blotting. The expression levels of them in cells treated with A $\beta$  alone was significantly upregulated compared to normal cells, while the expression in cells pretreated with aliskiren was less upregulated (Fig. 1D). These indicated that aliskiren can protect SH-SY5Y cell line from the damage caused by A $\beta$ .

## 2.2. Aliskiren reduces the apoptosis of A $\beta$ -induced SY5Y cells

The apoptosis levels of cells were determined using TUNEL assay and western blotting. Compared with the control group, the fluorescence, of cells treated with A $\beta$  alone was significantly enhanced, and the fluorescence intensity of cells pretreated with aliskiren was not that distinct (Fig. 2A). From the results of western blotting, the expression levels of Bcl2 were downregulated in cells treated with A $\beta$ , and aliskiren alleviated the downregulation of that in cells pretreated by aliskiren. The expression levels of Bax, cleaved caspase3, cleaved PARP were opposite to that of Bcl2 (Fig. 2B), which suggested that aliskiren could reduce the apoptosis of A $\beta$ -induced SY5Y cells.

## 2.3. Aliskiren inhibits PDE4B and activates CREB/BDNF signaling

The target of aliskiren, PDE4B, was predicted in SwissTargetPrediction website. And the association between them was verified *via* molecular docking, there are several intermolecular forces between aliskiren as a ligand and PDE4B as the receptor, which make them bind stably (Fig. 3A). Following the computer simulation of molecular docking, the expression levels of p-CREB, CREB, BDNF, PDE4B were determined using western blotting. The expression level of PDE4B was upregulated in cells undergone the treatment with A $\beta$  while expression levels of a couple of other proteins were downregulated. Novelty, aliskiren reversed the variation trends of expression levels in cells pretreated by aliskiren (Fig. 3B). These demonstrated that aliskiren may be capable of inhibiting PDE4B expression and activating CREB/BDNF signaling.

## 2.4. Aliskiren upregulates cell viability and reduces inflammatory damage by inhibiting PDE4B

The efficiency of Ov-PDE4B was determined using RT-qPCR and western blotting. The expression level of PDE4B in the Ov-PDE4B group was significantly increased compared with Ov-NC (Fig. 4A-B). Then, the transfected cells were pretreated with aliskiren at a concentration of 30  $\mu\text{mol/L}$  and then stimulated with A $\beta$  at a concentration of 2.5  $\mu\text{mol/L}$ , the expression levels of PDE4B, p-CREB, CREB, BDNF were determined using western blotting. The results indicated that the expression of PDE4B was upregulated in transfected cells compared with untransfected cells undergone the identical treatment (Fig. 4C). Then cell viability and

