A combination of curcumin, vorinostat and silibinin reverses Aβ-induced nerve cell toxicity via activation of AKT-MDM2-p53 pathway

Jia Meng [1], **Yan Li** [2], **Mingming Zhang** [1], **Wenjing Li** [1], **Lin Zhou** [1], **Qiujun Wang** [1], **Lin Lin** [1], **Hongli Jiang** Corresp., [1], **Wenliang Zhu** Corresp. [3]

[1] Department of General Medicine, Second Affiliated Hospital of Harbin Medical University, Harbin, China

[2] Department of Pharmacy, Fourth Affiliated Hospital of Harbin Medical University, Harbin, China

[3] Department of Pharmacy, Second Affiliated Hospital of Harbin Medical University, Harbin, China

Corresponding Authors: Hongli Jiang, Wenliang Zhu
Email address: jianglihong2006@163.com, zhuwenliang@hrbmu.edu.cn

Alzheimer's disease (AD) is a significant health-care issue for the elderly and is becoming increasingly common as the global population ages. Although many efforts have been made to elucidate the pathology of AD, there is still a lack of effective clinical anti-AD agents. Previous research has demonstrated the neuroprotective properties of curcumin and vorinostat when administered in combination; in this study, we added silibinin to the combination to investigate whether it would act synergistically with the two drugs to alleviate amyloid beta (Aβ)-induced nerve cell toxicity. Our initial experiments included a screen of nine candidate compounds; however, only silibinin reduced $A\beta_{25–35}$-induced toxicity in PC12 cells at 1 μM concentrations. The neuroprotective effects of silibinin at 1 μM in combination with 5 μM curcumin and 0.5 μM of vorinostat (CVS) was demonstrated in PC12 cells through a decrease in apoptosis and reduction in levels of oxidative stress markers that are induced by 20 μM of $A\beta_{25–35}$. Western blotting assays showed that CVS pretreatment significantly increased the phosphorylation of AKT, BAD, and MDM2, which resulted in decreased intracellular expression of p53. Further, immunofluorescence demonstrated reduced levels of p53 in the nuclei of PC12 cells following CVS treatment, which indicates a reduction in the p53-mediated transcriptional activity associated with $A\beta_{25–35}$ exposure. In conclusion, our findings suggest that pretreatment with CVS protects PC12 cells from $A\beta_{25–35}$-induced toxicity through modulation of the AKT/MDM2/p53 pathway. Thus, CVS may present a new therapeutic option for the treatment of AD.

A combination of curcumin, vorinostat and silibinin reverses Aβ-induced nerve cell toxicity via activation of AKT-MDM2-p53 pathway

Jia Meng[1], Yan Li[2], Mingming Zhang[1], Wenjing Li[1], Lin Zhou[1], Qiujun Wang[1], Lin Lin[1], Hongli Jiang[1,*], Wenliang Zhu[3,*]

[1]Department of General Medicine, Second Affiliated Hospital of Harbin Medical University, Harbin, China

[2]Department of Pharmacy, Fourth Affiliated Hospital of Harbin Medical University, Harbin, China

[3]Department of Pharmacy, Second Affiliated Hospital of Harbin Medical University, Harbin, China

Corresponding author information

*To whom correspondence should be addressed. Address: Xuefu Road 246, Harbin 150086, Heilongjiang, China; Hongli Jiang: Tel/Fax: 86-451-8629-7273; E-mail: jianglihong2006@163.com; Wenliang Zhu: Tel/Fax: 86-451-8660-5765; E-mail: zhuwenliang@hrbmu.edu.cn.

ABSTRACT

Alzheimer's disease (AD) is a significant health-care issue for the elderly and is becoming increasingly common as the global population ages. Although many efforts have been made to elucidate the pathology

of AD, there is still a lack of effective clinical anti-AD agents. Previous research has demonstrated the neuroprotective properties of curcumin and vorinostat when administered in combination; in this study, we added silibinin to the combination to investigate whether it would act synergistically with the two drugs to alleviate amyloid beta (Aβ)-induced nerve cell toxicity. Our initial experiments included a screen of nine candidate compounds; however, only silibinin reduced $A\beta_{25–35}$-induced toxicity in PC12 cells at 1 μM concentrations. The neuroprotective effects of silibinin at 1 μM in combination with 5 μM curcumin and 0.5 μM of vorinostat (CVS) was demonstrated in PC12 cells through a decrease in apoptosis and reduction in levels of oxidative stress markers that are induced by 20 μM of $A\beta_{25–35}$. Western blotting assays showed that CVS pretreatment significantly increased the phosphorylation of AKT, BAD, and MDM2, which resulted in decreased intracellular expression of p53. Further, immunofluorescence demonstrated reduced levels of p53 in the nuclei of PC12 cells following CVS treatment, which indicates a reduction in the p53-mediated transcriptional activity associated with $A\beta_{25–35}$ exposure. In conclusion, our findings suggest that pretreatment with CVS protects PC12 cells from $A\beta_{25–35}$-induced toxicity through modulation of the AKT/MDM2/p53 pathway. Thus, CVS may present a new therapeutic option for the treatment of AD.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by extracellular deposition of amyloid beta (Aβ) plaques and intracellular aggregation of neurofibrillary tangles (Sanabria-Castro, et al., 2017). AD is the most common form of dementia among the elderly (Mattson, 2004) and is characterized by severe cognitive impairment and memory loss. AD is the fifth-leading cause of death in

people over the age of 65, and it is estimated that 5.4 million people have AD in the United States (Alzheimer's Association, 2016). These issues are not specific to the United States though, as other countries are also facing the problems associated with an aging population (The main data announcement of sixth national census, 2011). In China, the most recent national population census demonstrated that approximately 13.3% of the total population are aged ≥ 60 years, which correlates with a high prevalence of AD. In fact, there are currently more than 7 million AD patients in China, and this number is growing (Chan et al., 2013; Jia et al., 2014). This global growth of AD patients has contributed to a worldwide increase in nursing demand and economic burden (Jia et al., 2018).

In spite of the rapidly increasing prevalence of AD, there is a severe lack of therapeutic strategies to prevent AD or to reverse the development of Aβ plaques (Agatonovic-Kustrin et al., 2018). To date, only three acetylcholinesterase inhibitors (donepezil, galantamine, and rivastigmine) and one non-competitive N-methyl-D-aspartate receptor antagonist (memantine) have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of AD (Guzior et al., 2015). Unfortunately, these approved drugs only elicit modest symptomatic improvement and temporary cognitive improvement in half of the patients with AD (Blennow et al., 2006). Given such limited options, a significant amount of research has gone into alternative therapies for AD. As a result, a number of natural compounds have shown promising neuroprotective effects that are mediated through the p53 pathway, making them promising candidate drugs for AD (Jazvinšćak et al., 2018).

