

Vincamine Alleviates Amyloid- β 25–35 Peptides-induced Cytotoxicity in PC12 Cells

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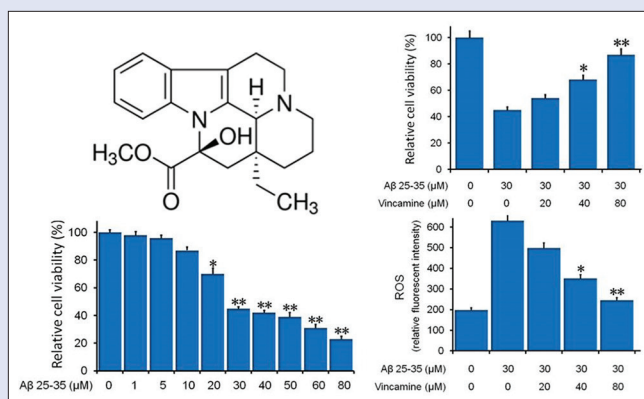
ABSTRACT

Objective: Vincamine is a plant alkaloid used clinically as a peripheral vasodilator that increases cerebral blood flow and oxygen and glucose utilization by neural tissue to combat the effect of aging. The main purpose of the present study is to investigate the influence of vincamine on amyloid- β 25–35 (A β 25–35) induced cytotoxicity, to gain a better understanding of the neuroprotective effects of this clinically used anti-Alzheimer's disease drug. **Materials and Methods:** Oxidative stress was assessed by measuring malondialdehyde, glutathione, and superoxide dismutase (SOD) levels. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell apoptosis detection was performed using an Annexin-V-FITC Apoptosis Detection Kit. The production of reactive oxygen species (ROS) was determined using a ROS Assay Kit. Western blot detection was carried out to detect the protein expression. **Results:** Our studies showed that pretreatment with vincamine could reduce A β 25–35 induced oxidative stress. Vincamine markedly inhibited cell apoptosis dose-dependently. More importantly, vincamine increased the phosphatidylinositol-3 kinase (PI3K)/Akt and Bcl-2 family protein ratios on preincubation with cells for 2 h. **Conclusion:** Above observation led us to assume that one possible mechanism of vincamine protects A β 25–35-induced cell death could be through upregulation of SOD and activation of the PI3K/Akt pathway.

Key words: Alzheimer's disease, amyloid- β , PC12 cells, phosphatidylinositol-3 kinase/Akt pathway, vincamine

SUMMARY

- Vincamine ameliorates amyloid- β 25–35 (A β 25–35) peptides induced cytotoxicity in PC12 cells
- Vincamine reduces A β 25–35 peptides induced apoptosis of PC12 cells
- Vincamine activates the phosphatidylinositol-3 kinase/Akt pathway
- Vincamine up-regulates the superoxide dismutase.



Abbreviations used: A β 25–35: Amyloid- β 25–35; AD: Alzheimer's disease; BCA: Bicinchoninic acid; GSH: glutathione; PBS: Phosphate buffered solution; SDS: Sodium dodecylsulphate; SOD: Superoxide dismutase

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INTRODUCTION

The number of people suffering from Alzheimer's disease (AD) increases as the world population ages, creating a huge socioeconomic burden. AD is the crucial cause of dementia in people over the age of 60,^[1,2] which is mainly characterized by three pathological hallmarks: Cholinergic system dysfunction, the amyloid- β (A β) peptide deposition, and the Tau protein hyperphosphorylation.^[3,4] The intercellular transfer of A β and tau proteins has received increasing attention in AD. Although the link of A β or tau protein to brain degeneration has remained elusive, the A β cascade hypothesis remains as one of the dominant hypotheses for AD etiology. Nevertheless, with the fact that many therapeutic approaches toward A β lowering/clearing fail to gain anticipated benefits in the clinical trials,^[5,6] it is of great importance to further understand and analyze the essence of A β cascade theory. Currently, the extracellular deposit of insoluble A β is no longer considered as the major contributor for AD pathogenesis^[7,8] whereas supports from numerous experimental paradigms have implicated the abnormal accumulation of intracellular

A β oligomers is responsible for the manifestations of AD pathology.^[9,10] Growing researches have been focused on studying the association between the intracellular A β cascade and the dysfunction of subcellular organelles, especially mitochondria.^[11–13] More interestingly, it is reported that mitochondrial A β levels were positively correlated with the extent of mitochondrial dysfunction in different brain regions in APP or APP/PS1 transgenic mice, and the degree of cognitive impairment in AD transgenic mice could be linked to the extent of synaptic

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mitochondrial dysfunction and mitochondrial A β levels.^[14,15] Hence, targeting A β -associated mitochondrial dysfunction, especially blocking mitochondrial A β accumulation is expected as a promising approach for AD-modifying.