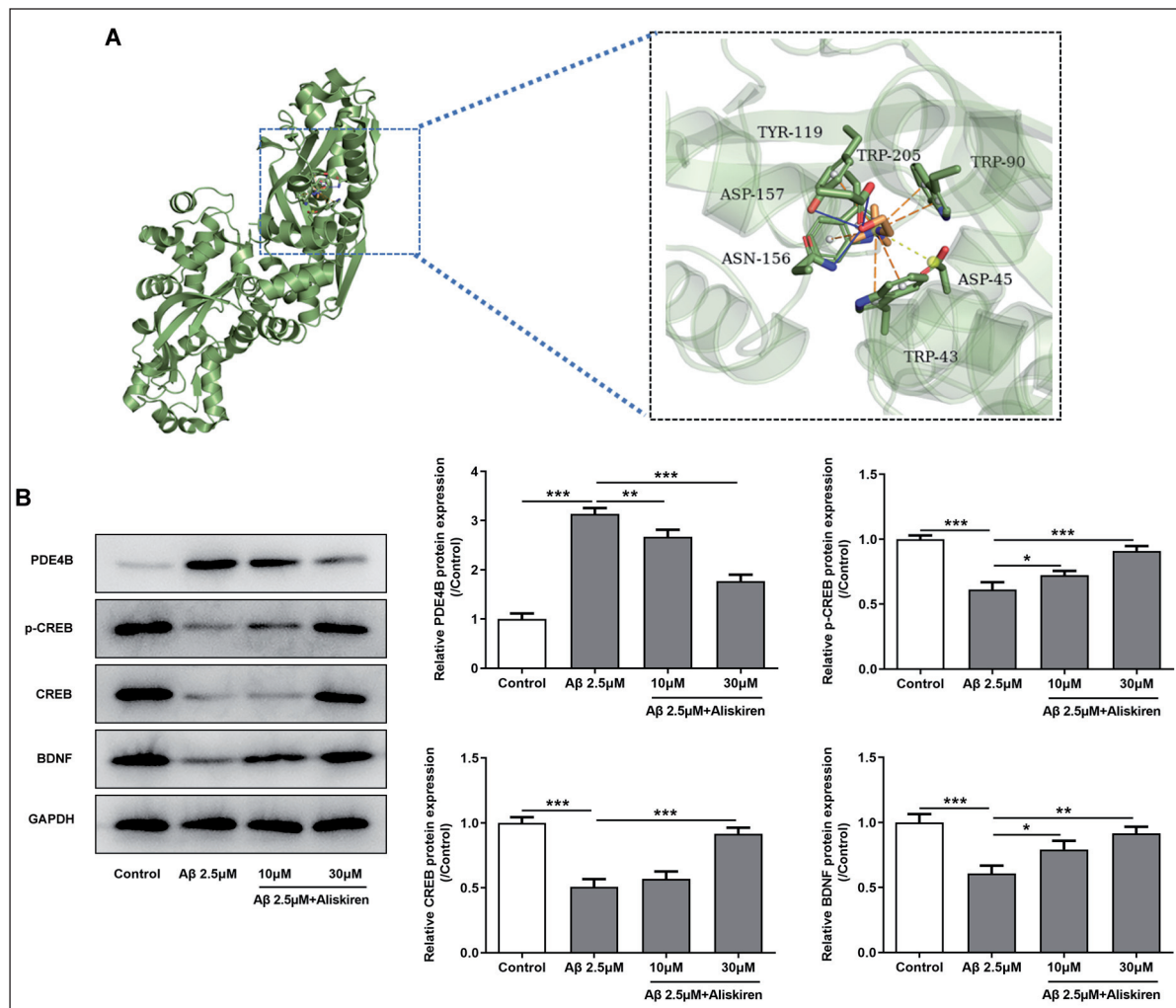


Fig. 3: Aliskiren inhibits PDE4B and activates CREB/BDNF signaling. (A) The association between aliskiren and PDE4B was verified *via* molecular docking. (B) The expression levels of p-CREB, CREB, BDNF, PDE4B were determined using western blotting. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