One such example is curcumin, a natural flavonoid isolated from *rhizoma curcumae longae*, which has demonstrated neuroprotective effects against Aβ-induced neurotoxicity in both cell and animal models (Potter, 2013). However, no clinical studies have shown the efficacy of oral curcumin for AD, which is

likely due to the limited bioavailability of curcumin in humans (Ringman et al., 2012). However, our research group has demonstrated that curcumin and vorinostat, a histone deacetylase inhibitor, have synergistic-neuroprotective effects against Aβ toxicity in PC12 cells (Meng et al., 2014). Our work also suggests that co-administration of curcumin and vorinostat protects neural cells from Aβ-induced apoptosis by maintaining the high phosphorylation of AKT serine/threonine kinase (AKT). Of note, the effect of the drug combination on the expression and transcriptional activity of tumor protein p53 was not investigated. Given that simultaneous accumulation, and cooperation, of Aβ and p53 has been observed in human AD, it is likely that p53 plays a critical role in AD progression (Ohyagi et al., 2005). Furthermore, we suspect that through these synergistic mechanisms, the bioavailability of curcumin may be increased.

In the present study, the effects of curcumin were assessed in combination with other natural compounds to identify if its neuroprotective effect is mediated through activation of the AKT/ MDM2 proto-oncogene 2 (MDM2)/p53 pathway. Furthermore, as many natural compounds, such as resveratrol, piceatannol, genistein, quercetin, kaempferol, luteolin, apigenin, daidzein, and silibinin, have been shown to have neuroprotective effects (Wang et al., 2018; Fu et al., 2016; You et al., 2017; Ansari et al., 2009; Pate et al., 2017; Sawmiller et al., 2014; Zhao et al., 2013; Westmark et al., 2013; Duan et al., 2015), we explored whether the addition of one of these compounds would further enhance the neuronal benefit of curcumin and vorinostat.

MATERIALS and METHODS

Materials

Amyloid beta-23-25 ($A\beta_{23-25}$) peptide was purchased from Chinapeptides Corporation (Shanghai, China),

diluted to a stock concentration of 1 mM in double-distilled H_2O, and incubated at 37 °C for seven days to induce aggregation. Following incubation, $A\beta_{23-25}$ was diluted in cell culture media to a final concentration of 20 μM. Unless noted otherwise, all additional chemicals and reagents were purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA).

Cell culture and treatment

Rat pheochromocytoma (PC12) cells were grown on tissue-culture treated plates with Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12) supplemented with 7% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. Cells were maintained at 37 °C in an incubator with 5% CO_2. Upon reaching 80% confluence, cells were cultured in serum-free medium for 12 h, followed by pretreatment with either a single compound of interest or a combination of compounds, for 1 h. After the pretreatment, cells were exposed to $A\beta_{23-25}$ peptide for 24 h.

Cell viability analysis

Cell viability was measured using the 3-[4,5-dimethylthiazol-3-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and treated with the compound(s) of interest, while control wells were treated with an equal volume of the vehicle. Following a 24-h incubation, 10 μL of MTT (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. The medium was then discarded and replaced with 100 μL dimethyl sulfoxide (DMSO) to dissolve the crystals. The optical density was then measured at 490 nm using a multi-mode microplate reader (SpectraMax M5, Molecular Devices Company, California, United States).

Determination of reactive oxygen species (ROS) levels

PC12 cells were pretreated with the following drug combinations for 1 h at 37 °C in DMEM; curcumin (5

μM) alone; a two-drug combination of curcumin (5 μM) and vorinostat (0.5 μM); or a three-drug combination of curcumin (5 μM), vorinostat (0.5 μM), and silibinin (1 μM). Following the pretreatment, $A\beta_{23-25}$ peptide was added to induce cell apoptosis, and intracellular ROS generation was assessed using a reactive oxygen species assay kit (Nanjing Jiancheng Institute of Biotechnology, China) according to the manufacturer's instructions. In brief, PC12 cells were washed twice with phosphate-buffered saline (PBS) and then incubated with 10 μM of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) dye in serum-free DMEM-F12 for 1 h at 37 °C. The cells were washed with PBS and fluorescence was observed at 488 nm and recorded using confocal microscopy. Semi-quantification of ROS levels was evaluated by using ImageJ software.

Measurement of superoxide dismutase (SOD) and malondialdehyde (MDA) levels

Briefly, the treated PC12 cells were harvested and lysed in lysis buffer, and the resulting cell lysates were centrifuged at 3,000×g for 10 min at 4 °C， the resulting supernatant was collected for enzyme activity analysis. The level of SOD and MDA were determined through the nitroblue tetrazolium using a total SOD Detection Kit (Beyotime Institute of Biotechnology, Shanghai, China) and a MDA assay kit (Jiancheng Institute of Biotechnology, Nanjing, China), according to the manufacturer's instruction. The protein concentration was determined using BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). One unit of SOD and MDA were defined as the amount of protein. The SOD results were expressed as enzyme activity per milligram protein (U/mg) and the MDA results were expressed as per milligram protein (U/mg).

Western blotting assay

The PC12 cells were washed with PBS and lysed with ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer added to phenylmethanesulfonyl fluoride (PMSF). The mixture was then centrifuged for 15 min at 14,000×g and 4 ºC. Supernatants containing the total cellular proteins were measured using a BCA

protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. The proteins were then separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein bands transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA, USA), and the membrane blocked with 5% non-fat milk diluted 1×Tris Buffered Saline with 0.1% Tween (TBS-T) for 1 h at room temperature(24 ºC). The membranes were then incubated overnight at 4°C with the following primary antibodies; rabbit polyclonal phospho-MDM2 antibody (*p*-MDM2, 1:1000, Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal p53 antibody (1:1000, Cell signaling Technology, Danvers, MA, USA), rabbit monoclonal phospho-AKT (ser473) antibody (*p*-AKT, 1:1000, Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal phospho-BAD (Ser112) antibody (*p*-BAD, 1:1000, Cell Signaling Technology, Danvers, MA, USA), and mouse monoclonal β-actin antibody (1:5000, Abcam Cambridge, USA). The membranes were then washed and incubated at room temperature for 1 h with goat anti-rabbit IRDye ® 800CW (1:10000, Li-Cor Biosciences, Lincoln, NE, USA) or goat anti-mouse IRDye ® 800CW (1:10000, Li-Cor Biosciences, Lincoln, NE, USA). The proteins were visualized using the Odyssey Infrared Imager System (LI-COR Biosciences, Lincoln, NE, USA), and the odyssey v1.2 software was used to quantify the western blot bands by measuring band intensity (area × optical density) in each group with β-actin as an internal control.

Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay

During apoptosis, cleavage of genomic DNA yields double-stranded DNA breaks that are identifiable by labeling the free 3'-OH termini with modified nucleotides in an enzymatic reaction; a TUNEL assay is based on this principle. In brief, cells were grown on confocal dishes (NEST, Wuxi, China) in DMEM-F12 for 24 h. PC12 cells were then pretreated with the compounds of interest alone or in combinations for 1 h.

Pretreated cells were then exposed to 20 μM of $A\beta_{23-25}$ for 24 h 37°C and 5% CO_2. Cells were washed twice with PBS followed by fixation with 4% paraformaldehyde (PFA) at room temperature. Fixed cells were washed twice with PBS buffer, treated with 0.1% TritonX-100 to permeate cells, and then washed twice with PBS. Next, the TUNEL reaction mixture (Beyotime Institute of Biotechnology, Shanghai, China) was added to the samples, and the cells were incubated for 60 min at 37°C in a dark, humidified atmosphere. Negative controls were prepared with equal volumes of labeling solution. After this incubation, the confocal dishes (NEST, Wuxi, China) were rinsed twice with PBS. Finally, samples were analyzed using a confocal Laser Scanning Biological microscope FV1000 (Olympus, Tokyo, Japan) at the excitation wavelength of 488 nm.

