Research article

Novel donepezil-tacrine hybrid (TAHB3) induces neurodifferentiation, neuroprotective effects, and activates the PI3K/AKT pathway on PC12 cells

Journal of Alzheimer's Disease Reports
Volume 9: 1–15

DOI: 10.1177/25424823241309268
journals.sagepub.com/home/alr

Renata Melo dos Santos Ono[1], Natália Chermont dos Santos Moreira[1], Ivone Carvalho[2], Geraldo Aleixo Passos[1,3] and Elza Tiemi Sakamoto-Hojo[1,4]

Abstract

Background: Alzheimer's disease (AD) is a neurodegenerative disease characterized by the impairment of cognitive functions and neuronal loss. AD has no cure; current treatments like acetylcholinesterase inhibitors (AChEI) alleviate symptoms but do not halt disease progression.

Objective: We aimed to evaluate the effects and mechanisms of two novel AChEI hybrid compounds (TAHB3 and TA8Amino), regarding cytotoxicity, neuroprotection and neurodifferentiation in PC12 cells.

Methods: The effects of TAHB3 and TA8Amino on neurodifferentiation were analyzed on PC12 cells which were treated with AChEI compounds for seven days, following morphological, and quantitative analyses to calculate the differentiation percentages, neurite length, and protein expression. Regarding cytotoxicity and neuroprotection assays, PC12 cells were differentiated into mature neurons, then treated with TAHB3 or TA8Amino, following a posttreatment with H_2O_2 (an inducer of oxidative damage); the analyses were performed using the XTT assay and flow cytometry.

Results: The hybrid compound TAHB3 induced differentiation of PC12 cells, but TA8Amino did not cause the same effect. Both compounds did not show cytotoxic effects to PC12 cells and did not change the cell cycle progression, nor induce cell death. Only TAHB3 showed neuroprotective potential against induced-oxidative damage, and TAHB3 increased the levels of p-AKT, suggesting its action through the activation of the PI3K/AKT pathway.

Conclusions: Our results showed that TAHB3 can induce neurodifferentiation, besides a neuroprotective activity, indicating the potential of AChEI hybrid compounds as novel candidates to be explored for the establishment of novel therapeutic strategies for patients with AD.

Keywords

acetylcholinesterase inhibitors, Alzheimer's disease, donepezil-tacrine hybrid, neurodifferentiation, neuroprotection

Received: 16 July 2024; accepted: 5 December 2024

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by cognitive impairment and changes in memory and behavior. These changes are associated with neuronal loss caused by various pathological mechanisms, including the presence of amyloid plaques formed by amyloid-β (Aβ) peptide accumulation and neurofibrillary tangles (NFTs) resulting from abnormal tau protein

[1]Department of Genetics, Ribeirão Preto Medical School, University of São Paulo-USP, Brazil
[2]School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil
[3]Laboratory of Genetics and Molecular Biology, Department of Basic and Oral Biology, Ribeirão Preto School of Dentistry, University of São Paulo-USP, Brazil
[4]Department of Biology, Faculty of Philosophy, Sciences and Letters at Ribeirão Preto, University of São Paulo-USP, Brazil

Corresponding author:
Elza Tiemi Sakamoto-Hojo, Department of Biology – Faculty of Philosophy, Sciences and Letters at Ribeirão Preto, University of São Paulo – USP, Av. Bandeirantes, 3900 – Monte Alegre 14040-901 – Ribeirão Preto, S.P., Brazil.
Email: etshojo@usp.br

phosphorylation.[1,2] Besides those alterations, several subjacent mechanisms are involved in the development of AD, as follows: oxidative stress, inflammatory processes, alterations in the neurotransmitters, excitotoxicity, and mitochondrial dysfunction.[3,4] AD has been considered a major health problem worldwide, and the available drug treatments may improve the symptoms but show only minor effects to slow down the progression of the disease.[5]

The main current drugs for patients with AD belong to the class of acetylcholinesterase enzyme inhibitors (AChEI), which were designed to protect brain tissues and slow down cognitive decline, through the increase of cholinergic transmission.[6] The AChEI reduce acetylcholinesterase (AChE) activity, increasing the availability of acetylcholine (ACh) in the synaptic gap; AChEI drugs have also been associated with non-cholinergic effects based on their capacity to reduce the levels of Aβ aggregation and induction of neuronal differentiation.[7,8] Only few drugs have been used for AD treatment; tacrine was the first one to be widely used in the treatment of AD, but it was suspended due to hepatotoxicity.[9,10] Currently, another three AChEI compounds (rivastigmine, galantamine, and donepezil) have been prescribed for AD treatment, but their efficacy is modest and limited to the initial and medium stages of the disease.[11,12] In this context, many studies have been developed on the mechanisms of disease progression, aiming to obtain relevant information that can support the search for new therapeutic modalities for patients with AD. Oxidative stress has been considered a central mechanism in the development and progression of AD, affecting AD hallmarks, such as Aβ aggregation and tau-hyperphosphorylation.[13–15] Targeting oxidative stress is a promising strategy for developing novel multi-target compounds for AD treatment, considering that the brain is continuously exposed to various cytotoxic agents that contribute to AD-related damage.

The ability to induce neuronal differentiation is also an important characteristic for new therapy strategies, considering that in AD, besides neuronal death, Aβ oligomers and low ACh availability can compromise neurogenesis, contributing to neuron loss.[16,17] In this context, strategies capable of compensating neuron loss are considered relevant, with a focus on the induction of neuronal differentiation.[18] Studies with donepezil have demonstrated that AChE inhibition can induce neuronal differentiation in human models, both *in vivo* and *in vitro* conditions,[19,20] thus demonstrating the potential of this approach as a therapeutic strategy to be considered for AD.

Therefore, for being a multifactorial disease, the treatments with single drugs that target mainly one mechanism involved in AD development, are still limited and provide insufficient information for AD therapy.[21,22] However novel strategies to improve the effectiveness and responses of patients to those drugs have been considered important tools, including the use of hybrid compounds.[23] Molecular hybridization is a strategy that consists of synthetizing novel prototypes, using subunits of already known molecules, allowing the creation of new structures that will preserve specific characteristics of the original compounds while losing others.[24] The benefits of studying hybrid compounds include lower costs and shorter time since characteristics of the original compounds are already known, such as toxicity, pharmacokinetics, and mechanisms of action.[21,24] Also, it seems promising the possibility of combining the mechanisms of action of two or more bioactive molecules, aiming to act on multiple targets and enhance the efficacy of the original compounds.[25]

In this context, research with hybrids which were synthesized from AChEI drugs, seeks to enhance the inhibition of AChE through simultaneous binding to multiple inhibition sites.[22,26] For this reason, Chierrito (2017)[27] synthesized new hybrid compounds (TAHB3 and TA8Amino) from tacrine and donepezil (Figure 1), aiming to obtain compounds with low toxicity but maintaining the binding capacity to the CAS site of AChE, in addition to conferring the binding capacity to the PAS site of the same enzyme. Tacrine hepatotoxicity is attributed to its acridine nucleus, which is not present in the structure of the novel hybrid compounds.[27] TA8Amino and TAHB3 demonstrated similar efficiency compared to the original compounds, in terms of AChE inhibition, by binding to an allosteric site of this protein.[27] These novel hybrids were tested by Moreira et al. (2020),[28] which demonstrated that different concentrations of the novel compounds were not toxic to SH-SY5Y and HepG2 cells, TAHB3 and TA8Amino also stimulated neurodifferentiation and neuritogenesis in SH-SY5Y cells, possibly through the activation of the PI3K/AKT pathway (phosphoinositide 3-kinase and Protein Kinase B).[28,29]

In the present study, we aimed to evaluate the effects and mechanisms of two novel hybrid AChEI compounds, TAHB3 and TA8Amino, regarding cytotoxicity, neuroprotection and neurodifferentiation in PC12 cells, which are considered a suitable neuronal model for studying the cholinergic system, and an *in vitro* model system for drug testing.

