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Research article

# Galantamine alleviates senescence of U87 cells induced by beta-amyloid through decreasing ROS production

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## HIGHLIGHTS

- The effects of galantamine on proliferation, senescence and ROS production in a U87 cell line treated with beta-amloid were investigated.
- Galantamine protects U87 cells from senescence induced by $A\beta_{1-40}$ through decreasing ROS levels and p53 expression.
- Neuroprotection of galantamine is due to its functioning as a nicotinic allosteric potentiating ligand (APL).

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## ABSTRACT

Galantamine, which is currently used in the treatment of Alzheimer's disease (AD), has been shown to exert a neuroprotective effect against beta-amyloid (Aβ) peptide-induced toxicity, a critical component involved in the pathogenesis of AD. The aim of this study was to examine the effects of galantamine on proliferation, senescence and ROS production in a U87 cell line treated with Aβ. With the use of a Cell Counting Kit-8 and β galactosidase staining assay, we observed that galantamine (0.3 μM) pretreatment significantly prevented Aβ1-40-induced cell degradation and senescence. Aβ1-40-induced ROS production and p53 expression were increased as determined by DCF-derived fluorescence using flow cytometry and Western blotting and reduced in response to galantamine pretreatment. Overall, we found that all alterations resulting from Aβ1-40 were reversed by galantamine pretreatment. In addition, we demonstrate that this neuroprotection from galantamine can be blocked by an α7 nAChR antagonist. Taken together, the findings of this study provide a better understanding of the mechanisms underlying the protective effects of galantamine, effects which include antioxidative properties.
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## 1. Introduction

Alzheimer's disease (AD) represents the most common basis for dementia affecting elderly populations worldwide. AD is characterized pathologically with the deposition of amyloid β (Aβ) peptides in brain parenchyma, hyperphosphorylation of the microtubule associated protein tau and a loss of cholinergic neurons [12]. Deposition of Aβ peptides triggers a series of inflammatory reactions, including activation of astrocytes and microglia, generation of reactive oxygen species (ROS) and cytochemokines [9]. P53, known as tumor suppressor, has important role in determining the cell fate. It is well known that p53 is up-regulated in AD brain and leads to neuronal loss [18].

Astrocytes are necessary for homoeostasis and neuronal defense and regeneration. Loss of astroglial function and astroglial reactivity contributes to brain aging and neurodegenerative diseases, including AD [4]. Astroglial changes in aging and neurodegeneration are highly heterogeneous and region-specific. Both the morphology and function of astrocytes can be regulated through environmental stimulation and/or medication suggesting that astrocytes can be regarded as targets for therapies aimed at the prevention and cure of neurodegenerative disorders [15].

Galantamine hydrobromide, a phenanthrene alkaloid, was initially isolated from a variety of plants [11]. It functions as a reversible, competitive inhibitor of acetylcholinesterase, which can then serve as a nicotinic allosteric potentiating ligand (APL) [16]. Galantamine possesses neuroprotectant capabilities, as indicated by its ability to defend against the cytotoxic effects of glutamate [7], hypoxia resulting from trophic factor deprivation [6] and Aβ neurotoxicity via APL [10]. In addition, galantamine plays an essential role in restoring cognitive function in APP/PS1 transgenic mice, reduces

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Aβ deposition and inhibits astrocyte activation and the intracellular expressions of TNF-α and IL-6 [21].

Increasing evidence for a causal role of cell senescence has been demonstrated in age-related dysfunctions and pathologies [3]. Astrocytes have been largely ignored as factors involved with AD progression and the actual role of astrocytes in AD remains elusive. The purpose of the present study was to observe the effects of galantamine on proliferation, senescent morphology and ROS production in a U87 cell line treated with Aβ. Our findings suggest some of the additional mechanisms of galantamine as can result from protecting astrocytes against Aβ damage.

## 2. Material and methods

### *2.1. Reagents*

Galantamine hydrobromide, with a purity >99%, was obtained from Melonepharma (MD1560, Dalian, P.R.China). The molecular formula is $C_{17}H_{21}NO_3HBr$ and molecular weight is 368.27. $A\beta_{1\text{-}40}$ was purchased from Sigma-Aldrich (A1075, St. Louis, USA). Methyllycaconitine citrate (MLA) was purchased from Santa Cruz Biotechnology (sc-253043, CA, USA). The Cell Counting Kit-8 was purchased from Dojindo Inc. (Kumamoto, Japan). The Senescence β-Galactosidase Staining Kit (C0602) and Lipid Peroxidation MDA Assay Kit (S0131) were purchased from Beyotime Biotechnology (Shanghai, P.R.China). The Reactive Oxygen Species Assay Kit was purchased from Sigma-Aldrich (MAK144-1KT, St. Louis, USA).

### *2.2. Cell culture*

The human U87 astrocytoma cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 10% (v/v) fetal bovine serum (FBS) (Zhejiang Tianhang Biotechnology Co., Ltd., China), 100U/ml penicillin and 10 μg/ml streptomycin (Beyotime Biotechnology, Shanghai, China) at 37 °C in a humidified incubator with 5% $CO_2$.