Immunofluorescence staining assay

Colocalization of p53 and MDM2 proteins was examined by immunofluorescence staining of treated PC12 cells. In brief, PC12 cells were cultured on confocal dishes (Nest, Wuxi, China) at 37 °C for 24 h. After treatments with $A\beta_{23-25}$, curcumin (5 μM), vorinostat (0.5 μM), and silibinin (1 μM) as motioned before, cells were fixed with 4% PFA for 15 min at room temperature, washed twice with PBS, and incubated with 0.1% Triton-X 100 for 15 min at room temperature. Cells were blocked with 1% bovine serum albumin in PBS for 30 min and subsequently incubated with antibodies against p53 (1:1000, Cell signaling, USA and MDM2 (1:400, Cell signaling, USA) at 4 °C overnight with mild shaking. The cells were washed twice with PBS, then incubated with the secondary antibody at room temperature for 1 h; the secondary antibodies were donkey anti-rabbit 488 (1:1000, Invitrogen, Camarillo, CA, USA) and donkey anti-mouse 594 (1:1000, Invitrogen, Camarillo, CA, USA). To stain the nuclei, the cells were then incubated with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Following two washes with PBS, the cells were observed

using a confocal Laser Scanning Biological microscope FV1000 (Olympus, Tokyo, Japan).

Statistical analysis

All the experiments were repeated five times, and all figures are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism v6.0. To assess for differences among three or more groups, one-way analysis of variance (ANOVA) with Dunnett test was used. Differences of $p < 0.01$ were considered to indicate statistically significant differences.

RESULTS

Silibinin enhanced the neuroprotective effects of the combination of curcumin and vorinostat

In the present study, nine natural compounds were investigated for synergistic activity with the drug combination of curcumin and vorinostat. The nine compounds had each been documented to have neuroprotective effects (Wang et al., 2018; Fu et al., 2016; You et al., 2017; Ansari et al., 2009; Pate et al., 2017; Sawmiller et al., 2014; Zhao et al., 2013; Westmark et al., 2013; Duan et al., 2015), and included, resveratrol, piceatannol, genistein, quercetin, kaempferol, luteolin, apigenin, daidzein, and silibinin. Our results indicated that at concentrations of 1 μM, only silibinin showed significant neuroprotection against $A\beta_{25–35}$ ($p < 0.01$, Figure 1A). Further, cell viability assays showed synergistic activity between silibinin and the combination treatment that consisted of 5 μM curcumin and 0.5 μM vorinostat (Figure 1B). This three-drug combination increased the ability of PC12 cells to resist $A\beta_{25–35}$-induced effects. No differences were observed between the control group and the curcumin-vorinostat-silibinin (CVS) group with regard to either CASP3 expression or the percentage of TUNEL positive cells (Figures 1C and D, $p > 0.01$).

Pretreatment of CVS significantly reduced oxidative stress following stimulation by $A\beta_{25–35}$

192 A significant increase in ROS production (3.6-fold relative to control) was observed when PC12 cells were
193 incubated with 20 μM of $A\beta_{25–35}$ (Figure 2A); however, when cells were pretreated with CVS for 1 h, there
194 was no significant increase in ROS generation following $A\beta_{25–35}$ stimulation ($p > 0.01$). Consistent with
195 these results, $A\beta_{25–35}$ significantly decreased the SOD concentration (1.9 fold compared to control) and
196 increased the MDA concentration (6.4 fold compared to control) in PC12 cells (Figures 2B and C); yet
197 when cells were pretreated with CVS, the SOD and MDA concentrations were not significantly affected by
198 $A\beta_{25–35}$ ($p > 0.01$).

199 **Pretreatment with CVS maintained an active Akt/MDM2/p53 pathway**

200 Treatment of PC12 cells with 20 μM $A\beta_{25–35}$ induced a significant decrease in the phosphorylation of AKT
201 ($p < 0.001$, Figure 3A), which correlated with significantly decreased expression of the downstream
202 proteins, *p*-BAD and *p*-MDM2 (Figures 3B and C). However, when the cells were pretreated with drugs,
203 the $A\beta_{25–35}$-mediated reduction of these proteins was inhibited (Figures 3A-C). Further, pretreatment with
204 the three-drug combination was more effective at reducing the effects mediated by $A\beta_{25–35}$ exposure than
205 pretreatment with curcumin alone or the two-drug combination (curcumin and vorinostat). Similarly,
206 pretreatment of PC12 cells with CVS reduced intracellular p53 expression, which was significantly
207 increased by 20 μM of $A\beta_{25–35}$ (Figure 3D). This was supported by a reduction in the $A\beta_{25–35}$-mediated
208 increase in transcriptional activity of p53 when cells were pretreated with CVS, which was demonstrated
209 by immunofluorescent microscopy (Figure 4).

210

211 # DISCUSSION

212 In the United States, approximately one in seven people aged ≥ 65 have AD, and this figure jumps to

213 approximately 50% in those over 85 (Alzheimer's Association, 2016). As the global population ages, these
214 numbers continue to grow, making prevention and treatment of AD one of the most important healthcare
215 issues of this century (Goedert and Spillantini, 2006). However, this is complicated by a severe lack of
216 therapeutic options.

217 It has been approximately 25 years since the amyloid hypothesis of AD was proposed; however, recent
218 studies have demonstrated that this is only part of the story (Hardy and Higgins, 1992; Armstrong, 2013),
219 rather Aβ aggregation should be considered a reaction to, rather than a cause of the pathological progression
220 of AD. The real impetus appears to be that sedentary, overindulgent lifestyles cause chronic stress on the
221 brain, which in turn accelerates brain aging (Caruso et al., 2018; Mattson and Arumugam, 2018). The
222 clinical failure of treatment strategies involved in scavenging Aβ from the brain partially supports this
223 hypothesis with regard to the pathological procession of AD (Citron, 2010). Further, clinical investigations
224 suggest that approximately one in four patients with AD are not diagnosed according to the discriminant
225 threshold levels of Aβ plaques and Tau tangles. Yet these patients still experience severe loss of
226 hippocampal pyramidal neurons (Mattson, 2015), suggesting that this pathology may not be the exclusive
227 result of advanced AD, and thus, that the Aβ scavenging strategy is too arbitrary and focused. As such, drug
228 target research from a new perspective is necessary for the development of effective AD drugs.