Herbal medicines have been proven to be a major source of novel agents with various pharmaceutical activities.^[16-21] Natural products have provided a rich source of drugs for many diseases, including AD. Vincamine [Figure 1] is an alkaloid of clinical use against the brain sclerosis, as well as in postoperative states of the central nervous system.^[22] It is employed today in the therapy of cerebral metabolic and circulatory disorders since it combines cerebrometabolic and hemodynamic properties.^[23-26] Twenty years before, it was used as a drug for treating memory impairments. Vinpocetine, a vincamine derivative, efficiently protects cells from reactive oxygen species (ROS) attack. Recently, the protective effect of vinpocetine was demonstrated using *in vitro* models of oxidative stress induced by the oxidant pair ascorbate/Fe²⁺ and by synthetic peptides of the AD-associated A β . Results obtained from these *in vitro* experiences support that additional clinical trials should be carried out using vincamine, or vincamine derivatives, to test its therapeutic or preventive effects in AD.^[26]

To the best of our knowledge, in the present study, we demonstrated for the first time that vincamine could alleviate A β 25–35 induced cytotoxicity in PC12 cells, thus providing basis for clinical application of vincamine in AD cases.

MATERIALS AND METHODS

Chemicals and preparation

Vincamine was purchased from Sigma Chemical Co., (St. Louis, MO, USA) and was dissolved with deionized water as stock solution. The drug stock solution was further diluted with deionized water to proper concentrations before usage. Unless otherwise stated, all other chemicals were purchased from Sigma (MO, USA).

Preparation and treatment with amyloid- β 25–35

A β 25–35 peptide (GenScript, Piscataway, NJ, USA) stock solutions were freshly prepared before each treatment at 1 mM in double distilled deionized water, considered the soluble form. The cells were then treated with A β 25–35 peptide in a range of 0–80 nM in serum-free medium containing 1% PS for 24 h. Then, the cells were incubated at 37°C in a humidified and sterile atmosphere containing 5% CO₂ for 24, 48, and 72 h.

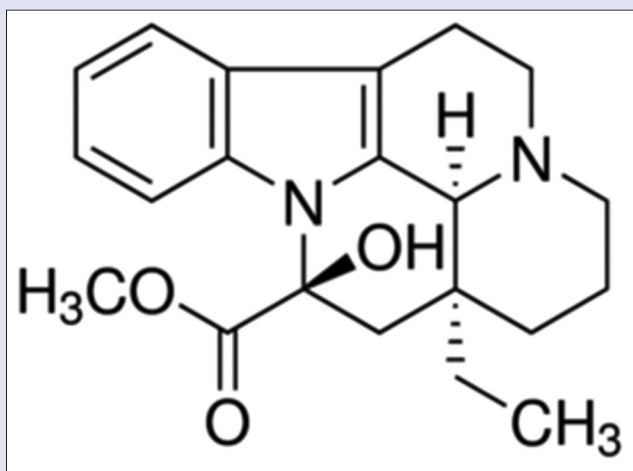


Figure 1: Chemical structure of vincamine

Oxidative stress assays

Oxidative stress was assessed by measuring malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) levels. PC12 cells cultured in 6-well plates (4×10^4 cells/well) for 24 h were treated with various concentrations of vincamine for 3 h before the addition of 30 μ M A β 25–35 and further 24 h incubation. Cells were then digested with trypsin and washed twice in phosphate buffered solution (PBS). Thereafter, cells were suspended in 500 μ l of PBS and lysed by ultrasonication in the presence of protease inhibitor before centrifugation at 4000 rpm for 5 min. The supernatant was collected for analysis. Supernatant protein concentrations were measured using a Bradford protein assay kit from Key Gen Biotech (Nanjing, China). The levels of MDA, GSH, and SOD were measured using appropriate kits purchased from Key Gen Biotech following the manufacturer's instructions.

Detection of reactive oxygen species

PC12 cells were cultured and treated as per the oxidative stress assays. The production of ROS was determined using an ROS Assay Kit in accordance with the manufacturer's instructions (Beyotime, Shanghai, China).