Methods

Cell lines and culture conditions

The PC12 cell line (rat pheochromocytoma) was acquired from the American Type Culture Collection (ATCC). Cells were grown in culture flasks (25 cm^2) with complete culture medium, consisting of DMEM (Dulbecco's Modified Eagle Medium) supplemented with HAM F10 (1:1 DMEM/F10) (Sigma-Aldrich, St Louis, MO, USA) and 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 1% penicillin (100 units /mL) and 1% streptomycin (10 mg/mL) (Sigma) solutions. The cells were

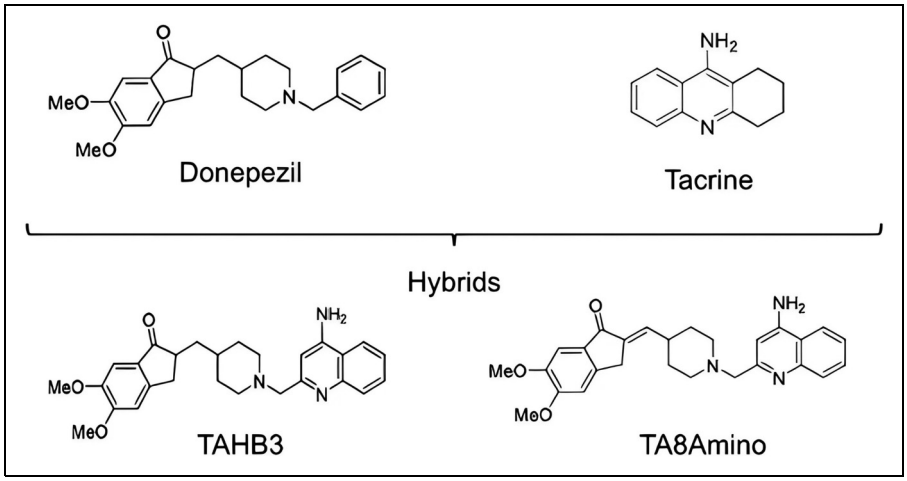

Figure 1. Representation of the molecular structures of AChEI compounds. Original compounds (donepezil and tacrine) and the new hybrid compounds, TAHB3 and TA8Amino (data extracted from Chierrito et al.).[27]

maintained in culture flasks until reaching a semi-confluent state (80%) and incubated at 37°C and 5% CO2. To carry out the experiments with the compounds, cells were detached from the flasks with trypsin-EDTA solution (0.05%) and then sub-cultured in 6 or 24-well plates, following incubation at 37°C and 5% CO2.

Differentiation of PC12 cells with nerve growth factor (NGF)

The differentiation of PC12 cells into mature neurons was achieved by using NGF 2.5S (nerve growth factor, Gibco) according to the seven days NGF (50 ng/mL) differentiation protocol of Greene and Tischler (1976),[30] with adaptations, which include the change of the medium culture from RPMI to DMEM/F10 and cells directly cultured in plastic dishes, instead of collagen-coated dishes. Cells were maintained in complete culture medium, DMEM/F10 containing 10% FBS (as described in the previous topic) and 50 ng/mL NGF for 7 days. The culture medium with NGF was changed every 48 h to carry out cell maintenance for the experiments.

Ache inhibitor compounds

The AChE inhibitor compounds were synthesized by Chierrito et al. (2017)[27] from the Medicinal Chemistry Laboratory of the Faculty of Pharmaceutical Sciences of Ribeirão Preto – USP, under the coordination of Prof[a]. Dr Ivone Carvalho. The donepezil-tacrine hybrid compounds are called TA8Amino and TAHB3 (AChE inhibitors). The compounds were initially diluted in dimethyl sulfoxide (DMSO) (Thermo-Fisher Scientific), following a second dilution in phosphate buffer solution (PBS). Subsequently, cells were treated with the AChE inhibitors using concentrations based on values of 50% AChE inhibition (IC_{50}) for TA8Amino and TAHB3, based on dose-response curves that were previously obtained in our previous studies, being 5 μM for the hybrid compounds;[29] For donepezil and tacrine, a concentration of 10 μM was used, based on the literature.[31,32]

Induction of oxidative damage

The compound used to induce oxidative damage was hydrogen peroxide (H_2O_2 – Sigma-Aldrich), which was tested at concentrations of 25, 50, 75, 100, 150, and 200 μM. We chose H_2O_2 150 μM to be used in the next experiments, since at this concentration, the cell viability was reduced by approximately 50%.

Neurodifferentiation assay in cells treated with AChEI compounds

To analyze the effects of AChEI compounds on neuronal differentiation, PC12 cells were cultured in 6-well plates (1×10^5 cells/well) and maintained in 2 mL of complete culture medium, following the treatment with TA8Amino or TAHB3 AChEI compounds (5 μM) and donepezil or tacrine (10 μM). For this assay, the same differentiation protocol established for NGF (50 ng/mL) was used, and the culture medium with AChEI drugs was changed every 48 h to maintain the treatments. NGF was used as a positive control for neuronal differentiation, while undifferentiated PC12 cells were used as a negative control. After 7 days of treatment, different cellular analyzes were performed to evaluate neurodifferentiation.

Neurodifferentiation analysis

To prove neurodifferentiation after the 7-day treatment protocol, cells were analyzed under an inverted microscope (EVOS XL Core- Invitrogen, Waltham, MA, USA); 20 images (40x magnification) were captured for each treatment. To measure the length of neurites, we used the Simple Neurite Tracer tool from the ImageJ software (Fiji, image processing package). The first step was setting a micrometer scale (μm), then, we randomly selected one neurite per cell to measure its successive points starting from the cell body until its end; the plugin use this points to generate a tracking path that determines the neurite length (μm unit). 100 neurites were measured per time-point.

To quantify the percentage of differentiated cells, 100 cells were counted per treatment, we considered as differentiated the cells that had at least one neurite longer than the diameter of the cell body; measurements were also performed using the Simple Neurite Tracer tool from ImageJ software (Fiji) as described.

Protein expression by western blotting

Protein extraction. For protein extraction the RIPA Lysis extraction buffer reagent (Thermo Fisher Scientific Inc., Waltham, MA, EUA), the samples were prepared according to the manufacturer's protocol and storage at −80°C. Protein quantification was carried out in a spectrophotometer, by comparison with a standard curve obtained for a known protein (bovine serum albumin) using the BCA kit (Pierce, Waltham, MA, USA).

Protein electrophoresis. To perform protein electrophoresis, the NuPage Bis-Tris Gelis gel (Invitrogen) was used. The molecular weight marker used was the Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific). Electrophoresis was performed at 100 V for 1.5 h in a vat (Xcel SureLock Mini Cell-Invitrogen). After electrophoresis, the proteins were transferred to a PVDF membrane (Invitrolon PVDF- Invitrogen), using the XCell II Blot Mobile system (Invitrogen). The voltage used was 25 V for 2 h, as recommended by the manufacturer. After this time, the membranes were stored in MilliQ water (ultrapure) at 4°C, following a subsequent immunostaining procedure.

Immunodetection and protein expression. For immunodetection and visualization of proteins, the Western Breeze Chromogenic kit (Invitrogen) was used and the following antibodies: Anti-rabbit β-III-tubulin (Abcam, Cambridge, UK, #ab18207) (1:1000); p-AKT (Cell Signaling Technology, #4060) (1:1000) and p-PTEN (Cell Signaling Technology, #9554) (1:1000), in addition to the endogenous anti-rabbit β-Actin (Cell Signaling Technology, #4967) (1:1000), which was used as a control for normalization. The membranes were scanned using the image system ImageQuant LAS 500 (GE Healthcare Life Sciences, Chicago, IL, USA), and the bands were quantified using the proper software for Western Blot Analysis - Image Studio program (LI-COR Biosciences GmbH, Bad Homburg, Germany); the quantification was performed from the results obtained in three independent experiments, and the values were normalized with the endogenous protein β-actin.

Analysis of neuronal markers by immunofluorescence

For the analysis of cell differentiation, PC12 cells were cultured on coverslips using a 6-well plate (1×10^6). The culture medium with the differentiation-inducing compounds was changed every two days, maintaining the treatments until 7 days. To carry out the immunofluorescence analysis, cells were fixed with methanol, permeabilized with PBS/0.2% Tween and blocked with BSA (1%); next, cells were incubated with the primary antibodies Anti-rabbit β-III-tubulin (Abcam, #ab18207) (1:500), Anti-rabbit MAP2 (Abcam, #ab183830) (1:500), anti-rabbit nestin (Abcam, #ab105389) (1:500) for 12 h at 4° C, and then incubated for 1 h with the secondary antibody Alexa fluor 488 anti-rabbit (Invitrogen, #A-11008) (1:400). Finally, cells were stained with Hoechst 33342 (0.15 μg/mL) and analyzed under a fluorescence microscope.

Cytotoxicity assay

For the analysis of cytotoxicity, PC12 cells were seeded in 24-well plates (3×10^4) and subjected to the differentiation protocol for 7 days with NGF (50 ng/mL). After obtaining mature neurons, AChEI compounds were added for 48 h, and H_2O_2 was added during the last 2 h, as a posttreatment to induce DNA damage. After these treatments, the medium was removed and a new complete DMEM/F10 medium with 10% FBS, containing NGF (50 ng/mL) was added, in which cells were maintained for 24 h (recovery time), and subsequent analyses (Figure 2).