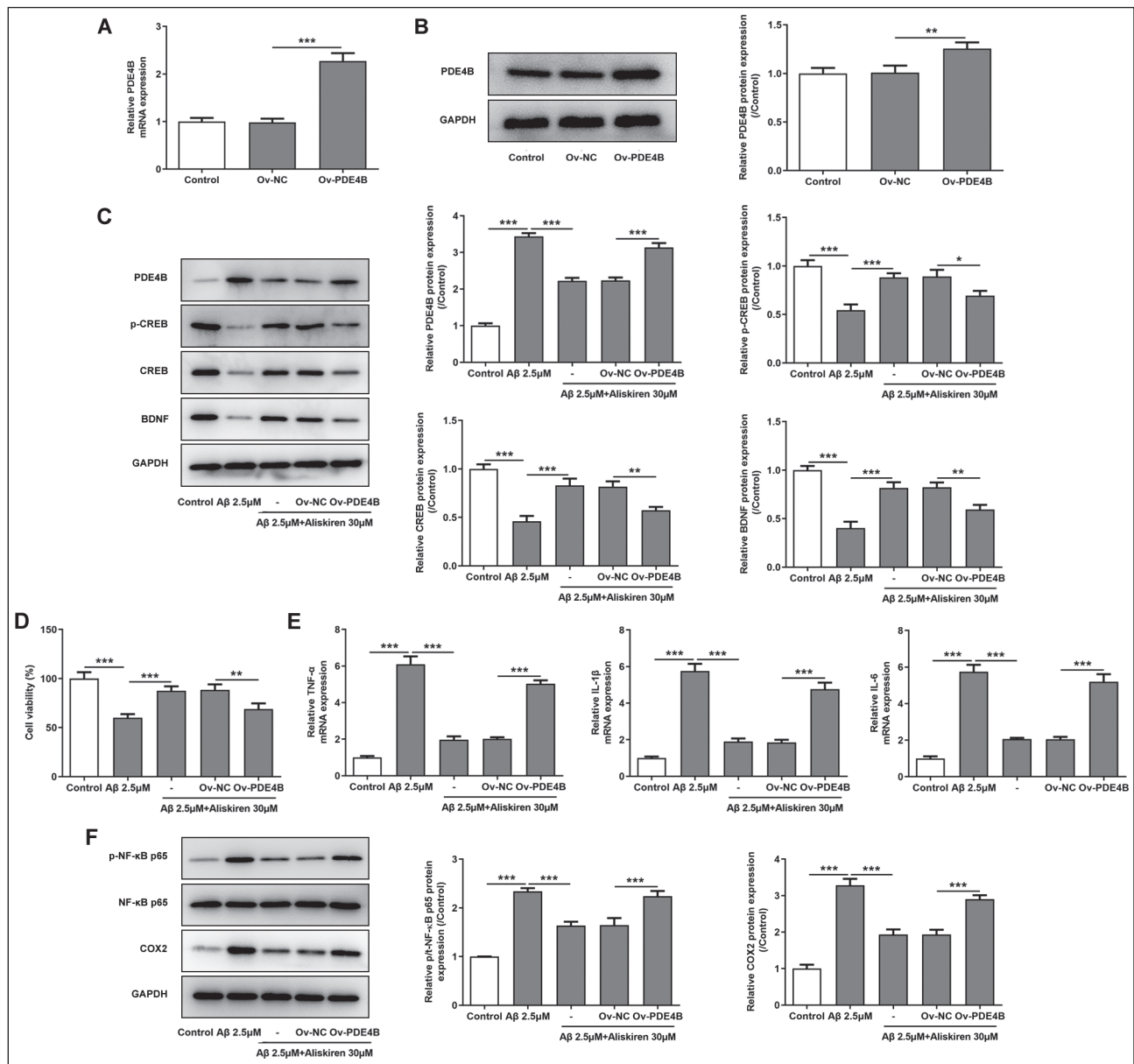


Fig. 4: Aliskiren upregulates cell viability and reduces inflammatory damage by inhibiting PDE4B. The efficiency of Ov-PDE4B was determined using (A) RT-qPCR and (B) western blotting. (C) The expression levels of PDE4B, p-CREB, CREB, BDNF were determined using western blotting. (D) Cell viability was determined using a CCK8 assay. (E) The expression levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 was determined using RT-qPCR. (F) The expression levels of p-NF- $\kappa$ B p65, NF- $\kappa$ B p65, Cox2 were examined using western blotting. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

inflammatory factors were examined and the performance of assays was consistent with the above. The result of the CCK8 assay illustrated PDE4B overexpression could reduce cell viability (Fig. 4D). Moreover, the results of RT-qPCR showed PDE4B overexpression upregulated the expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Fig. 4E). In addition, western blotting displayed that the expression levels of p-NF- $\kappa$ B p65, Cox2 were upregulated and that of NF- $\kappa$ B p65 remained invariable (Fig. 4F). To sum up, PDE4B overexpression broke the protective effect of aliskiren on cell viability and the inhibitory role in cell inflammatory damage.

### 2.5. Aliskiren reduces cell apoptosis by inhibiting PDE4B

In view of the effect of PDE4B overexpression on cell viability and inflammatory damage, the effect on cell apoptosis was explored using TUNEL assay and western blotting. The fluorescence of untransfected cells pretreated with aliskiren was weaker than that of cells treated with A $\beta$  alone, and the fluorescence intensity of transfected cells was significantly enhanced (Fig. 5A). The result of western blotting

demonstrated that the expression level of Bcl2 was downregulated when PDE4B was overexpressed in cells, even reversed the effect of aliskiren. And the expression levels of Bax, cleaved caspase3, cleaved PARP were simultaneously reversed in transfected cells (Fig. 5B). In general terms, these suggested that PDE4B may mediate the process of aliskiren restraining cell apoptosis.