Cell apoptosis assay

Cell apoptosis detection was performed using an Annexin-V-FITC Apoptosis Detection Kit (BD Company, USA) as described elsewhere.^[27,28] In brief, 24 h after A β 25–35 exposure, the PC12 cultures were washed with warm (37°C) Krebs–Ringer solution and fixed with 4% (w) paraformaldehyde in PBS for 30 min at room temperature. For vincamine-treated group, cells were collected after 24 h treatment with vincamine. The cells were washed twice with cold PBS then resuspended in $1 \times$ binding buffer at a concentration of 1×10^6 cells/ml. Then 500 μ l cell suspension was incubated with 5 μ l Annexin-V-FITC and 10 μ l propidium iodide (PI) for 15 min in the dark and analyzed by a FACS calibur instrument (Becton Dickinson, San Jose, USA) within 1 h. Apoptotic cells were those stained with Annexin V+/PI– (early apoptosis) plus Annexin V+/PI+ (late apoptosis).

Western blot analysis

Western blot detection was carried out to detect the protein expression.^[29,30] Briefly, following treatment of PC12 cells with vincamine at the corresponding concentration and for the indicated time, cells were harvested using trypsin ethylenediaminetetraacetic acid (EDTA), washed twice with PBS, and stored at –80°C. Cells were lysed in lysis buffer (1 mM EDTA, 150 mM NaCl, 100 μ g/ml phenyl methylsulfonyl fluoride 50 mM Tris-HCl, pH 7.5) for 30 min on ice and then a particle-free supernatant solution was obtained by centrifugation at 14,000 g for 15 min. All operations were at 0–4°C. A sample was taken for measurement of protein content by a bicinchoninic acid assay (Pierce). Equal amounts of protein were heated in sodium dodecylsulphate (SDS) sample buffer (Laemmli) for 15 min at 95°C, separated on an 8–12% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes. Membranes blocked with 5% nonfat milk powder (w/v) in TBST (10 mM Tris, 10 mM NaCl, 0.1% Tween 20) for 2–4 h at room temperature to prevent nonspecific antibody binding, and incubated with the corresponding primary antibody diluted in blocking buffer overnight at 4°C. After 3 min \times 10 min washes in TBST, blots were incubated for 1 h with corresponding peroxidase conjugated secondary antibody and developed employing a commercial kit (West Pico chemiluminescent

substrate). Blots were reprobed with an antibody against β -actin or GAPDH as control of protein loading and transfer.

Statistical analysis

Data were expressed as mean \pm standard error of mean Student's *t*-test was applied in the comparisons between two groups. Multiple comparisons between model group and different concentrations of vincamine-treated groups were analyzed by one-way ANOVA, followed by Dunnett test. Differences were considered significant at $P < 0.05$.

RESULTS

Determination of amyloid- β 25–35 cytotoxicity to PC12 cells

The relative survival rate of PC12 cells treated with A β 25–35 for 24 h decreased with increasing concentration of A β 25–35 [Figure 2]. The relative survival rate was 97.6% with 1 μ M A β 25–35 and 25.8% with 80 μ M A β 25–35. The survival rate was approximately 50% with exposure to 30 μ M A β 25–35; hence, this concentration was chosen in all subsequent experiments for the determination of survival in response to different treatments.

Vincamine alleviated amyloid- β 25–35 induced cytotoxicity in PC12 cells

The relative survival rate of A β 25–35-treated PC12 cells pretreated with vincamine increased with increasing vincamine concentration [Figure 3]. The relative survival rate was 43.5% without vincamine and 83.6% with 80 μ M vincamine ($P < 0.01$).

Vincamine decreased amyloid- β 25–35 induced oxidative stress

Intracellular MDA concentrations were significantly increased in A β 25–35-treated cells compared with the negative control group cells ($P < 0.05$) [Table 1]. Vincamine decreased intracellular MDA concentrations in A β 25–35-treated cells in a dose-dependent manner [Table 1]. The MDA concentrations in the vincamine-treated groups were significantly lower than the concentration in the model control group ($P < 0.05$). Intracellular GSH concentrations were significantly decreased in A β 25–35-treated cells compared with the negative control group cells ($P < 0.05$) [Table 1]. Vincamine increased intracellular GSH concentrations in A β 25–35-treated cells in a dose-dependent manner [Table 1]. The GSH concentrations in the vincamine-treated groups were significantly higher than the concentration in the model control group ($P < 0.05$). Intracellular SOD concentrations were significantly decreased in A β 25–35-treated cells compared with the negative control group cells ($P < 0.05$) [Table 1]. Vincamine increased intracellular SOD concentrations in A β 25–35-treated cells in a dose-dependent manner [Table 1]. The SOD concentrations in the vincamine-treated groups were significantly lower than the concentration in the model control group ($P < 0.05$).