Cell viability assay

Cytotoxicity assays were performed with the XTT-kit (Cell Proliferation Kit II – XTT, Roche Molecular Biochemicals). PC12 cells were seeded in 24-well plates (3×10^4) and treated with NGF for 7 days to achieve neuronal differentiation. Then, the neuroprotection protocol (described in the item 2.9) was followed. At the end of the steps, cells were incubated with the XTT solution, according to the

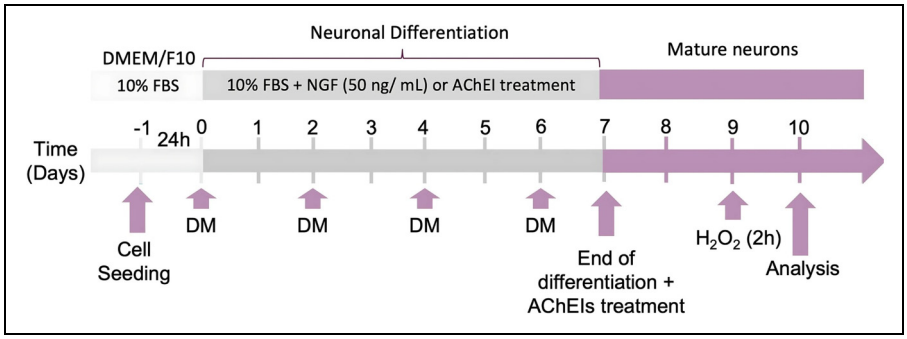

Figure 2. Protocol of differentiation and treatment with AChEI compounds. For cell viability analysis after differentiation of PC12 cells into mature neurons using NGF, cells were pre-treated with the AChEI compounds, followed by treatment with H_2O_2 for 2 h to induce DNA damage. After 24 h of recovery, cell cultures were analyzed for cell viability. To analyze the effects of hybrid compounds on neurodifferentiation, PC12 cells were treated with each AChEI compound alone, without NGF, which was used only as a positive control for neuronal differentiation. DM (Differentiation medium) containing complete DMEM/F10 culture medium + 10% FBS + 50 ng/ mL NGF.

instructions provided by the manufacturer. Absorbance was measured at 492 and 690 nm (Epoch Microplate Spectrophotometer device, BioTek, Winooski, VT, USA), obtaining results that were directly proportional to the number of viable cells for each sample.

Cell cycle kinetics analyzed by flow cytometry

To analyze cell cycle kinetics, cells were seeded and treated according to the protocol in item 2.9. On the day of collection, the culture medium was transferred to microcentrifuge tubes; the adhered cells were transferred to their respective microtubes. Then, cells were fixed in ice-cold 70% ethanol and kept at −20°C until performing flow cytometry reading. For this, samples were suspended in 200 µL of propidium iodide solution, composed of 1X PBS, propidium iodide (3 mg/ mL), Ribonuclease A (10 mg/mL), and Triton X-100 (0.2%), and the analysis was carried out on a Guava EasyCyte Mini System flow cytometer, using the Guava CytoSoft 4.2.1 Software Environment (Guava Technologies, Hayward, CA, USA); at least 5000 cells were analyzed for each sample. Values were expressed as percentages of cells distributed in each phase of the cell cycle and sub-G1 fraction.

Analysis of apoptosis and necrosis by flow cytometry

For cell death analysis, the eBioscience Annexin V Apoptosis kit (Invitrogen) was used. The cells were seeded, and treatments were carried out according to the protocol previously described in item 2.9. The samples were incubated in a solution containing annexin V, followed by suspension in a solution of 7-AAD, according to the manufacturer's instructions. The samples were then evaluated using flow cytometry, using the Guava EasyCyte Mini System equipment (Guava Technologies), with cells being analyzed using the Guava CytoSoft 4.2.1 Software Environment (Guava Technologies), and 5000 events were counted per sample. Cells undergoing apoptosis showed labeling with Annexin V and those that underwent necrosis showed positive labelling for 7-AAD, while viable cells did not show labeling for the dyes used. The values were expressed in percentages according to the marking by the dyes.

Statistical analyses

The statistical analyzes and graphs were constructed using the Prism v program. 5.0 (GraphPad software). To analyze the results of neurodifferentiation, the group treated with NGF was compared with the negative control, and the t-test was used. For the neurodifferentiation analyzes in cells treated with the four different AChEI compounds, as well as for the cytotoxicity and neuroprotection analyzes, in which only one variable between the analyzed groups was considered, such as cell viability and cell cycle kinetics, the One-Way ANOVA test with Dunnet post-test. For the cell death assay, two variables (death by apoptosis and necrosis) were analyzed between the different treatment groups, using the Two-Way ANOVA test with Dunnet post-test. The Dunnet post-test was used since it considers the significance between each treatment against the negative control. For all assays, a p-value of 0.05 was considered as a limit for significance. Data were expressed as mean and standard error (± SE). Before the application of the specific significance tests, all the results passed the Shapiro-Wilk test for normality.

Results

Characterization of PC12 differentiation into mature neurons

Morphological alterations. Neuronal differentiation was achieved by using NGF (50 ng/ mL) over a period of 7

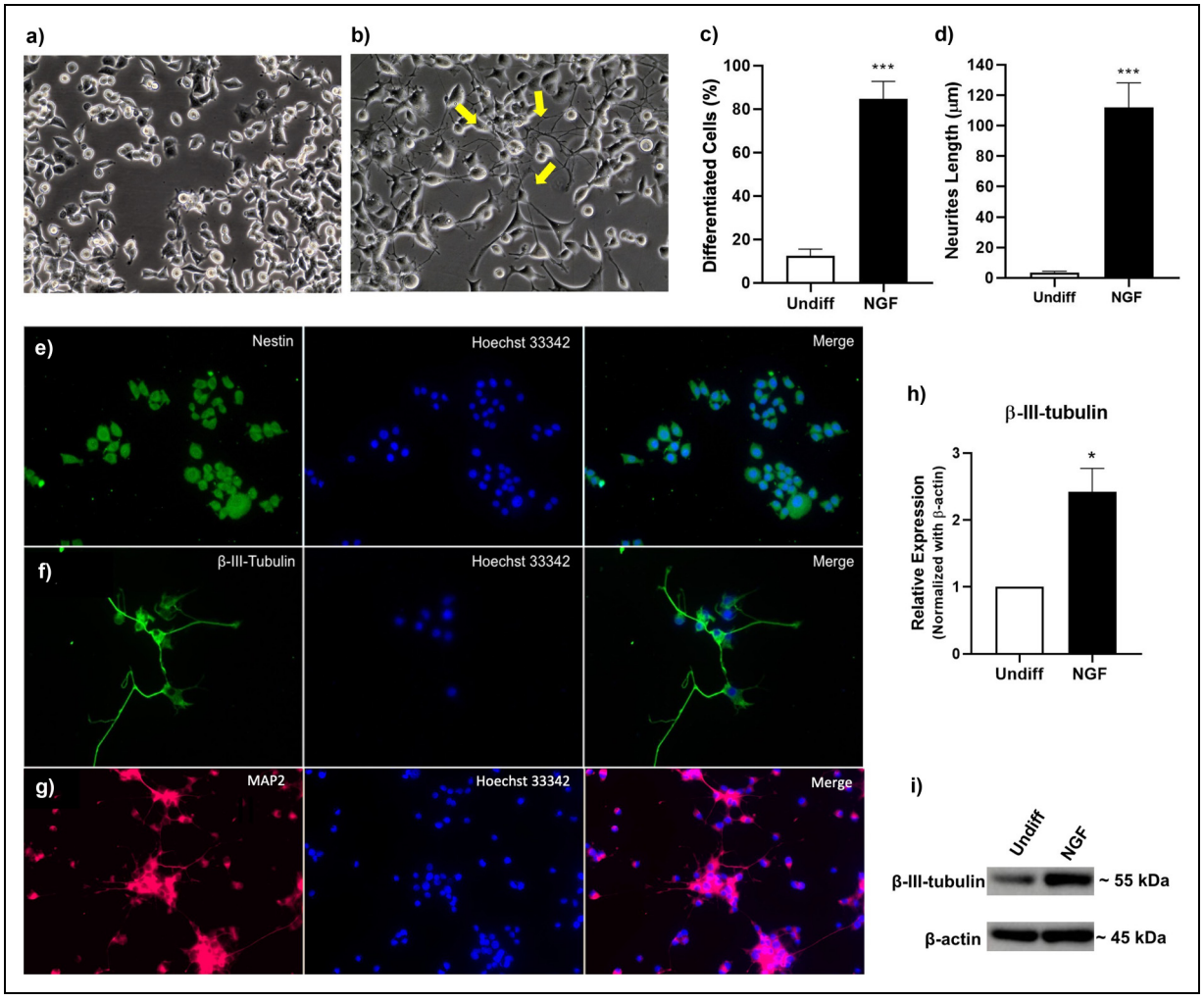

Figure 3. Neurodifferentiation of PC12 cells. (a) Cells were incubated with DMEM/F10 medium + 10% FBS for 7 days, or (b) in the presence of NGF 50 ng/mL at similar conditions. The morphological changes associated with neurodifferentiation were observed under an inverted microscope (EVOS XL Core- Invitrogen; 40× magnification). (c) Percentages of differentiated cells after NGF treatment compared to the negative control (d) Analysis of neurite length (µm) after NGF treatment compared to the negative control; 100 cells were randomly analyzed using the ImageJ software (Fiji). The results were obtained from three independent experiments, and the statistical analysis was performed using the t-test. Values expressed as mean ± SE. ***$p < 0.001$ indicates statistically significant differences between NGF-treated and untreated (undifferentiated) cells. (e) Undifferentiated PC12 cells labeled with anti-Nestin and Hoechst 33342 staining. (f) Differentiated PC12 cells into neurons, showing a positive labeling for anti-β-III-tubulin and Hoechst 33342. (g) PC12 differentiated neurons showing a positive labeling for anti-MAP2 and Hoechst 33342. Morphological changes were observed at 20× magnification using a fluorescence microscope (Zeiss, Axio Imager). (h) Expression levels of β-III-tubulin in undifferentiated and differentiated cells. Values were calculated by normalization with the endogenous β-actin protein expression, using the Image Studio Lite V. 5.0 software (Lite Software) Three independent experiments were carried out, and the statistical analysis was performed by the t-test. (i) Expression of β-III-tubulin (~55 kDa) and β-actin (~45 kDa) proteins analyzed by western blot, before and after neuronal differentiation. Data are expressed as mean ± SE. *$p < 0.05$ indicates a statistically significant difference for the comparison between differentiated and undifferentiated cells.