### *2.3. Treatment of cells*

Cells grown to 80% confluence were washed with serum-free medium and maintained in serum-free medium for 2 h. $A\beta_{1\text{-}40}$ was prepared by incubating freshly solubilized peptides in double deionized water at 37 °C for 24 h [10]. Galantamine and MLA were dissolved in distilled water and stored at −20 °C. To examine the effects of galantamine on $A\beta_{1\text{-}40}$-induced neurotoxicity, 0.3 μM galantamine was incubated with U87 cells for 24 h prior to $A\beta_{1\text{-}40}$ exposure, followed by the addition of 5 μM $A\beta_{1\text{-}40}$ for an additional 24 h of incubation. To test the effects of galantamine on α7 nAChR activation, U87 cells were treated simultaneously with 0.3 μM galantamine and 10 nM MLA for 24 h prior to $A\beta_{1\text{-}40}$ exposure, followed by treatment with 5 μM $A\beta_{1\text{-}40}$ for an additional 24 h. The experiment consisted of four groups: A. control (normal U87cells), B. U87cells + $A\beta_{1\text{-}40}$, C. U87cells + $A\beta_{1\text{-}40}$+ galantamine and D. U87cells + $A\beta_{1\text{-}40}$+ galantamine + MLA.

### *2.4. Measurement of cell proliferation*

U87 cells were seeded in 96-well culture plates ($5 \times 10^4$cells/well) and incubated at 37 °C for 24 h. As described above, after U87 cells were treated with 5 μM $A\beta_{1\text{-}40}$ with fresh medium (containing 10% FBS) for 24, 48, 72 or 96 h, the cells were then collected for determination of cell proliferation.Cell proliferation was measured using the Cell Counting Kit-8. Briefly, 10 μl of Cell Counting Kit-8 solution was added to the medium and incubated for 2 h in an incubator with 5% $CO_2$. The amount of orange formazan dye produced was then calculated by measuring the absorbance at 450 nm on a microplate reader (Awareness Technology, Inc., Palm City, FL).

### *2.5. β- galactosidase staining*

β galactosidase staining was performed according to the instructions provided in the Senescence β-Galactosidase Staining Kit. Images of senescent stained cells were captured using a microscope (CX31; Olympus Corp., Tokyo, Japan) and photographed with use of a digital camera (WAT-221S; WATEC, Japan). The number of SA-β-Gal positive cells in four fields per sample was counted using a 40 × objective. The proportion of cells positive for β galactosidase activity was determined by counting the number of blue cells within the total number of cells as determined at 48 h after treatment with $A\beta_{1\text{-}40}$. Each analysis was performed by a single examiner blinded as to sample identities. Results shown are averages obtained from three separate experiments and error bars indicate the standard error of the mean.

### *2.6. ROS production and MDA level*

Intracellular production of ROS in U87 cells following $A\beta_{1\text{-}40}$ and galantamine treatment were determined by measuring DCF-derived fluorescence with use of flow cytometry. U87 cells ($4 \times 10^5$ cells/sample) were pretreated for 24 h with galantamine (0.3 μM) and MLA (10 nM), followed by $A\beta_{1\text{-}40}$ (5 μM) in serum-free DMEM at 37 °C for an additional 24 h. After treatment, U87 cells were incubated with 10 μM DCFH-DA in serum-free DMEM at 37 °C in a humidified incubator with 5% $CO_2$ for 1 h. The treated cells were then washed and resuspended in PBS. The cells were analyzed by flow cytometry measuring DCF fluorescence ($\lambda_{ex} = 540/\lambda_{em} = 570$ nm). The content of malondialdehyde (MDA), a naturally occurring product of lipid peroxidation which can be used as an indicator of oxidative stress, was determined within each sample, according to the instructions sp sp="0.25"/>540/$\lambda_{em} = 570$ nm). The content of malondialdehyde (MDA), a naturally occurring product of lipid peroxidation which can be used as an indicator of oxidative stress, was determined within each sample, according to the instructions provided in the Lipid Peroxidation MDA Assay Kit.

### *2.7. Western blot analysis*

U87 cells were seeded in 6-well plates at a density of $4 \times 10^5$ cells/well. After various treatment (as indicated), cells were lysed on ice in a cell-lysis RIPA buffer (20101ES60, Beyotime Biotechnology, Shanghai, China) and centrifuged at 12,000*g* for 10 min at 4 °C. The supernatants were collected as cell lysates and assayed for protein contentusing the BCA protein assay kit (P0012 BCA, Beyotime Biotechnology, Shanghai, China). SDS-polyacrylamide gel electrophoresis (PAGE; 7.5% gel) (Beyotime Biotechnology, Shanghai, China) was performed on each sample (30 μg of protein). Isolated proteins were subsequently transferred to 0.2-μmnitrocellulose membranes at 16 V for 30 min. The membranes were blocked with 5% fat-free milk in Tris-buffered saline containing 0.05% Tween 20 (TBST; 2 h at RT) and washed in TBST. The blots were probed with rabbit polyclonal antibodies to p53 (1:500) (ab131442, Abcam Inc. MA, USA) and GAPDH (1:1000) (TA309157, ZhongshanGoldbridge Biotechnology Co., Ltd., Beijing, China) at 4 °C overnight, washed for 30 min in TBST, incubated with horseradish peroxidase conjugated goat anti-mouse IgG and goat anti-rabbit IgG (both at 1:2000) (ZK-9600 and ZB-2301, ZhongshanGoldbridge Biotechnology Co., Ltd., Beijing, China) for 2 h at RT, and visualized using a DAB kit. Densitometric quantification of blots was performed using

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