Vincamine reduced reactive oxygen species levels

ROS levels were significantly increased in A β 25–35-treated cells (667.5 vs. 192.6 fluorescence intensity units, $P < 0.05$) [Figure 4]. Vincamine significantly reduced ROS level in a dose-dependent manner [Figure 4]. ROS levels in the vincamine 40 and 80 μ M groups were significantly

Table 1: Effects of vincamine on A β 25-35-treated PC12 cells

	Negative control	A β 25-35	Vincamine (20 μ M)	Vincamine (40 μ M)	Vincamine (80 μ M)
MDA (nmol/mg)	13.3 \pm 1.5	59.2 \pm 5.3	51.2 \pm 3.2*	24.2 \pm 1.3*	18.9 \pm 4.1**
GSH (U/mg)	745 \pm 46	255 \pm 49	411 \pm 23**	501 \pm 55**	709 \pm 38***
SOD (U/mg)	28.37 \pm 0.3	12.17 \pm 1.3	14.56 \pm 2.5	24.58 \pm 3.6**	25.22 \pm 0.7**

* $P < 0.05$ compared to A β 25-35-treated group; ** $P < 0.01$ compared to A β 25-35-treated group; *** $P < 0.001$ compared to A β 25-35-treated group. MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Glutathione

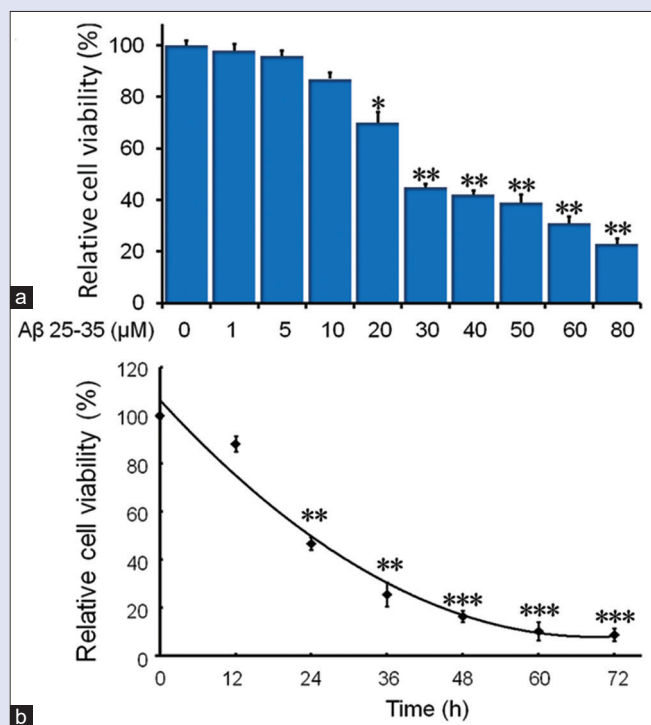


Figure 2: Effect of amyloid- β 25–35 on cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in PC12 cells. (a) Cells treated with amyloid- β 25–35 (0–80 μ M) for 24 h. (b) Cells treated with 30 μ M amyloid- β 25–35 for 0, 12, 24, 36, 48, 60 and 72 h. All results are expressed as mean \pm standard deviation ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the control group

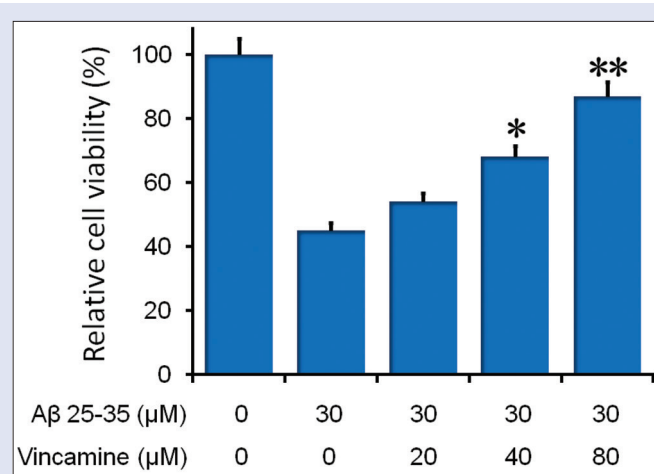


Figure 3: Effect of vincamine on cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in amyloid- β 25–35-treated PC12 cells. The results are expressed as mean \pm standard deviation ($n = 3$). * $P < 0.05$, ** $P < 0.01$ as compared with the amyloid- β 25–35-treated group