days. After treatment, cells started to show morphological changes, such as neurite growth (yellow arrows), reduction of the cytoplasm, and a decrease in the number of dividing cells (Figure 3(b)); all these alterations were not observed in undifferentiated PC12 cells (negative control) (Figure 3(a)). We observed a significant increase ($p < 0.001$) in the percentage of differentiated cells after NGF treatment for 7 days, 84.6% of differentiated cells, compared to 12% presented by undifferentiated cells (control group, without NGF) (Figure 3(c)). Regarding the neurite length, after treatment with NGF, cells significantly increased ($p < 0.001$) the average length (111.9 µm) of their neurites, compared to 3.5 µm observed in undifferentiated cells (Figure 3(d)). These results demonstrate that NGF can

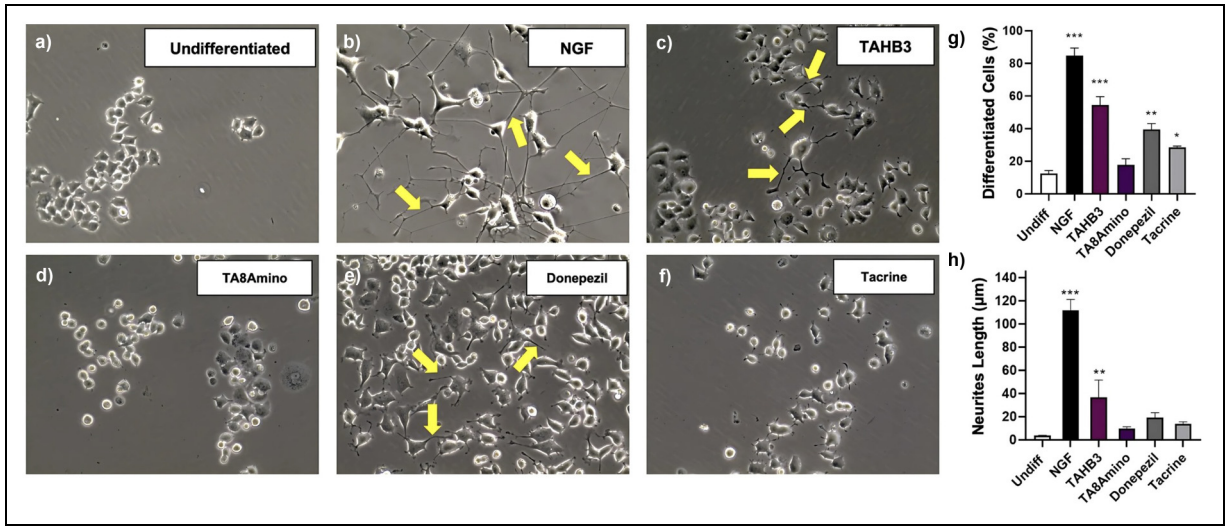

Figure 4. PC12 cells after 7 days of treatment with AChEI compounds. (a) Undiff (Undifferentiated PC12 cells- negative control); (b) Treatment with 50 ng/mL NGF (Positive control); (c) Treatment with TAHB3 (5 μM); (d) TA8Amino treatment (5 μM); (e) Donepezil treatment (10 μM); (f) Tacrine treatment (10 μM). The morphological changes were associated with neuronal differentiation, such as the formation of neurites (indicated by arrows) were observed under an inverted microscope (EVOS XL Core- Invitrogen), at 40× magnification. (g) Percentage of neurodifferentiated cells after treatment with AChEI; 100 cells were analyzed per treatment; Cells that showed at least one neurite with length greater than the diameter of the cell body were considered as neurodifferentiated. (h) The length of neurites was measured in micrometers (μm) for 100 cells evaluated per treatment, using the Simple Neurite Tracer tool in ImageJ software (Fiji app).

induce morphological changes associated with neuronal differentiation in PC12 cells.

Expression of neuron-specific proteins. After 7 days of treatment with NGF (50 ng/mL) we analyzed the expression of neuron-specific markers, through an immunofluorescence assay. Undifferentiated PC12 cells were labeled with the anti-Nestin antibody, a marker of neuronal progenitor cells (Figure 3(e)), while differentiated PC12 cells were labeled with anti-β-III-tubulin and anti-MAP2 antibodies, which are neuron-specific proteins present in the cytoplasm and neuronal axons. Thus, we demonstrated that cells collected after 7 days of NGF treatment showed a positive labeling for β-III-tubulin and MAP2 (Figure 3(f) and (g)). The quantification of β-III-tubulin expression levels demonstrated a two-fold increase ($p < 0.01$) after NGF treatment when compared to undifferentiated PC12 cells, thus confirming the differentiation of PC12 cells into mature neurons (Figure 3(h) and (i)).

Novel hybrid TAHB3 induces PC12 neurodifferentiation

To check whether the AChEI hybrid compounds would induce neurodifferentiation of PC12 cells, cells were treated with each of the compounds alone for seven days; NGF was used only as a positive control for neurodifferentiation. We observed morphological changes that are typical of neurons, such as reduction of cytoplasm content, neurite formation, interconnections between neurons, and reduction of cell proliferation after treatments with the positive control (NGF) and the TAHB3 hybrid compound (Figure 4(a)-(f)).

Each cell was considered as differentiated when presenting one neurite with length longer than the cell body. The negative control (undifferentiated PC12 cells) showed 12% of differentiated cells, NGF treated cells, exhibited 81% of differentiated neurons. For the experiments with AChEI, TAHB3 significantly increased ($p < 0.001$) the differentiation rate (50% increase); Donepezil and tacrine induced respectively 37.5% ($p < 0.01$) and 28.3% ($p < 0.05$) of differentiated neurons, while TA8Amino did not cause a significant increase in the percentage of neurons (Figure 4(g)). Regarding measurements of neurite length, the negative control presented a mean neurite length of 3.57 μm. Cells treated with NGF suffered a significant increase in neuritogenesis, showing neurite length = 111.9 μm ($p < 0.001$); TAHB3 was the only AChEI compound that significantly increased the neurite length, 36.7 μm ($p < 0.01$) (Figure 4(h)).

Since we demonstrated that TAHB3 induced morphological changes associated with neuronal differentiation, we analyzed the expression of the neuronal marker β-III-tubulin for all group treatments. Cells treated with NGF and AChEI compounds were found positively stained for β-III-tubulin after 7 days, as analyzed by fluorescence microscopy (Figure 5(a)-(f));