### 3. Discussion

The primary component of neural plaque in AD is deposited A $\beta$  gel strands. A $\beta$  plays a critical role in the continuous damage of nerve cells (Schedin-Weiss et al. 2014). The neurotoxicity of A $\beta$  and its deposition in neurons is essential in the occurrence and development of AD (Boese et al. 2020). The association between its neurotoxicity and AD is principally manifested in the following aspects: breaking the calcium balance and generating oxygen free radicals (Reiss et al. 2018), enhancing the toxicity of glutamate (Hynd et al. 2004), inducing the production of nitric oxide (Oliveira et al. 2011), cerebral vascular dysfunction (Klohs 2019), inducing neuronal apoptosis (Han et al. 2020), central nervous system inflammatory

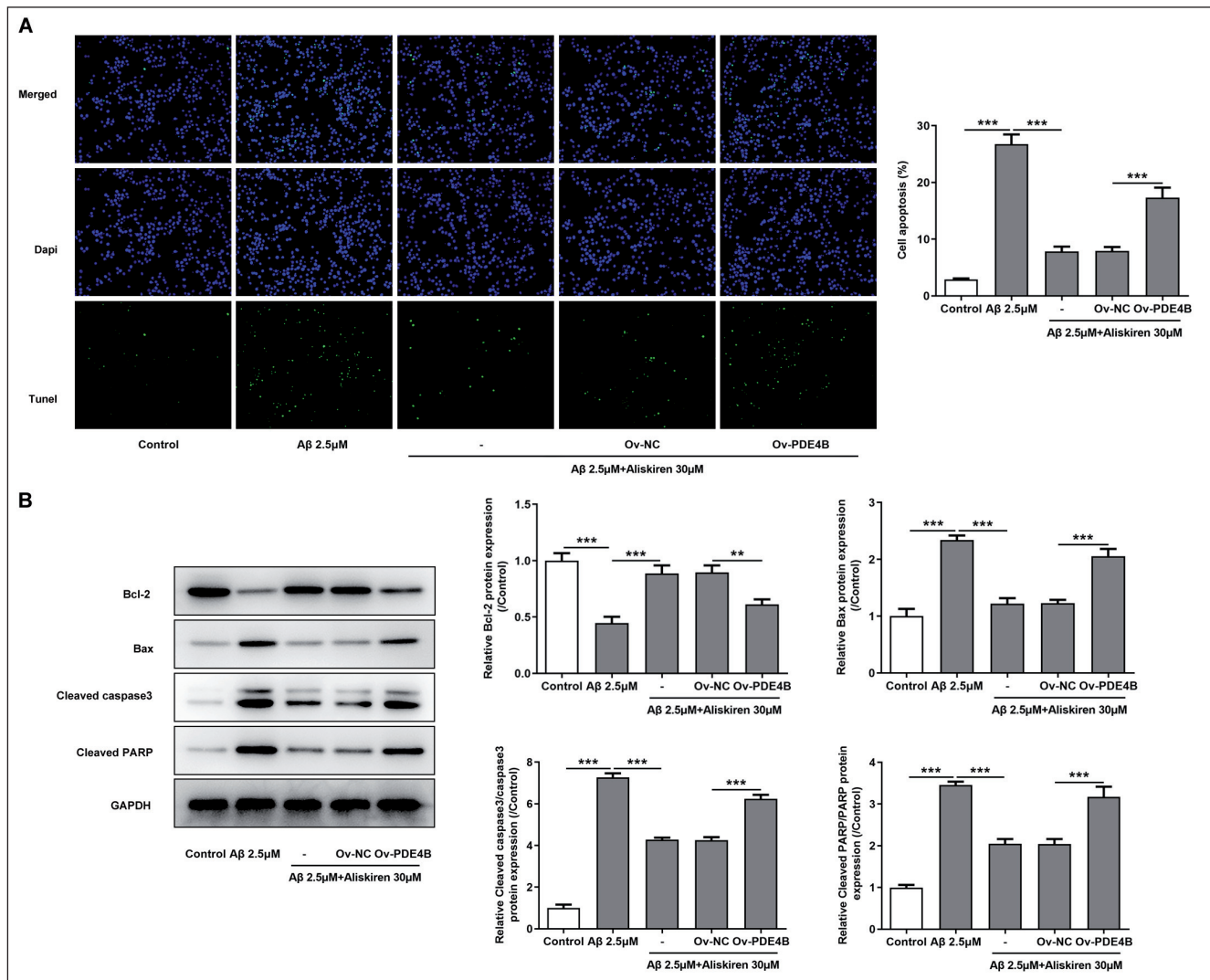


Fig. 5: Aliskiren reduces cell apoptosis by inhibiting PDE4B. (A) Cell apoptosis was explored using TUNEL assay. (B) The expression levels of protein relevant to apoptosis were examined using western blotting. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

response (Delaby et al. 2015) and toxic effect in the body (Osorio et al. 2019). Aβ protein has a variety of toxic effects, which can give rise to mitochondrial stress, oxidative stress, and membrane structure damage. This leads to impaired synaptic function, cognition, memory and nerve cell apoptosis (Mizushima and Komatsu 2011). At present, the theory of Aβ has been widely recognized and has been used in the establishment of a variety of animal models. In view of the fact that Aβ<sub>1-42</sub> protein is the dominant form of Aβ protein (Yao et al. 2018), the study of Aβ<sub>1-42</sub> protein as the target is of great significance for the development of drugs for AD. Herein, Aβ<sub>1-42</sub> was employed for inducing SH-SY5Y cells to establish the model of neuronal toxic damage. The result of the CCK8 assay indicated Aβ<sub>1-42</sub> accumulation could significantly downregulate the viability of SH-SY5Y cells. In addition, Aβ<sub>1-42</sub> accumulation could promote neuronal inflammatory damage and apoptosis. Aliskiren is a drug utilized to lower blood pressure, and hypertension is generally relevant to the increased