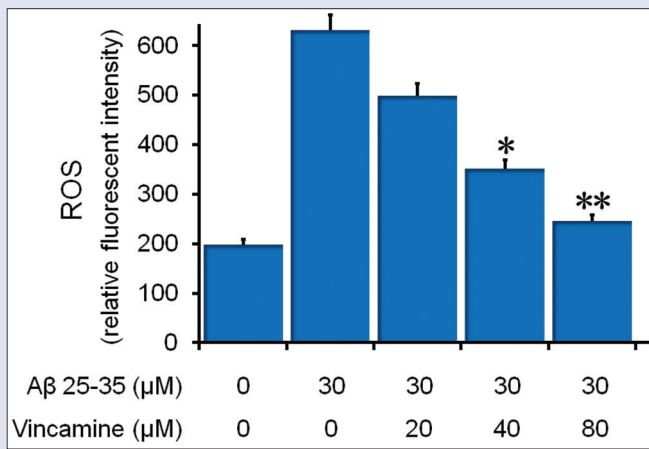


Figure 4: Intracellular reactive oxygen species produced after amyloid- β 25-35 induced oxidative stress in PC12 cells with and without vincamine. Student's *t*-test was performed to evaluate the significance of the results. **P* < 0.05, ***P* < 0.01, vincamine-treated cells compared with respective control. Results are mean \pm standard deviation (*n* = 3)

lower than the level in the model control group (365.2 and 286.6 vs. 667.5 fluorescence intensity units, *P* < 0.05).

Anti-apoptotic effect of vincamine

An Annexin V-FITC and PI double stain were used to evaluate the percentages of apoptosis. The rate of apoptosis was significantly increased in A β 25-35-treated cells compared with the negative control group cells (75.8 vs. 4.8%, *P* < 0.05) [Figure 5]. Vincamine significantly reduced the rate of apoptosis in a dose-dependent manner [Figure 5]. The rate of apoptosis in the three vincamine groups was significantly lower than the rate in the model control group (57.3, 35.6, and 25.3 vs. 75.8%, *P* < 0.05).

Vincamine regulated Akt and phospho-Akt levels in PC12 cells

To gain a better insight into anti-apoptotic effect of vincamine, we detected protein expression of apoptosis marker molecular. Akt, and phospho-Akt were examined by Western blotting. Results showed that A β 25-35 increased the phospho-Akt/Akt ratio. Vincamine (20, 40, and 80 μ M) increased the phospho-Akt/Akt ratio after preincubation for 2 h.