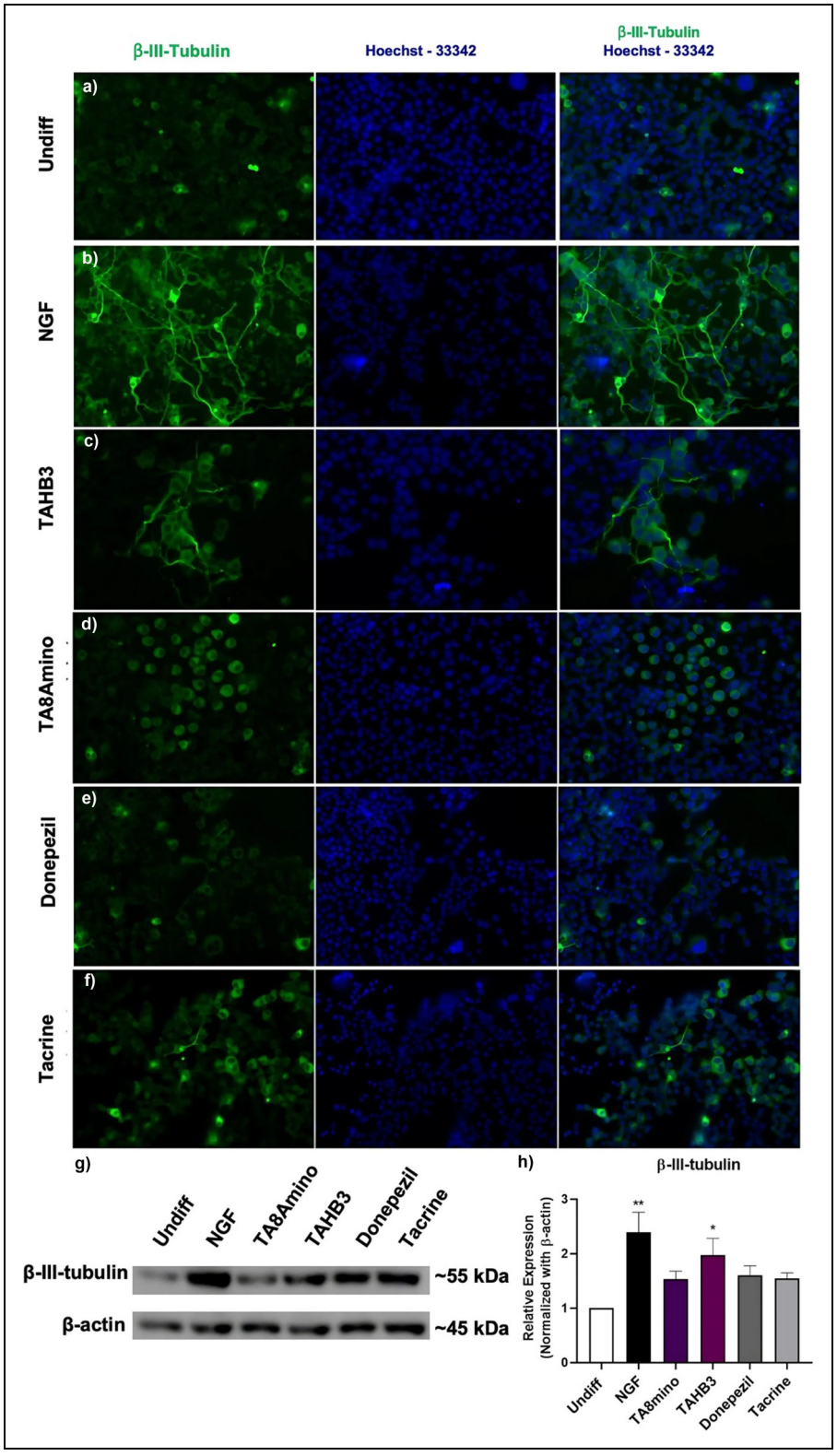

Figure 5. Expression of the neuronal marker β-III-tubulin in PC12 cells treated with AChEI compounds. (a–f) labeling with β-III-tubulin, and nuclear Hoechst-33342 staining. Morphological changes were observed at 20× magnification using a Zeiss fluorescence microscope (Axio Imager). (g) Effects of the AChEI compounds on the expression of β-III-tubulin and the endogenous protein β-actin. Treatments: Cells were incubated in DMEM/F10 culture medium + 10% SBF (negative control: undifferentiated cells); NGF 50 ng/mL (positive control); compound TAHB3 (5 μM); TA8Amino (5 μM); donepezil (10 μM); tacrine (10 μM). Three independent experiments were carried out, with statistical analysis performed using the One-way ANOVA test, with Dunnet's post-test. Data are expressed as mean ± SE. *$p < 0.05$; **$p < 0.01$ indicate statistically significant differences when results were compared with the negative control.

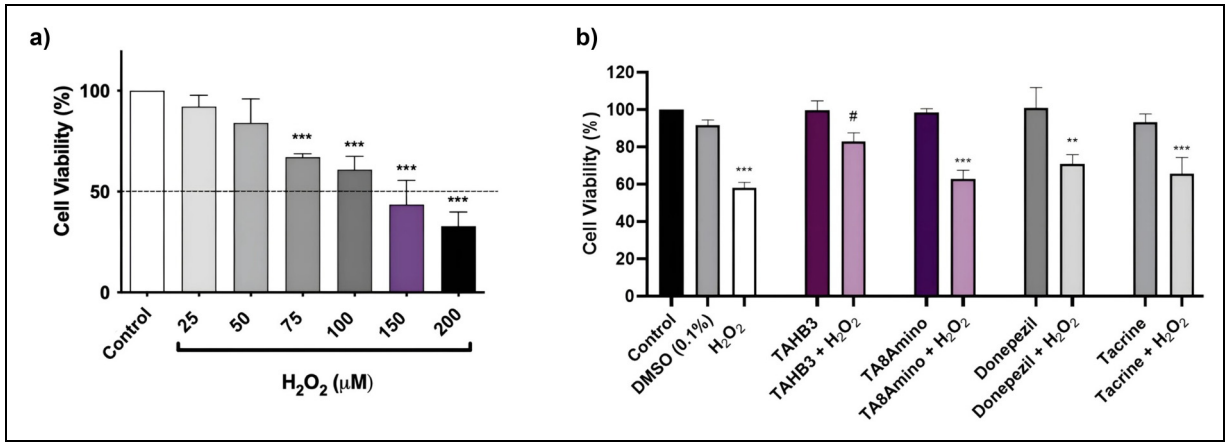

Figure 6. Cell viability analyses. The assay was carried out using the XTT-kit (Cell Proliferation Kit II – XTT, Roche Molecular Biochemicals) and analyzed by spectrophotometry (Epoch Microplate Spectrophotometer device, BioTek). (a) H_2O_2 action curve, cells were cultured in DMEM/F10 culture medium + 10% FBS (negative control) and different concentrations of hydrogen peroxide (H_2O_2) were tested. The results were obtained from three independent experiments. (b) Cytotoxicity and neuroprotection analysis of AChEI in neurodifferentiated PC12 Cells. Percentages of viable PC12 cells after neuronal differentiation, obtained in cytotoxicity and neuroprotection assays against damage induced by H_2O_2. The results were obtained from four independent experiments and are expressed as mean ± SE. One-Way Anova statistical analysis, with Dunnet post-test. $^{**}p < 0.005$, $^{***}p < 0.001$ indicates statistically significant differences compared to the negative control. $^{\#}p < 0.05$ indicates a statistically significant difference compared to the positive control. Cells were cultured in DMEM/F10 culture medium + 10% FBS (negative control); Hydrogen peroxide (H_2O_2, positive control); Dimethyl sulfoxide (DMSO 0.1%, solvent control); compound TAHB3 (5 μM); TA8Amino (5 μM); donepezil (10 μM); tacrine (10 μM).

morphological changes were also observed in cells treated with NGF and TAHB3 (Figure 5(b) and (c)). NGF-treated cells showed a two-fold significant increase ($p < 0.01$) in β-III-tubulin protein expression, and similar result ($p < 0.05$) was found for TAHB3-treated cells. However, the other AChEI drugs did not induce significant differences regarding β-III-tubulin expression ((Figure 5(g) and (h)).

TAHB3 was not cytotoxic to PC12-differentiated neurons and exhibited neuroprotective potential against oxidative damage induced by H_2O_2

The cell viability assay was performed in PC12-differentiated neurons, which were treated with the AChEI for 48 h. The results showed that none of the AChEI compounds alone reduced cell viability; the compounds were not cytotoxic to differentiated PC12 cells under the concentrations and conditions of our experiments (Figure 6).

To test the neuroprotective effects of the compounds, after the differentiation and 48 h treatment with the AChEI drugs, cells were treated with H_2O_2 for 2 h. First, we tested different concentrations of H_2O_2 alone, aiming to use the one that reduced cell viability closer to 50%, therefore the concentration of 150 μM, which reduced cell viability to 43%, was chosen to induce damage in the following neuroprotection analysis (Figure 6(a)). It was observed that H_2O_2 alone significantly ($p < 0.001$) reduced the cell viability to 58%, while cells pre-treated with TAHB3 followed by H_2O_2 showed 82% of viability, and a significant ($p < 0.05$) difference when compared to the H_2O_2 single treatment. In opposite, cells treated with TA8Amino, or donepezil or tacrine, followed by H_2O_2 treatment, showed 63%, 70%, and 65% of cell viability, respectively. These results demonstrate a neuroprotective potential of TAHB3 against H_2O_2-induced damage (Figure 6(b)).

TAHB3 did not induce cell death or changes in cell cycle kinetics in PC12-differentiated neurons

Percentages of cell death (apoptosis or necrosis) and cell cycle kinetics were analyzed by flow cytometry assays after differentiation of PC12 cells into neurons, and a later treatment with AChEI compounds and H_2O_2. The treatments with each AChEI compound tested alone, or combined with H_2O_2, did not significantly increase cell death levels by apoptosis or necrosis (Figure 7(a)). It was also found a lack of alterations in the distribution of cells at cell cycle phases. These results demonstrate that none of the AChEI compounds were toxic to PC12-differentiated neurons, either in the presence or absence of induced-oxidative stress damage (Figure 7(b)).