229 One of the most promising avenues is the up-regulation of p53, which has been found to be crucial to the
230 development of AD (Jazvinšćak et al., 2018). Conveniently, p53 is encoded by *tp53*, one of the most
231 thoroughly investigated genes in the human genome (Dolgin, 2017). There are, therefore, numerous
232 potential drug candidates already available to modulate the p53 pathway that may be of use as targeted AD
233 therapeutics. Recently, Jazvinšćak *et al.* suggested that natural compounds may be a source of suitable drug

234 candidates, as many natural compounds are known to regulate this pathway (Jazvinšćak et al., 2018). In our
235 study, nine natural compounds were screened for their ability to inhibit the cytotoxic effects of excessive
236 Aβ deposits in PC12 cells. Accordingly, 1 μM silibinin demonstrated the greatest activity in the drug screen.
237 Subsequent experiments revealed that the addition of silibinin to our previously established drug
238 combination (curcumin and vorinostat) enhanced the neuroprotective effects of this combination by
239 activating the AKT/MDM2/p53 axis. The potent activity of this three-drug combination at low
240 concentrations suggests synergistic drug interactions and is consistent with the results in our previous study
241 (Meng et al., 2014). Compared to monotherapies, synergistic drug combinations possess many inherent
242 advantages, including lower doses, multi-target regulation, and a reduced risk of drug resistance
243 development (Zimmermann et al., 2007; Lehár et al., 2009; Jia et al., 2009). More importantly, the three
244 drugs investigated in this study are all commercially available. Thus, the CVS treatment regimen is
245 economically feasible for late-stage drug development.
246 Curcumin is a flavone chemical found naturally, and studies have demonstrated that it is a promising
247 anti-AD drug (Venigalla et al., 2015). However, poor human bioavailability limits its further clinical
248 application (Chin et al., 2013). Multiple strategies have been assessed to solve the issue of low
249 bioavailability. For example, the formulation of curcumin-loaded nanoparticles increased bioavailability
250 but was associated with higher costs (Tiwari et al., 2014). Other studies have indicated that a curcuminoid
251 mixture (instead of curcumin alone) has greater efficacy in the potential treatment of AD (Ahmed and
252 Gilani, 2014), suggesting that combination therapies may be an effective strategy for curcumin in the
253 treatment of AD. Vorinostat, or suberoylanilidehydroxamic acid (SAHA), is a histone deacetylase inhibitor
254 that has FDA approval for the treatment of cutaneous T cell lymphoma. Vorinostat may also have potential

255 value for the treatment of AD through its effects on the CREB binding protein (CBP)/ E1A binding protein
256 p300 (EP300) signaling pathway (Rouaux et al., 2004). In a previous study, we demonstrated synergistic
257 properties between vorinostat and curcumin for the protection of PC12 cells against Aβ toxicity; however,
258 exposure of high concentrations of vorinostat was found to be cytotoxic (Meng et al., 2014). Given this
259 potential cytotoxicity, we reduced its concentration from 1 μM to 0.5 μM. Recently, several studies have
260 demonstrated the neuroprotective effects of silibinin in a model of Aβ-treated rats (Song et al., 2017; Song
261 et al., 2018). Consistent with these findings, our results showed that silibinin increased the effects of the
262 two-drug combination by protecting neural cells from Aβ toxicity.

263 In our study, an experimental design was adopted such that the PC12 cells were pretreated with the drug
264 combination prior to simulation of Aβ aggregation. According to the clinical features of AD, three
265 developmental stages can be defined, pre-clinical AD, prodromal AD, and AD-type dementia (De-Paula et
266 al., 2012). The former two phases constitute the pre-symptomatic phase of AD, while the latter is considered
267 symptomatic. Further, the pre-symptomatic phases of AD last longer than the symptomatic phase (De-Paula
268 et al., 2012) and are associated with lower economic costs (Jia et al., 2018). Therefore, the prevention of
269 dementia via the treatment of early-stage AD is more economically feasible than treating AD-type dementia
270 (Goedert and Spillantini, 2006).

271

272 CONCLUSIONS

273 Through a cell viability screen of 9 natural compounds, we successfully identified a low concentration
274 combination drug, CVS, that has potential as an AD therapeutic. Our results demonstrated the strong
275 neuroprotective ability of CVS against Aβ toxicity *in vitro* and that pretreatment of CVS increased the

tolerance of nerve cells to Aβ toxicity. We also demonstrate that simulated Aβ aggregation led to inactivation of the AKT/MDM2/p53, which has a critical role in the progression of AD. However, pretreatment with CVS maintained the active state of the pathway and ensured low transcriptional activity of p53. As a direct result of the protection provided by CVS, no significant cell apoptosis or oxidative stress occurred when PC12 cells were exposed to Aβ aggregation. However, further studies in animal models are necessary to evaluate the therapeutic value of CVS for this disease. In conclusion, our findings suggest that CVS is a promising prophylaxis for AD treatment.

ACKNOWLEDGEMENT

We thank Dr. Songbin Fu for reviewing the manuscript and giving suggestions.

REFERENCES

Agatonovic-Kustrin S, Kettle C, Morton DW. 2018. A molecular approach in drug development for Alzheimer's disease. *Biomedicine & Pharmacotherapy* **106:**553-565 DOI 10.1016/j.biopha.2018.06.147.

Ahmed T, Gilani AH. 2014. Therapeutic potential of turmeric in Alzheimer's disease: curcumin or curcuminoids? *Phytotherapy Research* **28(4):**517-525 DOI 10.1002/ptr.5030.

Alzheimer's Association. 2016. 2016 Alzheimer's disease facts and figures. *Alzheimer's & Dementia* **12(4):**459-509 DOI 10.1016/j.jalz.2016.03.001.

Ansari MA, Abdul HM, Joshi G, Opii WO, Butterfield DA. 2009. Protective effect of quercetin in primary neurons against Abeta(1-42): relevance to Alzheimer's disease. *Journal of Nutritional Biochemistry* **20(4):**269-275 DOI 10.1016/j.jnutbio.2008.03.002.

297 **Armstrong RA. 2013.** What causes alzheimer's disease? *Folia Neuropathologica* **51(3):**169-188 DOI
298 10.5114/fn.2013.37702.

299 **Blennow K, de Leon MJ, Zetterberg H. 2006.** Alzheimer's disease. *Lancet* **368(9533):**387-403 DOI
300 10.1016/S0140-6736(06)69113-7.

301 **Caruso A, Nicoletti F, Mango D, Saidi A, Orlando R, Scaccianoce S. 2018.** Stress as risk factor for
302 Alzheimer's disease. *Pharmacological Research* **132:**130-134 DOI 10.1016/j.phrs.2018.04.017.

303 **Chan KY, Wang W, Wu JJ, Liu L, Theodoratou E, Car J, Middleton L, Russ TC, Deary IJ, Campbell**
304 **H, Wang W, Rudan I; Global Health Epidemiology Reference Group (GHERG). 2013.** Epidemiology
305 of Alzheimer's disease and other forms of dementia in China, 1990-2010: a systematic review and analysis.
306 *Lancet* **381(9882):**2016-2023 DOI 10.1016/S0140-6736(13)60221-4.

307 **Chin D, Huebbe P, Pallauf K, Rimbach G. 2013.** Neuroprotective properties of curcumin in Alzheimer's
308 disease--merits and limitations. *Current Medicinal Chemistry* **20(32):**3955-3985 DOI
309 10.2174/09298673113209990210.

310 **Citron M. 2010.** Alzheimer's disease: strategies for disease modification. *Nature Reviews Drug Discovery*
311 **9(5):**387-398 DOI 10.1038/nrd2896.

312 **De-Paula VJ, Radanovic M, Diniz BS, Forlenza OV. 2012.** Alzheimer's disease. *Subcellular*
313 *Biochemistry* **65:**329-352 DOI 10.1007/978-94-007-5416-4_14.

314 **Dolgin E. 2017.** The most popular genes in the human genome. *Nature* **551(7681):**427-431 DOI
315 10.1038/d41586-017-07291-9.