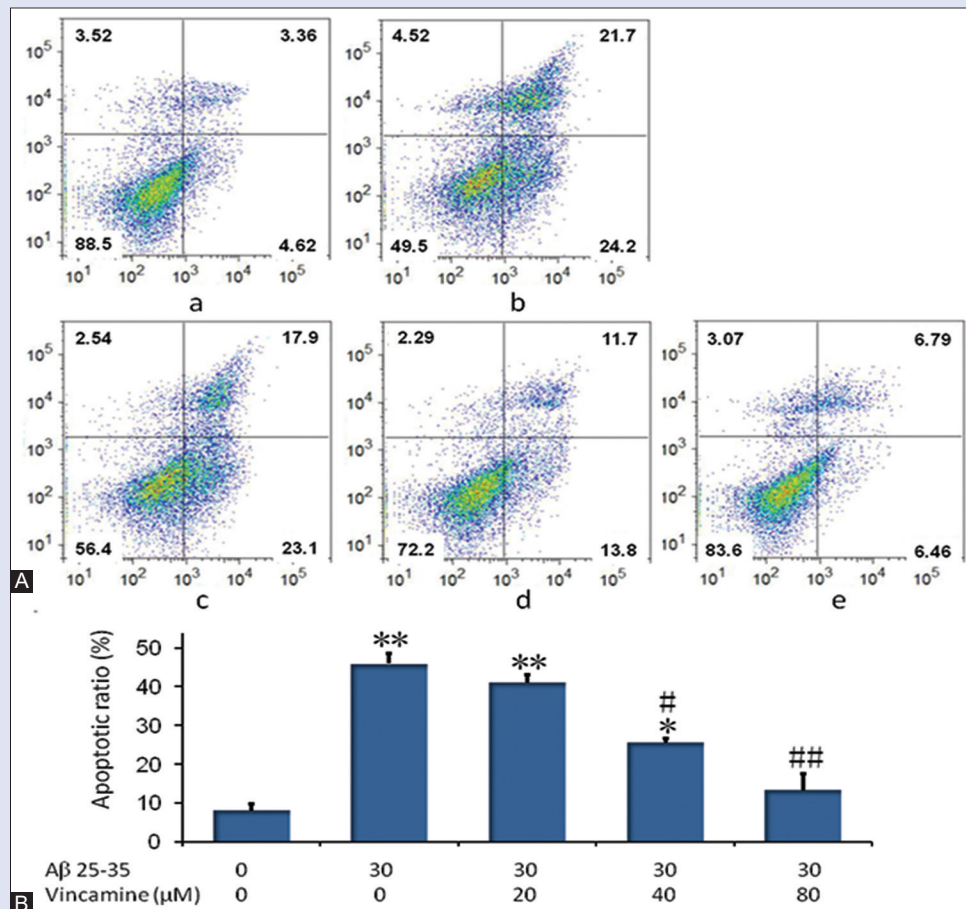


Figure 5: Vincamine attenuated amyloid- β 25-35 induced neurotoxicity in PC12 cells. (A) Apoptosis analysis of PC12 cells treated with amyloid- β 25-35, vincamine, or a combination of them. (a) Untreated cells; (b) 30 μ M amyloid- β 25-35-treated cells; (c) 30 μ M amyloid- β 25-35 + 20 μ M vincamine-treated cells; (d) 30 μ M amyloid- β 25-35 + 40 μ M vincamine-treated cells; (e) 30 μ M amyloid- β 25-35 + 80 μ M vincamine-treated cells. Cells were exposed for 48 h. Double staining was used to distinguish between viable (lower left quadrant, annexin V-negative, propidium iodide-negative), early apoptosis (lower right quadrant, annexin V-positive, propidium iodide-negative), late apoptosis and necrotic (upper right quadrant, annexin V-positive, propidium iodide-positive) and cell debris (upper left quadrant). Statistical analysis is shown in (B). **P* < 0.05, ***P* < 0.01, amyloid- β 25-35, vincamine or both treated cells compared with untreated control cells. #*P* < 0.05, ##*P* < 0.01, vincamine-treated cells compared with 30 μ M amyloid- β 25-35-treated cells. Results are mean \pm standard deviation (*n* = 3)

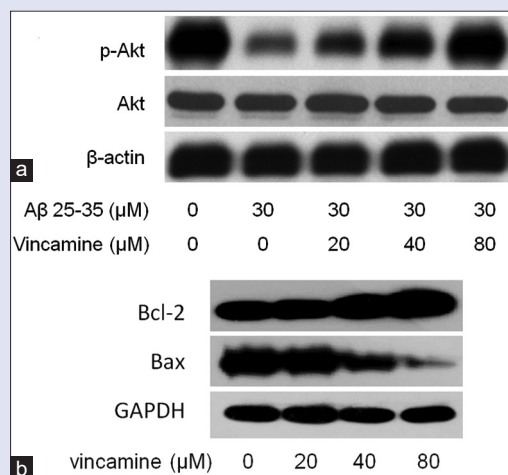


Figure 6: Phosphatidylinositol-3 kinase/Akt pathway was involved in the anti-apoptotic effects of vincamine. (a) PC12 cells were treated with vincamine for 2 h. Akt and its phosphorylation were detected by Western blotting. β -actin served as a control for loading. (b) Protein expression of Bcl-2, Bax in PC12 cells after indicated treatments was also measured by Western blots. GAPDH served as a control for loading

These data suggested that vincamine could provide neuroprotection from A β 25–35 induced PC12 apoptosis through the phosphatidylinositol-3 kinase/Akt signaling pathway [Figure 6a].