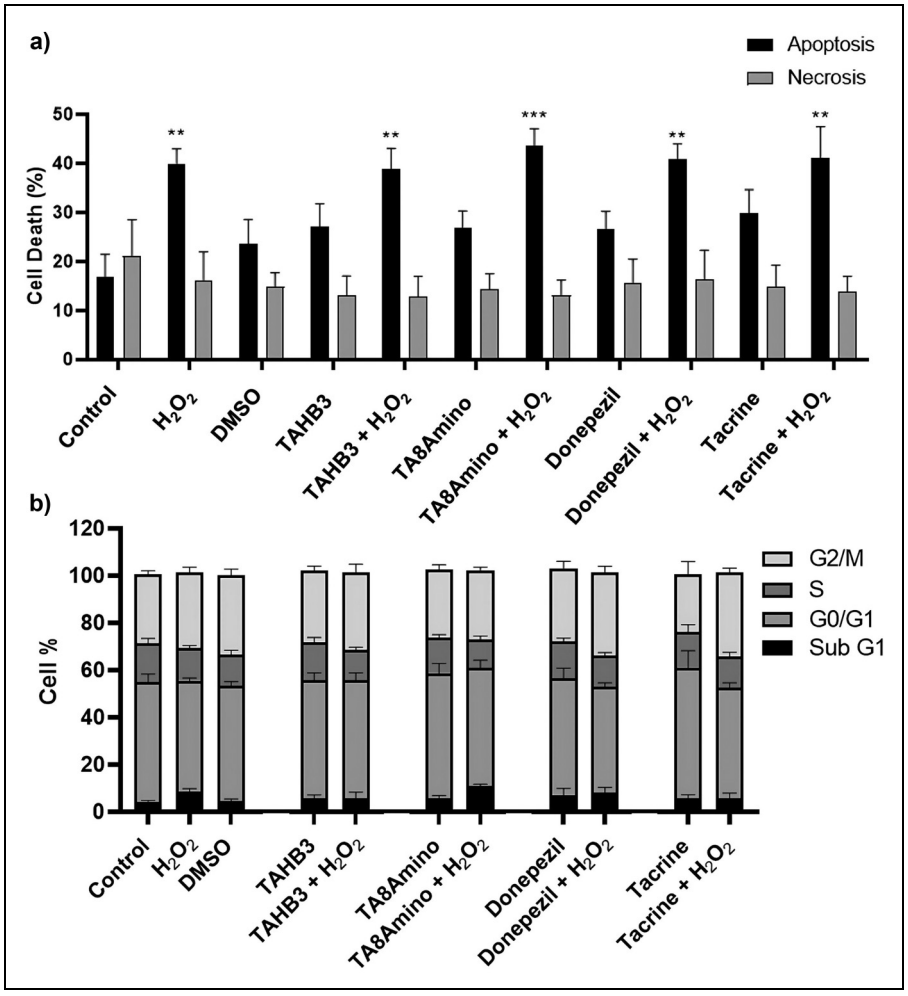

Figure 7. Flow cytometric assays performed for the analysis of cell death by apoptosis and necrosis (a) and cell cycle kinetics (b) in cells submitted to oxidative damage induced by H_2O_2 (150 μM) and under treatment with AChEI compounds. The analysis of cell death was performed using the Guava Nexin kit (Guava Technologies), the values were obtained from four independent experiments. For cell cycle kinetics, the analysis was performed by the Guava Cell Cycle software (Guava technologies) using propidium iodide for DNA labeling; the values were obtained from three independent experiments. Statistical analysis: For cell death analysis, we applied the Two-Way ANOVA with Dunnet post-test; for cell cycle kinetics, the results were analyzed by One-Way ANOVA with Dunnet post-test. Values expressed as mean ± SE. $^{**}p < 0.005$, $^{***}p < 0.001$ indicates statistically significant differences compared to the negative control. Cells were cultured in DMEM/F10 culture medium + 10% FBS (negative control); Hydrogen peroxide (H_2O_2, positive control); Dimethyl sulfoxide (DMSO 0.1%, solvent control); compound TAHB3 (5 μM); TA8Amino (5 μM); donepezil (10 μM); tacrine (10 μM).

TAHB3 increases the activation of PI3K/AKT pathway

Aiming to investigate the involvement of the PI3K/AKT pathway in cell responses to drug treatments, after seven days of treatment with AChEI compounds, we analyzed the phospho-PTEN and phospho-AKT expression, which would indicate the status of PI3K/AKT activation. Our results showed that TAHB3 significantly ($p < 0.05$) increased the levels of phospho-AKT (Figure 8(a) and (c)), compared to undifferentiated cells, and demonstrated a tendency to increase the phospho-PTEN expression (Figure 8(b) and (c)), thus suggesting that TAHB3 induces the activation of this pathway.

Discussion

AD is a major health problem worldwide and still requires novel efficient therapies that can act on multiple targets involved in the development of the disease. In our work, we tested two novel hybrid AChEI compounds, TA8Amino and TAHB3, which were synthetized from donepezil and tacrine. Our results demonstrated that TAHB3 could induce neurodifferentiation of PC12 cells, it also showed a neuroprotective potential against oxidative damage. Both compounds were not cytotoxic to PC12 neurons, either in the presence or absence of induced-oxidative stress. These results are relevant since TAHB3 displayed better results for all parameters,

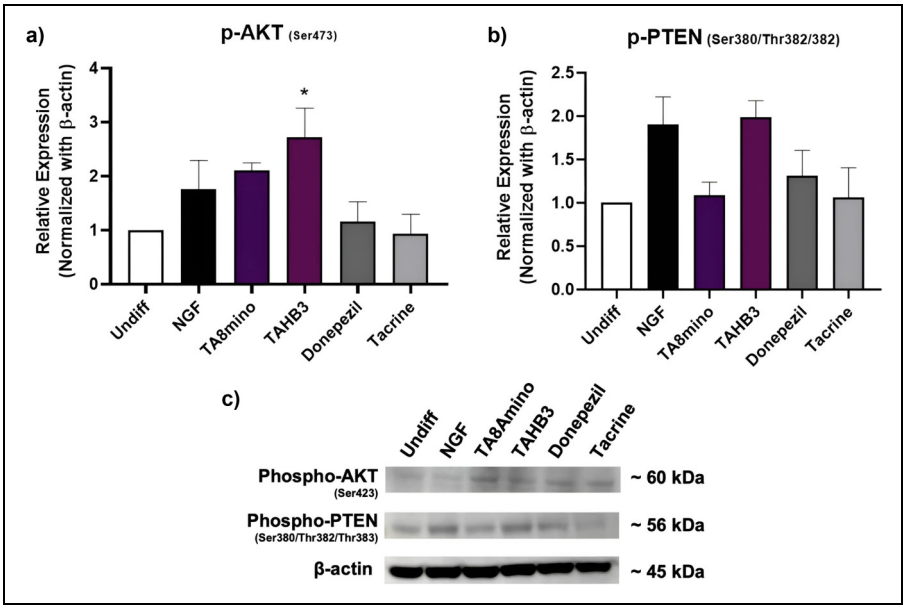

Figure 8. Expression of PI3K/AKT proteins after treatment with AChEI compounds. (a) Effects of AChEI in the expression of p-AKT, and endogenous control (β-actin). (b) p-PTEN expression levels after 7 days of treatment were calculated by normalizing the values against the endogenous β-actin protein expression, using the Image Studio Lite Ver 5.0 software (Lite Software). Three independent experiments were carried out, with statistical analysis performed using the One-way ANOVA test, and Dunnet's post-test. Data are expressed as mean ± SE. *$p < 0.05$; **$p < 0.01$ indicates statistically significant differences when results were compared to the negative control. (c) Effects of AChEI treatments: Undiff (Undifferentiated PC12 cells - negative control); NGF 50 ng/mL (positive control); compound TAHB3 (5 μM); TA8Amino (5 μM); donepezil (10 μM); tacrine (10 μM).

compared to the original compounds. Considering that donepezil is currently the main drug treatment for patients in the initial stage of AD, this study provides a novel potential strategy to be explored in the search for novel therapies for AD.

Neuronal differentiation is an important approach to be evaluated aiming to searching for new drug candidates for the treatment of AD.[33,34] In fact, it has been demonstrated that donepezil can induce neurogenesis in animal models for different neurodegenerative diseases, which could be related to the activation of downstream pathways from ACh receptors.[35,36] Regarding the effects of tacrine on neuronal differentiation, some authors reported that tacrine inhibits neurite outgrowth in primary cultured cortical cells,[37] while others described that the same compound induced neurogenesis in rat cortical cells.[38] Studies with hybrid compounds have demonstrated that a novel donepezil-flavonoid hybrid presented neurogenic properties, being able to induce maturation of rat stem cells with neuronal-like phenotype, with increased expression of β-III-tubulin, but the authors did not compare the effects between the hybrid compound and donepezil.[39]

Under the conditions of the present study, donepezil and tacrine tested alone showed a tendency to increase neurite development, but these compounds did not significantly induce neuronal differentiation. On the other hand, the hybrid compound TAHB3 induced neuritogenesis, being observed the development of neurites, as well as neuronal differentiation, which was confirmed by the expression of the neuronal marker β-III-tubulin, showing a greater efficacy on neurodifferentiation than the original compounds. These results suggest that probably, the mechanisms might be related to the activation of the PI3K/AKT pathway, since TAHB3 induced a significant increase in the activation of phospho-AKT, a key component of this pathway, culminating in the neurodifferentiation process.