316 **Duan S, Guan X, Lin R, Liu X, Yan Y, Lin R, Zhang T, Chen X, Huang J, Sun X, Li Q, Fang S, Xu**
317 **J, Yao Z, Gu H. 2015.** Silibinin inhibits acetylcholinesterase activity and amyloid β peptide aggregation:

a dual-target drug for the treatment of Alzheimer's disease. *Neurobiology of Aging* **36(5):**1792-1807 DOI 10.1016/j.neurobiolaging.2015.02.002.

Fu Z, Yang J, Wei Y, Li J. 2016. Effects of piceatannol and pterostilbene against β-amyloid-induced apoptosis on the PI3K/Akt/Bad signaling pathway in PC12 cells. *Food & Function* **7(2):**1014-1023 DOI 10.1039/c5fo01124h.

Goedert M, Spillantini MG. 2006. A century of Alzheimer's disease. *Science* **314(5800):**777-781 DOI 10.1126/science.1132814.

Guzior N, Wieckowska A, Panek D, Malawska B. 2015. Recent development of multifunctional agents as potential drug candidates for the treatment of Alzheimer's disease. *Current Medicinal Chemistry* **22(3):**373-404 DOI 10.2174/0929867321666141106122628.

Hardy JA, Higgins GA. 1992. Alzheimer's disease: the amyloid cascade hypothesis. *Science* **256(5054):**184-185 DOI 10.1126/science.1566067.

Jazvinšćak Jembrek M, Slade N, Hof PR, Šimić G. 2018. The interactions of p53 with tau and Aß as potential therapeutic targets for Alzheimer's disease. *Progress in Neurobiology* **168:**104-127 DOI 10.1016/j.pneurobio.2018.05.001.

Jia J, Zhu F, Ma X, Cao Z, Cao ZW, Li Y, Li YX, Chen YZ. 2009. Mechanisms of drug combinations: interaction and network perspectives. *Nature Reviews Drug Discovery* **8(2):**111-128 DOI 10.1038/nrd2683.

Jia J, Wang F, Wei C, Zhou A, Jia X, Li F, Tang M, Chu L, Zhou Y, Zhou C, Cui Y, Wang Q, Wang W, Yin P, Hu N, Zuo X, Song H, Qin W, Wu L, Li D, Jia L, Song J, Han Y, Xing Y, Yang P, Li Y, Qiao Y, Tang Y, Lv J, Dong X. 2014. The prevalence of dementia in urban and rural areas of China. *Alzheimer's & Dementia* **10(1):**1-9 DOI 10.1016/j.jalz.2013.01.012.

Jia J, Wei C, Chen S, Li F, Tang Y, Qin W, Zhao L, Jin H, Xu H, Wang F, Zhou A, Zuo X, Wu L, Han Y, Han Y, Huang L, Wang Q, Li D, Chu C, Shi L, Gong M, Du Y, Zhang J, Zhang J, Zhou C, Lv J, Lv Y, Xie H, Ji Y, Li F, Yu E, Luo B, Wang Y, Yang S, Qu Q, Guo Q, Liang F, Zhang J, Tan L, Shen L, Zhang K, Zhang J, Peng D, Tang M, Lv P, Fang B, Chu L, Jia L, Gauthier S. 2018. The cost of Alzheimer's disease in China and re-estimation of costs worldwide. *Alzheimer's & Dementia* **14(4):**483-491 DOI 10.1016/j.jalz.2017.12.006.

Lehár J, Krueger AS, Avery W, Heilbut AM, Johansen LM, Price ER, Rickles RJ, Short GF 3rd, Staunton JE, Jin X, Lee MS, Zimmermann GR, Borisy AA. 2009. Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nature Biotechnology* **27(7):**659-666 DOI 10.1038/nbt.1549.

Mattson MP. 2004. Pathways towards and away from Alzheimer's disease. *Nature* **430(7000):**631-639 DOI 10.1038/nature02621.

Mattson MP. 2015. Late-onset dementia: a mosaic of prototypical pathologies modifiable by diet and lifestyle. *NPJ Aging Mechanism & Disease* **1. pii: 15003** DOI 10.1038/npjamd.2015.3.

Mattson MP, Arumugam TV. 2018. Hallmarks of Brain Aging: Adaptive and Pathological Modification by Metabolic States. *Cell Metabolism* **27(6):**1176-1199 DOI 10.1016/j.cmet.2018.05.011.

Meng J, Li Y, Camarillo C, Yao Y, Zhang Y, Xu C, Jiang L. 2014. The anti-tumor histone deacetylase inhibitor SAHA and the natural flavonoid curcumin exhibit synergistic neuroprotection against amyloid-beta toxicity. *PLoS One* **9(1):**e85570 DOI 10.1371/journal.pone.0085570.

Ohyagi Y, Asahara H, Chui DH, Tsuruta Y, Sakae N, Miyoshi K, Yamada T, Kikuchi H, Taniwaki T, Murai H, Ikezoe K, Furuya H, Kawarabayashi T, Shoji M, Checler F, Iwaki T, Makifuchi T,

Takeda K, Kira J, Tabira T. 2015. Intracellular Abeta42 activates p53 promoter: a pathway to neurodegeneration in Alzheimer's disease. *FASEB Journal* **19(2):**255-257 DOI 10.1096/fj.04-2637fje.

Pate KM, Rogers M, Reed JW, van der Munnik N, Vance SZ, Moss MA. 2017. Anthoxanthin Polyphenols Attenuate Aβ Oligomer-induced Neuronal Responses Associated with Alzheimer's Disease. *CNS Neuroscience & Therapeutics* **23(2):**135-144 DOI 10.1111/cns.12659.

Potter PE. 2013. Curcumin: a natural substance with potential efficacy in Alzheimer's disease. *Journal of Experimental Pharmacology* **5:**23-31 DOI 10.2147/JEP.S26803.

Ringman JM, Frautschy SA, Teng E, Begum AN, Bardens J, Beigi M, Gylys KH, Badmaev V, Heath DD, Apostolova LG, Porter V, Vanek Z, Marshall GA, Hellemann G, Sugar C, Masterman DL, Montine TJ, Cummings JL, Cole GM. 2012. Oral curcumin for Alzheimer's disease: tolerability and efficacy in a 24-week randomized, double blind, placebo-controlled study. *Alzheimer's Research & Therapy* **4(5):**43 DOI 10.1186/alzrt146.

Rouaux C, Loeffler JP, Boutillier AL. 2004. Targeting CREB-binding protein (CBP) loss of function as a therapeutic strategy in neurological disorders. Biochemical Pharmacology **68(6):**1157-1164 DOI 10.1016/j.bcp.2004.05.035.

Sanabria-Castro A, Alvarado-Echeverría I, Monge-Bonilla C. 2017. Molecular Pathogenesis of Alzheimer's Disease: An Update. *Annals of Neurosciences* **24(1):**46-54 DOI 10.1159/000464422.

Sawmiller D, Li S, Shahaduzzaman M, Smith AJ, Obregon D, Giunta B, Borlongan CV, Sanberg PR, Tan J. 2014. Luteolin reduces Alzheimer's disease pathologies induced by traumatic brain injury. *International Journal of Molecular Sciences* **15(1):**895-904 DOI 10.3390/ijms15010895.