Bcl-2 family proteins were involved with the anti-apoptotic effect of vincamine

We next investigated the expression of Bcl-2 families, which regulated mitochondrial apoptosis and could be separated into pro-survival members (such as Bcl-2, BclxL, and Mcl-1), as well as pro-apoptotic proteins (such as Bax). As shown in Figure 6b, after vincamine treatment, Bcl-2 was upregulated significantly and Bax was downregulated on the contrary. These results are consistent with the general notion that Bcl-2 and Bax play pivotal role in regulating mitochondrial apoptosis pathway.

DISCUSSION

AD, the most prevalent form of dementia in older adults, is a chronic progressive neurodegenerative disorder.^[31] AD patients have severe progressive cognitive dysfunction, memory impairment, behavioral symptoms and loss of independence.^[32] According to AD International, at least 35.6 million people had dementia in 2010, with the numbers nearly doubling every 20 years.^[33] Many factors contribute to the etiology of AD, elevated A β and loss of nicotinic acetylcholine receptors being prominent.^[34] Although the neuroprotective effects of vincamine have attracted intense interest in recent years, the exact molecular mechanisms underlying have not yet been clarified. The major impressive characteristics of the present study are novel anti-apoptotic and antioxidant effects of vincamine.

A β plays a pivotal role in the mitochondrial dysfunctions because mitochondrial deficits like oxidative stress, energy deficiency, and mitochondrial depolarization were frequently seen in A β -treated cell models and A β over expression animal models.^[11,35,36] Therefore, reversing A β -associated oxidative stress may provide an opportunity to recover AD.

In the current study, we found that vincamine administration could effectively reduce A β induced cytotoxicity, which is the first report of vincamine reducing A β induced cytotoxicity to our knowledge.

Vincamine has been reported to probably hydrolyze in the rat plasma into vincamic acid (a hydroxycarboxylic acid) that could possibly form a complex with Fe and excreted in urine and subsequently the Fe level reduced in the brain.^[37] Vincamine was completely metabolized and excreted in urine as sulfates and glucuronide conjugates.^[38] Vincamine could be useful in aged people because it reduces the brain Fe concentration and subsequently prevents the oxidative damage of Fe on neural cells. Vincamine has been reported to cross the blood–brain barrier, and its antioxidant scavenging capacity to inactivate hydroxyl free radicals was actually ranked in part with Vitamin E.^[39] Iron is believed to accumulate in high concentration in neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's diseases and contribute to oxidative stress and subsequently lead to neuronal death.^[40]

Dietary phytochemicals consist of a wide variety of biologically active compounds that are ubiquitous in plants, many of which have been reported to have pharmaceutical properties. Epidemiological studies have shown that natural components may play an important role in preventing human diseases.^[41–45] Among them, vincamine, which is abundant in *Vinca minor* L., has been reported to have therapeutic potential for treating many human diseases.^[46,47]

In addition to looking at indicators of oxidative damage, we also examined the concentrations of several important antioxidants, GSH, and SOD. In keeping with oxidative stress findings, we found that concentrations of both GSH and SOD were significantly increased in PC12 cells pretreated with vincamine before A β 25–35 treatment. This is an important finding, given that decreases in the expression of both GSH and SOD have been implicated in the development of AD and other neurological diseases.^[48,49]

Unsurprisingly, given the decreased oxidative damage and higher concentrations of antioxidants, we found that survival was significantly increased and apoptosis was significantly decreased in PC12 cells pretreated with vincamine before A β 25–35 treatment compared with PC12 cells treated with A β 25–35 alone. Our data showed that pretreatment with vincamine markedly increased the survival percentage of PC12 cells subsequently treated with A β 25–35. The finding of decreased apoptosis is noteworthy because increased apoptotic signaling/neuronal death is thought to contribute to the pathology of neurodegenerative disorders, including AD.^[50]

CONCLUSION

Our research for the first time found that vincamine, a natural alkaloid, ameliorated the deleterious effects of A β 25–35 in PC12 cells. Specifically, vincamine increased cell survival, decreased apoptosis and cytotoxicity, and decreased the concentrations/activities of a variety of indicators of oxidative stress. We believe that these are promising findings that support the continued investigation of vincamine as a potential treatment for AD. Our data might shed more light on the clinical benefits gained by vincamine and provide useful clues for future AD drug development, however, the precise mechanisms underlying the beneficial effect of the drug needs further investigation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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