There is evidence that PI3K/AKT pathway plays important roles in the central nervous system, being associated with processes such as neuronal survival, differentiation, autophagy, neurodifferentiation, neurogenesis and neuronal plasticity.[40,41] In vitro studies with donepezil have shown that its neuroprotective effects may be directly related with the activation of the PI3K/AKT pathway.[32,42] It has also been demonstrated that the activation of PI3K/AKT pathway leads to the activation of neuron-specific transcription factors, culminating in the neurodifferentiation process.[43,44] Furthermore, there is evidence that when this pathway is blocked, the above-mentioned processes are impaired in neuronal cells and AD models.[28,32,45]

In previous studies performed by our research group using the SH-SY5Y cell line, we found a greater potential of TA8Amino to induce neurodifferentiation through the

activation of PI3K/AKT pathway, compared to TAHB3, donepezil and tacrine.[28,29] Most probably, this discrepancy observed between the results obtained in SH-SY5Y cells and those from the present study might be due to the intrinsic difference between the two cell lines, and can be related to the chemical interaction between each AChEI compound (TAHB3 or TA8Amino) with the enzyme (AChE) sites, either in PC12 cells (derived from rats), or SH-SY5Y (human cells). This assumption can be supported by the fact that the conformation of AChE differs between humans and rodents, also taking into consideration that this enzyme potentially has several structural variants that diverge among different species.[46,47] Therefore, the difference observed in the results obtained in human and rat models highlights the importance of testing new compounds in different in vitro cellular models, considering that genetic variations between species can generate different results regarding the effects of these compounds.

Another aspect analyzed in the present study refers to the cytotoxicity of the new hybrid compounds TAHB3 and TA8Amino, in the presence or absence of oxidative stress. The hybrid compounds did not reduce cell viability compared to the negative control, indicating that the new hybrid compounds are not toxic to neurons derived from PC12 cells. Furthermore, the results obtained in the cell cycle kinetics and cell death assays also demonstrate that none of the AChEI compounds induced changes in the cell cycle kinetics, nor increased cell death by apoptosis or necrosis. In our previous study, TAHB3 and TA8Amino were not cytotoxic to SH-SY5Y cells when tested at different concentrations of 0.71 μM and 0.014 μM respectively, and at 5 μM.[28,29] Therefore, the results obtained in the present work corroborate with these previous data in another widely used neuronal in vitro model.

Regarding the neuroprotection potential of the novel AChEI, the results obtained in the cell viability assay demonstrated the potential of TAHB3 to protect neurons against damage induced by H_2O_2, which was not observed in treatments with the other compounds. Our results also indicate that the hybrid compound TAHB3 showed a greater neuroprotective potential for PC12 cells, than the original compounds donepezil and tacrine.

Other studies carried out with different methods of hybridization (such as those involving the synthesis from donepezil and butylated hydroxytoluene) also demonstrated a neuroprotective effect on PC12 cells against damage induced by H_2O_2, when tested at concentrations of 7.5 μM and 10 μM,[48] which are higher than those tested for the hybrid compounds, TAHB3 and TA8Amino, by reducing intracellular ROS levels and apoptosis, but its mechanisms of action were not explored by the authors. The research reported by Estrada-Valencia (2018)[39] with donepezil-flavonoid compounds also showed the neuroprotective potential of novel hybrids against oxidative stress, suggesting the activation of endogenous antioxidant responses.

The neuroprotective effects of donepezil can be related to the PI3K/AKT pahthway,[32,42] since it is known that this pathway has an important role in the antioxidant response, probably due to the NRF2 regulation.[49,50] In the study reported by Estrada-Valencia (2018)[39] the authors did not evaluate the effects of donepezil-flavonoid hybrids on PI3K/AKT pathway activation. Differently, in the present work, we observed that the hybrid (donepezil-tacrine) TAHB3 could induce this pathway, suggesting a consequent increase in the antioxidant response. Therefore, our results also demonstrate the potential of donepezil as a relevant component in the research with hybrid AChEI compounds designed for the treatment of patients with AD.

Our study provided relevant information about novel donepezil-tacrine hybrids TAHB3 and TA8Amino regarding their antioxidant effects and capacity to induce neurodifferentiation. But challenges remain, considering the multifactorial aspect of AD, the neuroprotective effects of those drugs still need to be tested against other neurotoxic agents involved in AD development, such as tau-hyperphosphorylation, Aβ toxicity, and neuroinflammation, also to clarify their mechanisms of action. This is the third study to analyze the effects of the novel compounds TAHB3 and TA8Amino; it certainly has limitations, considering that PC12 cells are derived from rats, requiring additional studies in human cell models that would be a closer representation of the human brain, such as human induced pluripotent stem cells (IPSCs) derived 3D organoids; this model would benefit the advance in the knowledge with those compounds, especially when we consider the difference between animal and human models, as we could observe in the present work. Our research group is already working with 2D and 3D neural models derived from IPSCs, aiming to obtain a more accurate information in the field of AD pathophysiology and therapeutic approaches.

In addition, the experiments were performed in *in vitro* conditions, which is only the first stage of the trial of novel potential drugs and does not fully represent the effects of those compounds on humans. We cannot rule out the need for in vivo studies to better characterize the effects of hybrid compounds in mammalian systems. However even if the compounds can show some efficacy in all these models, there would still be a major challenge for clinical trials since most AD drugs are not approved for further use.[23] The models available to study potential new drug candidates for AD still show limitations to provide reliable information for real patients, taking into account the complexity of the human body, especially the brain, and also inter-individual variations between patients, different genetic backgrounds, physical and environmental conditions of each patient and the complexity of the AD disease.[51] However, despite all the limitations, we have

expectations about the development of technologies in the development of more accurate drugs and models to study AD, seeking and increasing the quality of information generated about AD and new treatments, which can be trusted to contribute to the development of more effective treatments for AD.

Conclusions

Thus, the results obtained in the present study showed that the new hybrid compounds TAHB3 and TA8Amino were not cytotoxic to PC12 cells, they did not alter cell cycle progression, nor induced cell death under the conditions of induced oxidative stress. The compound TAHB3 showed neuroprotective potential in response to oxidative damage induced by H_2O_2, as well as ability to induce differentiation in PC12 cells, most probably through the activation of PI3K/AKT pathway. Our results demonstrate the potential of the research with hybrid compounds as novel candidates for AD treatment, since the hybrid compounds exhibited more relevant effects for all parameters evaluated when compared to the original compounds (donepezil and tacrine). This study potentially contributes as support for the characterization of biological properties of the hybrid compounds, which has been designed aiming future therapeutic applications for AD. Future directions of the study with the novel hybrids TAHB3 and TA8Amino would include testing the neuroprotective effects of those compounds against different neurotoxic agents associated with AD pathology. Besides, it can be valuable the evaluation of their effects in human AD models, such as IPSC-derived neuronal 2D and 3D cultures and in vivo models, aiming to confirm the findings, and provide reliable data regarding the mechanisms of action.

Acknowledgments

We would like to thank Dr Talita Perez Chierrito for the partnership by the donation of the hybrid compounds.

ORCID iDs

Renata Melo dos Santos Ono https://orcid.org/0000-0002-3363-2253

Elza Tiemi Sakamoto-Hojo https://orcid.org/0000-0002-1383-3314

Statements and declarations

Author contributions

Renata Melo dos Santos Ono (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing – original draft); Natália Chermont dos Santos Moreira (Conceptualization; Investigation; Methodology; Validation; Writing – review & editing); Ivone Carvalho (Resources); Geraldo Aleixo Passos (Resources); Elza Tiemi Sakamoto-Hojo, PhD (Conceptualization; Resources; Supervision; Writing – review & editing)

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The present research received funding from the Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (Proc. 2017/15123-1 e 2018/21709-1), Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brasil – CNPq (Proc. 309854/2017-2, 142223/2019-0 e 311533/2021-3), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil - CAPES (Finance code 001).

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability

All data presented in this study are available, to any qualified researcher, from the authors upon reasonable request.

References

1. Masters CL, Bateman R, Blennow K, et al. Alzheimer's disease. *Nat Rev Dis Primers* 2015; 1: 15056.
2. Ferrari C and Sorbi S. The complexity of Alzheimer's disease: an evolving puzzle. *Physiol Rev* 2021; 10: 1047–1081.
3. Newcombe EA, Camats-Perna J, Silva ML, et al. Inflammation: the link between comorbidities, genetics, and Alzheimer's disease. *J Neuroinflammation* 2018; 15: 276.
4. Tönnies E and Trushina E. Oxidative stress, synaptic dysfunction, and Alzheimer's disease. *J Alzheimers Dis* 2017; 57: 1105–1121.
5. Alzheimer's Disease International. *World Alzheimer Report 2021: Journey through the diagnosis of Dementia*. London: Alzheimer's Disease International, 2021.
6. Vesey R, Birrell JM, Bolton C, et al. Cholinergic nicotinic systems in Alzheimer's disease: prospects for pharmacological intervention. *CNS Drugs* 2022; 16: 485–500.
7. Grossberg GT. Cholinesterase inhibitors for the treatment of Alzheimer's disease: getting on and staying on. *Curr Ther Res Clin Exp* 2003; 64: 216–235.
8. Camps P, Formosa X, Galdeano C, et al. Tacrine-based dual binding site acetylcholinesterase inhibitors as potential disease-modifying anti-Alzheimer drug candidates. *Chem Biol Interact* 2010; 187: 411–415.
9. Watkins PB, Zimmerman HJ, Knapp MJ, et al. Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. *JAMA* 1994; 271: 992–998.
10. Shao D, Zou C, Luo C, et al. Synthesis and evaluation of tacrine-E2020 hybrids as acetylcholinesterase inhibitors for the treatment of Alzheimer's disease. *Bioorg Med Chem Lett* 2004; 14: 4639–4642.