Song X, Liu B, Cui L, Zhou B, Liu W, Xu F, Hayashi T, Hattori S, Ushiki-Kaku Y, Tashiro SI,

Ikejima T. 2017. Silibinin ameliorates anxiety/depression-like behaviors in amyloid β-treated rats by upregulating BDNF/TrkB pathway and attenuating autophagy in hippocampus. *Physiology & Behavior* **179:**487-493 DOI 10.1016/j.physbeh.2017.07.023.

Song X, Liu B, Cui L, Zhou B, Liu L, Liu W, Yao G, Xia M, Hayashi T, Hattori S, Ushiki-Kaku Y, Tashiro SI, Ikejima T. 2018. Estrogen Receptors Are Involved in the europrotective Effect of Silibinin in Aβ(1-42)-Treated Rats. *Neurochemical Research* **43(4):**796-805 DOI 10.1007/s11064-018-2481-3.

The main data announcement of sixth national census, 2011. Available at: http://www.stats.gov.cn/ztjc/zdtjgz/zgrkpc/dlcrkpc/dcrkpcyw/201104/t20110428_69407.htm. Accessed March 18, 2017.

Tiwari SK, Agarwal S, Seth B, Yadav A, Nair S, Bhatnagar P, Karmakar M, Kumari M, Chauhan LK, Patel DK, Srivastava V, Singh D, Gupta SK, Tripathi A, Chaturvedi RK, Gupta KC. 2014. Curcumin-loaded nanoparticles potently induce adult neurogenesis and reverse cognitive deficits in Alzheimer's disease model via canonical Wnt/β-catenin pathway. *ACS Nano* **8(1):**76-103 DOI 10.1021/nn405077y.

Venigalla M, Gyengesi E, Münch G. 2015. Curcumin and Apigenin - novel and promising therapeutics against chronic neuroinflammation in Alzheimer's disease. *Neural Regeneration Research* **10(8):**1181-1185 DOI 10.4103/1673-5374.162686.

Wang H, Jiang T, Li W, Gao N, Zhang T. 2018. Resveratrol attenuates oxidative damage through activating mitophagy in an in vitro model of Alzheimer's disease. *Toxicology Letters* **282:**100-108 DOI 10.1016/j.toxlet.2017.10.021.

Westmark CJ, Westmark PR, Malter JS. 2013. Soy-based diet exacerbates seizures in mouse models of

neurological disease. *Journal of Alzheimers Disease* **33(3):**797-805 DOI 10.3233/JAD-2012-121426.

You F, Li Q, Jin G, Zheng Y, Chen J, Yang H. 2017. Genistein protects against Aβ(25-35) induced apoptosis of PC12 cells through JNK signaling and modulation of Bcl-2 family messengers. *BMC Neuroscience* **18(1):**12 DOI 10.1186/s12868-016-0329-9.

Zhao L, Wang JL, Wang YR, Fa XZ. 2013. Apigenin attenuates copper-mediated β-amyloid neurotoxicity through antioxidation, mitochondrion protection and MAPK signal inactivation in an AD cell model. *Brain Research* **1492:**33-45 DOI 10.1016/j.brainres.2012.11.019.

Zimmermann GR, Lehár J, Keith CT. 2007. Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discovery Today* **12(1-2):**34-42 DOI 10.1016/j.drudis.2006.11.008.

FIGURE LEGENDS

Figure 1. CVS treatment inhibits $A\beta_{25–35}$-induced PC12 cell apoptosis. A. Individual effects of 9 candidate compounds on cell viability. **B-D.** Effects of CVS on cell viability (B), reversion of CASP3 expression (C), and apoptotic cell levels (D) in $A\beta_{25–35}$-treated PC12 cells. Each experiment was completed with a minimum of 5 replicates. Statistical significance is presented as; ## $p < 0.001$ versus Ctrl; * $p < 0.01$, ** $p < 0.001$ versus Aβ. Abbreviations: Aβ, $A\beta_{25–35}$ treatment group; C, curcumin alone; CASP3, Caspase 3, Ctrl, control group; CV, curcumin and vorinostat; CVS, curcumin, vorinostat, and silibinin.

Figure 2. CVS pretreatment prevents $A\beta_{25–35}$-induced oxidative stress in PC12 cells. A-C. Effects of CVS on ROS (A), SOD (B), and MDA levels (C). Each experiment was completed with a minimum of 5 replicates. Statistical significance is presented as; ## $p < 0.001$ versus Ctrl; * $p < 0.01$, ** $p < 0.001$ versus Aβ. Ctrl: control group; Aβ: $A\beta_{25–35}$ treatment group. Abbreviations: Aβ, $A\beta_{25–35}$ treatment group; C,

423 curcumin alone; Ctrl, control group; CV, curcumin and vorinostat; CVS, curcumin, vorinostat, and silibinin.

424 **Figure 3. Effects of CVS pretreatment on the expression of critical proteins. A-C.** Effects of CVS on

425 the expression of *p*-AKT (A), *p*-BAD (B), *p*-MDM2 (C), and p53 (D) in activated $A\beta_{25–35}$-treated PC12

426 cells. Each experiment was completed with a minimum of 5 replicates. Statistical significance is presented

427 as; ## $p < 0.001$ versus Ctrl; * $p < 0.01$, ** $p < 0.001$ versus Aβ. Abbreviations: Aβ, $A\beta_{25–35}$ treatment group;

428 AKT, AKT serine/threonine kinase; BAD, BCL2 associated agonist of cell death; C, curcumin alone; Ctrl,

429 control group; CV, curcumin and vorinostat; CVS, curcumin, vorinostat, and silibinin; MDM2, MDM2

430 proto-oncogene 2; p53, tumor protein p53.

431 **Figure 4. CVS reduced the intranuclear content of p53 in $A\beta_{25–35}$-treated PC12 cells.** Each experiment

432 was completed with a minimum of 5 replicates. Statistical significance is presented as; ## $p < 0.001$ versus

433 Ctrl; * $p < 0.01$, ** $p < 0.001$ versus Aβ.

Figure 1

CVS treatment inhibits $A\beta_{25-35}$-induced PC12 cell apoptosis.

A. Individual effects of 9 candidate compounds on cell viability. **B-D.** Effects of CVS on cell viability (B), reversion of CASP3 expression (C), and apoptotic cell levels (D) in $A\beta_{25-35}$-treated PC12 cells. Each experiment was completed with a minimum of 5 replicates. Statistical significance is presented as; ## $p < 0.001$ versus Ctrl; * $p < 0.01$, ** $p < 0.001$ versus Aβ. Abbreviations: Aβ, $A\beta_{25-35}$ treatment group; C, curcumin alone; CASP3, Caspase 3, Ctrl, control group; CV, curcumin and vorinostat; CVS, curcumin, vorinostat, and silibinin.