risk of future cognitive impairment in hypertensive patients (Nagai et al. 2017). This suggests that there may be a potential association between aliskiren and neurological diseases. As expected, there have been several studies reporting the roles of aliskiren in nerves. For example, aliskiren and captopril improve the hippocampal P-ERK, GSK3 and P-GSK3 pathways to improve the cognitive deficits in STZ-induced diabetic rats (Youssef et al. 2020). In addition, aliskiren can improve the brain damage and working memory deficit of chronic cerebral ischemia model by reducing oxidative stress (Dong et

al. 2011). And the occurrence of oxidative stress can induce cell damage, leading to a variety of neurodegenerative diseases, such as Parkinson's disease and AD (Ganguly et al. 2017). As a novelty, a recent study has shown that aliskiren has a neuroprotective effect on Aβ-induced rat cortical neurons in AD (Chen et al. 2012). Therefore, this paper proposes the hypothesis that aliskiren can reduce the neurotoxicity induced by Aβ accumulation in AD. The results of the current study demonstrated that aliskiren has no effect on the viability of SH-SY5Y cells, and the reduction effect on cell inflammatory damage and apoptosis.

Given the above findings, the underlying mechanism of action of aliskiren remained unclear. The target of aliskiren, PDE4B, was predicted on the SwissTargetPrediction website and the association between them was validated *via* molecular docking. A previous study displayed that the PDE4B inhibitor roflumilast could inhibit the cognitive and depressive behaviors of dementia APP<sup>swe</sup>/PS1-dE9 mice, and reduce the expression of PDE4B and PDE4BD in the hippocampus and cortex, and activate the expression of CREB/BDNF in the cyclic adenosine monophosphate signal (Wang et al. 2020). Following confirmation of the high expression level of PDE4B in damaged cells and the fact that aliskiren can downregulate PDE4B expression, the PDE4B overexpression vector was constructed. And it was found that PDE4B overexpression can reverse the influence of aliskiren on cell viability, inflammatory damage and apoptosis, indicating that PDE4B mediates the action mechanism of aliskiren.