11. McShane R, Westby MJ, Roberts E, et al. Memantine for dementia. *Cochrane Database Syst Rev* 2019; 3: CD003154.
12. Long JM and Holtzman DM. Alzheimer disease: an update on pathobiology and treatment strategies. *Cell* 2019; 179: 312–339.
13. Fracassi A, Marcatti M, Zolochevska O, et al. Oxidative damage and antioxidant response in frontal cortex of demented and nondemented individuals with Alzheimer's neuropathology. *J Neurosci* 2021; 41: 538–554.
14. Ionescu-Tucker A and Cotman CW. Emerging roles of oxidative stress in brain aging and Alzheimer's disease. *Neurobiol Aging* 2021; 107: 86–95.
15. Bai R, Guo J, Ye XY, et al. Oxidative stress: the core pathogenesis and mechanism of Alzheimer's disease. *Ageing Res Rev* 2022; 77: 101619.
16. Choi SH, Bylykbashi E, Chatila ZK, et al. Combined adult neurogenesis and BDNF mimic exercise effects on cognition in an Alzheimer's mouse model. *Science* 2018; 361: eaan8821.
17. Walgrave H, Balusu S, Snoeck S, et al. Restoring miR-132 expression rescues adult hippocampal neurogenesis and memory deficits in Alzheimer's disease. *Cell Stem Cell* 2021; 28: 1805–1821.
18. Kaneko N and Sawamoto K. Adult neurogenesis and its alteration under pathological conditions. *Neurosci Res* 2009; 63: 155–164.
19. Kaneko N, Okano H and Sawamoto K. Role of the cholinergic system in regulating survival of newborn neurons in the adult mouse dentate gyrus and olfactory bulb. *Genes Cells* 2006; 11: 1145–1159.
20. Imamura O, Arai M, Dateki M, et al. Donepezil-induced oligodendrocyte differentiation is mediated through estrogen receptors. *J Neurochem* 2020; 155: 494–507.
21. Jana A, Bhattacharjee A, Das SS, et al. Molecular insights into therapeutic potentials of hybrid compounds targeting Alzheimer's disease. *Mol Neurobiol* 2022; 59: 3512–3528.
22. Gulcan HO and Kosar M. The hybrid compounds as multitarget ligands for the treatment of Alzheimer's disease: considerations on donepezil. *Curr Top Med Chem* 2022; 22: 395–407.
23. Zhang J, Zhang Y, Wang J, et al. Recent advances in Alzheimer's disease: mechanisms, clinical trials and new drug development strategies. *Signal Transduct Target Ther* 2024; 23: 211.
24. Viegas-Junior C, Danuello A, da Silva Bolzani V, et al. Molecular hybridization: a useful tool in the design of new drug prototypes. *Curr Med Chem* 2007; 14: 1829–1852.
25. Zang WB, Wei HL, Zhang WW, et al. Curcumin hybrid molecules for the treatment of Alzheimer's disease: structure and pharmacological activities. *Eur J Med Chem* 2024; 265: 116070.
26. Rodrigues Simões MC, Dias Viegas FP, Moreira MS, et al. Donepezil: an important prototype to the design of new drug candidates for Alzheimer's disease. *Mini Rev Med Chem* 2014; 14: 2–19.
27. Chierrito TPC, Pedersoli-Mantoani S, Roca C, et al. From dual binding site acetylcholinesterase inhibitors to allosteric modulators: a new avenue for disease-modifying drugs in Alzheimer's disease. *Eur J Med Chem* 2017; 139: 773–791.
28. Moreira NCDS, Lima JEBF, Chierrito TPC, et al. Novel hybrid acetylcholinesterase inhibitors induce differentiation and neuritogenesis in neuronal cells in vitro through activation of the AKT pathway. *J Alzheimers Dis* 2020; 78: 353–370.
29. Moreira NCDS, Tamarozzi ER, Lima JEBF, et al. Novel dual AChE and ROCK2 inhibitor induces neurogenesis via PTEN/AKT pathway in Alzheimer's disease model. *Int J Mol Sci* 2022; 23: 14788.
30. Greene LA and Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A* 1976; 73: 2424–2428.
31. Taheri R, Hadipour E and Tayarani-Najaran Z. Crocin protects against beta-amyloid peptide-induced apoptosis in PC12 cells via the PI3K pathway. *Curr Mol Pharmacol* 2021; 14: 627–634.
32. Noh MY, Koh SH, Kim Y, et al. Neuroprotective effects of donepezil through inhibition of GSK-3 activity in amyloid-beta-induced neuronal cell death. *J Neurochem* 2009; 108: 1116–1125.
33. Sung PS, Lin PY, Liu CH, et al. Neuroinflammation and neurogenesis in Alzheimer's disease and potential therapeutic approaches. *Int J Mol Sci* 2020; 21: 701.
34. Gao J and Li L. Enhancement of neural regeneration as a therapeutic strategy for Alzheimer's disease (review). *Exp Ther Med* 2023; 26: 444.
35. Kwon KJ, Kim MK, Lee EJ, et al. Effects of donepezil, an acetylcholinesterase inhibitor, on neurogenesis in a rat model of vascular dementia. *J Neurol Sci* 2014; 347: 66–77.
36. Man J, Cui K, Fu X, et al. Donepezil promotes neurogenesis via Src signaling pathway in a rat model of chronic cerebral hypoperfusion. *Brain Res* 2020; 1736: 146782.
37. Page M, Pacico N, Ourtioualous S, et al. Procognitive compounds promote neurite outgrowth. *Pharmacology* 2015; 96: 131–136.
38. Jin K, Xie L, Mao XO, et al. Alzheimer's disease drugs promote neurogenesis. *Brain Res* 2006; 1085: 183–188.
39. Estrada-Valencia M, Herrera-Arozamena C, de Andrés L, et al. Neurogenic and neuroprotective donepezil-flavonoid hybrids with sigma-1 affinity and inhibition of key enzymes in Alzheimer's disease. *Eur J Med Chem* 2018; 156: 534–553.
40. Long HZ, Cheng Y, Zhou ZW, et al. PI3K/AKT signal pathway: a target of natural products in the prevention and treatment of Alzheimer's disease and Parkinson's disease. *Front Pharmacol* 2021; 12: 648636.
41. Moreira NCDS, Lima JEBF, Marchiori MF, et al. Neuroprotective effects of cholinesterase inhibitors: current scenario in therapies for Alzheimer's disease and future perspectives. *J Alzheimers Dis Rep* 2022; 6: 177–193.

42. Arias E, Gallego-Sandín S, Villarroya M, et al. Unequal neuroprotection afforded by the acetylcholinesterase inhibitors galantamine, donepezil, and rivastigmine in SH-SY5Y neuroblastoma cells: role of nicotinic receptors. *J Pharmacol Exp Ther* 2005; 315: 1346–1353.
43. Zeng J, Ji Y, Luan F, et al. Xiaoyaosan ethyl acetate fraction alleviates depression-like behaviors in CUMS mice by promoting hippocampal neurogenesis via modulating the IGF-1Rβ/PI3K/akt signaling pathway. *J Ethnopharmacol* 2022; 288: 115005.
44. Jastrzębski MK, Wójcik P, Stępnicki P, et al. Effects of small molecules on neurogenesis: neuronal proliferation and differentiation. *Acta Pharm Sin B* 2024; 14: 20–37.
45. Wang Y, Lin Y, Wang L, et al. TREM2 Ameliorates neuroinflammatory response and cognitive impairment via PI3K/AKT/FoxO3a signaling pathway in Alzheimer's disease mice. *Aging* 2020; 12: 20862–20879.
46. Rudakova EV, Boltneva NP and Makhaeva GF. Comparative analysis of esterase activities of human, mouse, and rat blood. *Bull Exp Biol Med* 2011; 152: 73–75.
47. Massoulié J, Pezzementi L, Bon S, et al. Molecular and cellular biology of cholinesterases. *Prog Neurobiol* 1993; 41: 31–91.
48. Cai P, Fang SQ, Yang HL, et al. Donepezil-butylated hydroxytoluene (BHT) hybrids as anti-Alzheimer's disease agents with cholinergic, antioxidant, and neuroprotective properties. *Eur J Med Chem* 2018; 157: 161–176.
49. Chaudhary MR, Chaudhary S, Sharma Y, et al. Aging, oxidative stress and degenerative diseases: mechanisms, complications and emerging therapeutic strategies. *Biogerontology* 2023; 24: 609–662.
50. Zhang Q, Yao M, Qi J, , et al. Puerarin inhibited oxidative stress and alleviated cerebral ischemia-reperfusion injury through PI3K/akt/Nrf2 signaling pathway. *Front Pharmacol* 2023; 14: 1134380.
51. Sierksma A, Escott-Price V and De Strooper B. Translating genetic risk of Alzheimer's disease into mechanistic insight and drug targets. *Science* 2020; 370: 61–66.