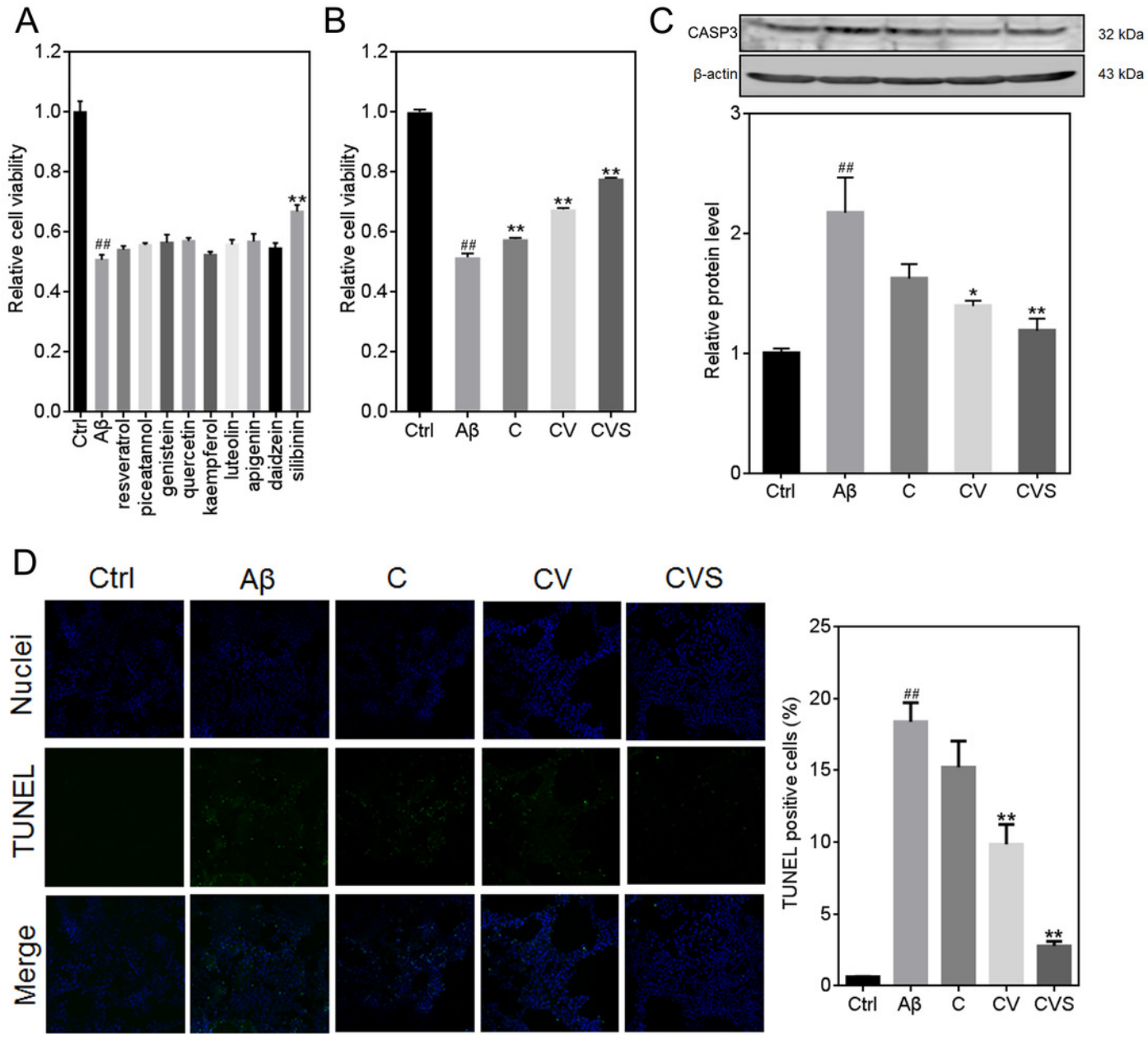
A
Relative cell viability
1.2
1.0
0.8
0.6
0.4
0.2
0.0
##
**
Ctrl
Aβ
resveratrol
piceatannol
genistein
quercetin
kaempferol
luteolin
apigenin
daidzein
silibinin
B
Relative cell viability
1.2
1.0
0.8
0.6
0.4
0.2
0.0
##
**
**
**
Ctrl
Aβ
C
CV
CVS
C
CASP3
32 kDa
β-actin
43 kDa
Relative protein level
3
2
1
0
##
*
**
Ctrl
Aβ
C
CV
CVS
D
Ctrl
Aβ
C
CV
CVS
Nuclei
TUNEL
Merge
TUNEL positive cells (%)
25
20
15
10
5
0
##
**
**
Ctrl
Aβ
C
CV
CVS

Figure 2

CVS pretreatment prevents $A\beta_{25-35}$-induced oxidative stress in PC12 cells.

Effects of CVS on ROS (A), SOD (B), and MDA levels (C). Each experiment was completed with a minimum of 5 replicates. Statistical significance is presented as; ## $p < 0.001$ versus Ctrl; * $p < 0.01$, ** $p < 0.001$ versus Aβ. Ctrl: control group; Aβ: $A\beta_{25-35}$ treatment group. Abbreviations: Aβ, $A\beta_{25-35}$ treatment group; C, curcumin alone; Ctrl, control group; CV, curcumin and vorinostat; CVS, curcumin, vorinostat, and silibinin.