In conclusion, aliskiren reduces the cell viability, inflammatory damage and apoptosis induced by A $\beta$  accumulation in AD via inhibiting PDE4B. These findings have expanded the scope of clinical application of aliskiren and provided a theoretical basis. However, this article is limited to *in vitro* study; abundant researches are needed before the clinical use of aliskiren in neurological diseases.

## 4. Experimental

### 4.1. Cell culture and reagents

Human neuroblastoma SH-SY5Y cells were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China) and were cultured in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. The cells were maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. Aliskiren (Aladdin Co., Ltd., Shanghai) was dissolved in dimethyl sulfoxide (DMSO; Solarbio Co., Ltd., Beijing) and diluted with serum-free medium to 1, 10 and 30  $\mu$ mol/L. A $\beta$ <sub>1-42</sub> was placed in an incubator containing 5% CO<sub>2</sub> and incubated at 37°C for 24 h one day in advance, then dissolved in DMSO and diluted with PBS to a concentration of 2.5  $\mu$ mol/L.

### 4.2. Cell transfection

Phosphodiesterase 4B (PDE4B) transcript cDNA was inserted into the pCDNA3.1 to achieve PDE4B overexpression (Ov-PDE4B; Shanghai GenePharma Co., Ltd.) and an empty vector was used as a negative control (Ov-NC; Shanghai GenePharma Co., Ltd.). The Ov-PDE4B and Ov-NC were transfected into SH-SY5Y cells (5 $\times$ 10<sup>5</sup> cells/well) using Lipofectamine<sup>®</sup> 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

### 4.3. Cell counting Kit-8 assay (CCK8)

SH-SY5Y cells were seeded in 96-well plates (5 $\times$ 10<sup>4</sup> cells/ml) and incubated until a density of 80%. Cells were treated with different doses of aliskiren for 1 h and then medium was removed. A $\beta$ <sub>1-42</sub> was added and cells were continued to incubate for 20 h at 37°C. Following this, CCK-8 reagent (10  $\mu$ l; Beyotime Institute of Biotechnology) was added into each well and the plates were subsequently incubated at 37°C for another 2 h. The optical density at 450 nm was measured using a microplate reader (Bio-Rad, USA).

### 4.4. Real time quantitative PCR (RT-qPCR)

Total RNA from each group of SH-SY5Y cells was extracted using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using a Reverse Transcription kit (Thermo Fisher Scientific, Inc.) according to manufacturer's instruction. RT-qPCR was performed, obtained cDNA as a template, using Roche SYBR Green PCR kits (Roche Diagnostics) and carried out in an ABI 7500 system (Applied Biosystems, USA). The cycling conditions were as follows: one cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The relative mRNA quantity was calculated by the 2<sup>- $\Delta\Delta$ C<sub>t</sub></sup> method (Livak and Schmittgen 2001) and normalized against GAPDH. The sequences of primer are listed as follows.  
TNF- $\alpha$  forward: 5'-CAGAC-CCTCACACTCAGATCATC-3',  
Reverse: 5'-AGCCTGTCCCTTGAAGAGAAC-3';  
IL-6 Forward 5'-ATGAAGTTTCTCTCCGCAA-3',  
Reverse 5'-TAGGCATAGCAGACTAGGTT-3';  
IL-1 $\beta$  Forward 5'-TGTGGCAGCTACCTATGTCT-3',  
Reverse 5'-GGGAACATCACACATAGCA-3';  
PDE4B Forward 5'-CGCAGGAGTCGTTCTCTA-3',  
Reverse 5'-CTCCTGTGTCGACACTTG-3';  
GAPDH Forward 5'-TCAAGGCTGAGAACGGGAAG-3',  
Reverse 5'-TGGACTCCACGACTACTCA-3'.