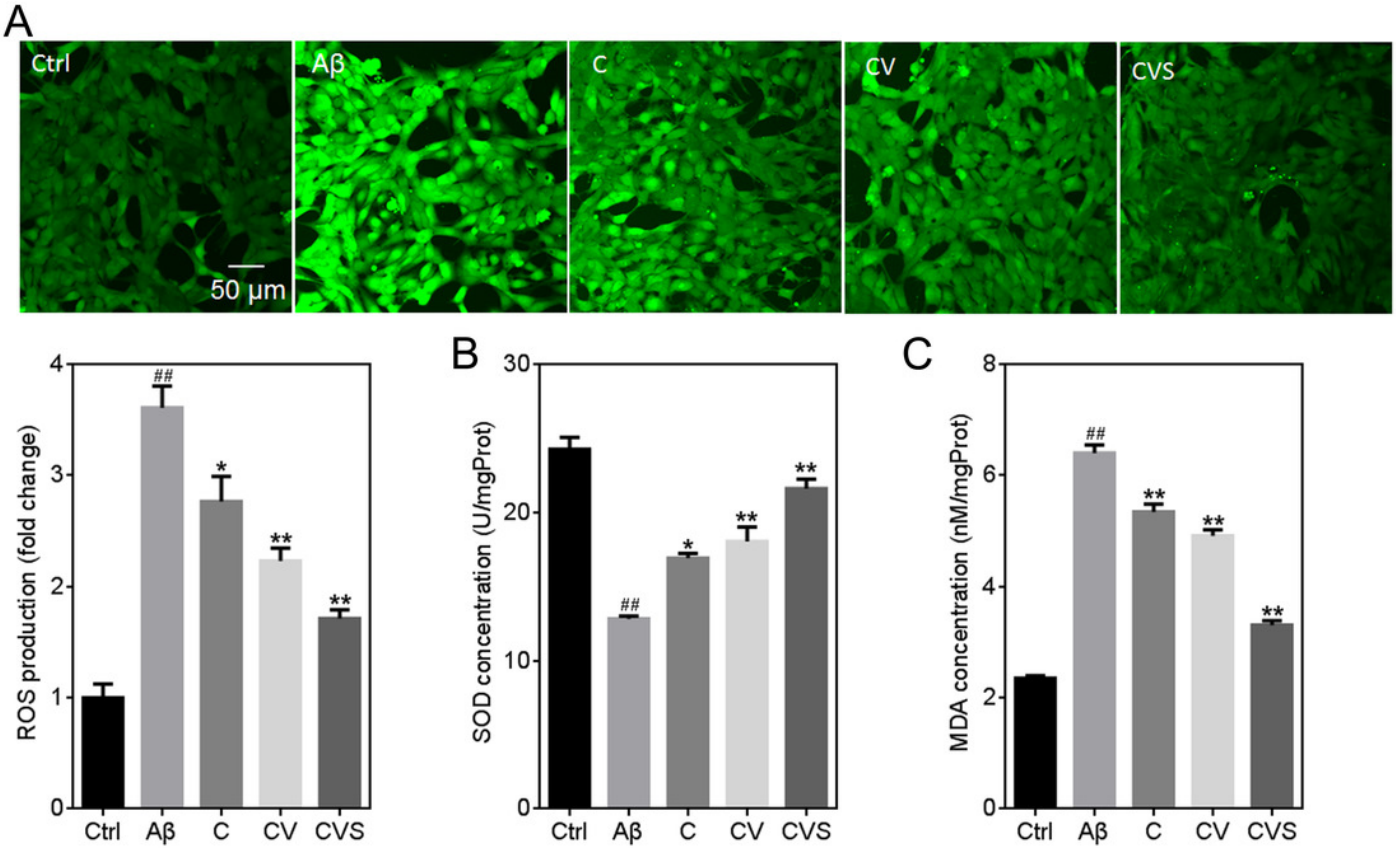

Figure 3

Effects of CVS pretreatment on the expression of critical proteins.

A-C. Effects of CVS on the expression of *p*-AKT (A), *p*-BAD (B), *p*-MDM2 (C), and p53 (D) in activated $A\beta_{25-35}$-treated PC12 cells. Each experiment was completed with a minimum of 5 replicates. Statistical significance is presented as; ## $p < 0.001$ versus Ctrl; * $p < 0.01$, ** $p < 0.001$ versus Aβ. Abbreviations: Aβ, $A\beta_{25-35}$ treatment group; AKT, AKT serine/threonine kinase; BAD, BCL2 associated agonist of cell death; C, curcumin alone; Ctrl, control group; CV, curcumin and vorinostat; CVS, curcumin, vorinostat, and silibinin; MDM2, MDM2 proto-oncogene 2; p53, tumor protein p53.

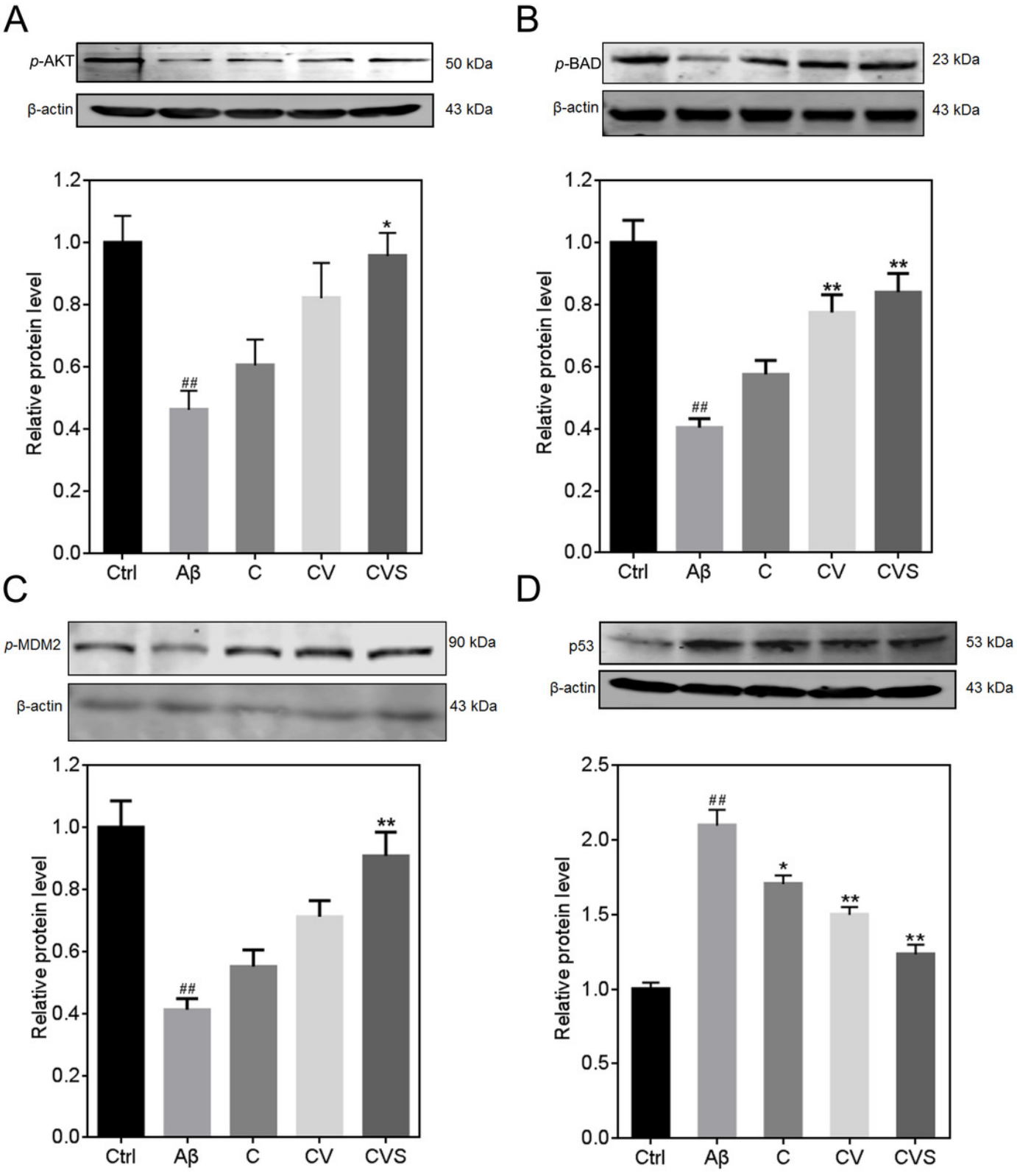
A
p-AKT
50 kDa
β-actin
43 kDa
Relative protein level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
##
*
Ctrl
Aβ
C
CV
CVS
B
p-BAD
23 kDa
β-actin
43 kDa
Relative protein level
##
**
**
Ctrl
Aβ
C
CV
CVS
C
p-MDM2
90 kDa
β-actin
43 kDa
Relative protein level
##
**
Ctrl
Aβ
C
CV
CVS
D
p53
53 kDa
β-actin
43 kDa
Relative protein level
2.5
2.0
1.5
1.0
0.5
0.0
##
*
**
**
Ctrl
Aβ
C
CV
CVS

Figure 4

CVS reduced the intranuclear content of p53 in $A\beta_{25-35}$-treated PC12 cells.

Each experiment was completed with a minimum of 5 replicates. Statistical significance is presented as; ## $p < 0.001$ versus Ctrl; * $p < 0.01$, ** $p < 0.001$ versus Aβ.