### 4.5. Western blotting

The cells were seeded on a 6-well plate at a density of 5 $\times$ 10<sup>5</sup> cells/ml, and each group was added with aliskiren or serum-free medium and cultured in an incubator for 1 hours. Following the addition of A $\beta$ <sub>1-42</sub>, the cells were incubated for another 20 h. Then cells were washed with cold PBS and lysed in a lysis buffer for 30 min. The protein extract was collected after centrifugation (12000 r/min) at 4 °C for 20min. A BCA kit (cat. no. ab102536; abcam) was applied to quantify the protein concentration according to the operating instructions. Proteins (25  $\mu$ g/lane) were separated by 10% SDS-PAGE gel, and then transferred onto PVDF membranes, which were later blocked with skimmed milk for 2 h at room temperature and incubated with primary antibodies against TNF- $\alpha$  (#ab183218; 1:1000), IL-1 $\beta$  (#216995; 1:1000), IL-6 (#ab233706; 1:1000), p-NF-kB (ab76302; 1:1000), P65 (ab16502; 1:1000), Cox2 (ab179800; 1:1000), Bcl-2 (#ab32124; 1:1000), Bax (#ab32503; 1:1000), Cleaved caspase3 (#ab2302; 1:500; Abcam), Cleaved PARP (#ab32064; 1:1000), p-CREB (#ab32096; 1:500), CREB (#ab32515; 1:5000), BDNF (#ab108319; 1:1000), PDE4B (#ab170939; 1:1000) at 37°C overnight. GAPDH (#ab8245; 1:1000) was used as a loading control. Following being washed with PBS three times, the PVDF membranes were incubated into HRP-goat anti-rabbit secondary antibody (#G-21234; 1:50000; Invitrogen) for 2h at room temperature, and the intensities of bands were analyzed using ImageJ software (v1.6; National Institutes of Health).

### 4.6. TdT-mediated dUTP nick-end labeling (TUNEL) assay

Each group of SH-SY5Y cells (5 $\times$ 10<sup>5</sup> cells/ml) were treated as described above and then fixed in 4% paraformaldehyde for 10 min at room temperature. After permeabilization with 0.1% Triton X-100 (Elabscience) for 5 min, cells were stained according to the procedure provided by the Elabscience<sup>®</sup> TUNEL assay kit (#E-CK-A334, Elabscience). Nuclei were labeled with DAPI and quantified under a fluorescence microscope (200 $\times$ ; Leica Microsystems GmbH, Wetzlar, Germany).

### 4.7. Molecular docking

The structure of aliskiren was drawn in the ChemDraw software, and then imported into OpenBabel software (v2.2.1) for hydrogenation and converted into a mol2 format file. The structure of PDE4B (PDB: 3FRG) was downloaded from the RCSB PDB webpage (<https://www.rcsb.org/>). The protein PDB file was opened in PyMOL software (v2.2.0) to remove the excess water molecules, delete any irrelevant small ligands originally carried, and only keep the protein structure. Since the downloaded protein structure comes with a ligand, the original ligand was deleted and the position of the original ligand was set as the docking site. After the running in AutoDock (v4.2) was completed, the specific docking energy values were displayed. And Protein-Ligand Interaction Profiler (PLIP; <https://plip-tool.biotech.tu-dresden.de/plip-web>) was used to analyze the results.

### 4.8. Bioinformatics and statistical analysis

SwissTargetPrediction (Daina et al. 2019) website (<http://www.swisstargetprediction.ch/>) can estimate the most probable macromolecular targets of a small molecule. The structure of aliskiren was entered and Homo sapiens were selected, then a report of probable targets was available. The data are presented as the mean $\pm$ standard error of mean (SEM) of at least three experiments. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Tukey's *post hoc* test was performed to determine the differences in the means among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: ML and JL designed the study, and performed the experiments alongside LL. ML wrote the manuscript and analyzed the data alongside HL. PY supervised the experiments and revised the manuscript. All authors read and approved the final manuscript. ML and JL confirm the authenticity of all the raw data.

Conflicts of interest: The authors declare that they have no competing interests.

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