Journal of Clinical Toxicology
ISSN: 2161-0495

Burgess et al. J Clinic Toxicol 2011, S:6
DOI: 10.4172/2161-0495.S6-003

Research Article Open Access

Cotinine Inhibits Amyloid- β Peptide Neurotoxicity and Oligomerization

Sarah Burgess[1], Ross Zeitlin[1,2] and Valentina Echeverria[1,2,3]*

[1]*Research Chemist, Research and Development, Department of Veterans Affairs, Bay Pines VA Healthcare System, Bay Pines, FL, USA*
[2]*Tampa VA Healthcare System, Tampa, FL, USA*
[3]*Department of Molecular Medicine, University of South Florida, Tampa, FL 33647, USA*

Abstract

Alzheimer's disease, the main cause of dementia, correlates with an increase in the brain levels of aggregated forms of amyloid-β (Aβ) peptide. We investigated the effect of cotinine, the main metabolite of nicotine, on $A\beta_{1-42}$ neurotoxicity. Compared to nicotine, cotinine has a longer plasma half-life, lower toxicity and is a poor agonist of the nicotinic acetylcholine receptors (nAChRs). We found that cotinine promoted the survival of primary cortical neurons exposed to $A\beta_{1-42}$. This neuroprotective effect was independent of the agonistic activation of the nAChRs and it was not prevented by the general nAChR antagonist mecamylamine. However, it was prevented by the pre-aggregation of Aβ in absence of cotinine, suggesting that inhibition of $A\beta_{1-42}$ aggregation by cotinine is a key mechanism mediating its neuroprotective activity. The analysis of $A\beta_{1-42}$ aggregation using dot blot immunoassay showed that cotinine inhibits its oligomerization. These results suggest that cotinine is neuroprotective at least in part by preventing the aggregation of the amyloid peptide. We previously found that cotinine prevented memory loss and reduced plaques in the brain of Tg6799 mice. This study suggests that cotinine can also inhibit neuronal cell death induced by Aβ neurotoxicity.

Keywords: Alzheimer's disease; Tobacco; Oligomerization

Abbreviations: Aβ: Amyloid beta; ANOVA: Analysis of variance; AD: Alzheimer's disease; DIV: Days *in vitro*; nAChR: Nicotinic Acetylcholine Receptor; ns: Not Significant; PI: Propidium Iodide; h: Hours; min: Minutes; PBS: Phosphate Buffered Saline; RT: Room Temperature; sec: Seconds; TBS: Tris-Buffered Saline; TBST: TBS with 0.05% Tween 20

Introduction

Numerous evidence suggest that cognitive impairment in Alzheimer's disease (AD) is associated with increased levels of Aβ oligomers in the brain [1]. Recently, it has been shown that cotinine prevented cognitive impairment and Aβ burden in a mouse model of AD [2].

In this study, we investigated the effect of cotinine on Aβ toxicity and oligomerization *in vitro*. Because the protective actions of nicotine against Aβ toxicity have been attributed to the action of nicotine on the nAChRs [3], the effect of mecamylamine, a general inhibitor of the nAChRs on cotinine's neuroprotective activity was also investigated. The potential of cotinine as a new therapeutic agent against AD is discussed in light of these results.

Materials and Methods

Reagents

Cotinine and mecamylamine were purchased from Sigma-Aldrich (Saint Louis, MO, USA). $A\beta_{1-42}$ peptide was obtained from American Peptide (Sunnyvale, CA, USA). Propidium Iodide, calcein-AM (dichlorodihydrofluorescein diacetate, CM-H2DCFDA) and cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

Cortical cells

Embryonic rat cortical cells were prepared as described [4]. Embryonic cortical tissues (Brain Bits LLC, Springfield, IL, USA) were dissociated by trypsin digestion and repeated passages through a pipette tip. Cortical cells (2×10^5 cells) were plated in Neurobasal E medium, supplemented with 2% B27 and 1 mM glutamax (Invitrogen), onto tissue culture wells coated with poly-D-lysine. Cells were incubated at 37°C in a humidified incubator with 95% air/5% CO_2 for 7-10 days, before analysis.

Preparation of Aβ oligomers

To obtain $A\beta_{1-42}$ oligomers, we used a protocol that results in solutions containing stable oligomers, but not fibrils as previously described [5]. The lyophilized $A\beta_{1-42}$ peptide (American Peptide) was dissolved in 1 volume of 1 mM NaOH and then diluted in PBS pH 7.4 to the desired concentrations.

Aβ toxicity assay

After 7 days *in vitro* (DIV) the cell conditioned media was replaced with Neurobasal E medium, supplemented with 2% B27 without antioxidants (Invitrogen), and cells were exposed to $A\beta_{1-42}$ oligomers solutions in the presence or absence of cotinine (10 μM), cotinine plus mecamylamine or mecamylamine alone for 24 h. To form Aβ oligomers the peptide solutions were incubated at 4°C for 3 h before being added to the culture media. To test for specificity some experiments were performed using the reverse $A\beta_{42-1}$ peptide.

MTT assay

This assay measures the viability of cells by analyzing the mitochondrial conversion of the tetrazolium salt MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to formazan [6]. Cell culture medium was replaced with Neurobasal

***Corresponding author:** Valentina Echeverria, Ph.D, 10,000 Bay Pines Blvd., Bldg. 23, Rm. 123, Bay Pines, FL 33744, USA, Tel: +727-398-6661 (Ext. 4425); Fax: +727-319-1161; E-mail: valentina.echeverria@va.gov

Received March 21, 2012; **Accepted** April 17, 2012; **Published** April 20, 2012

Citation: Burgess S, Zeitlin R, Echeverria V (2012) Cotinine Inhibits Amyloid-β Peptide Neurotoxicity and Oligomerization. J Clinic Toxicol S6:003. doi:10.4172/2161-0495.S6-003

medium containing freshly-dissolved MTT (0.5 mg/mL). Following 1-3 h incubation at 37°C, formazan crystals were dissolved in DMSO and absorbance at 590 nm was measured.

Double calcein-AM and propidium iodide staining viability assay

This assay was performed as described [6], calcein-AM and propidium iodide (PI) were added to the conditioned media of the cells to reach the desired final concentrations (calcein-AM, 2 μM; PI, 1 μM) and incubated for 30 min. The number of calcein- and PI (red) stained cells were analyzed by fluorescence microscopy. More than 600 cells per condition were analyzed in several 20 x focal planes.

Analysis of cotinine on Aβ oligomerization

For the analysis of $A\beta_{1-42}$ oligomers, the peptide solutions (100 μM, pH 7.4) were prepared in the absence or presence of ascending concentrations of cotinine (100, 200, 500 μM) and incubated for 2 and 6 days at 25°C. After centrifugation, Aβ oligomers were analyzed in the solutions by dot-blot analysis as described [5]. 4-8 μl of the oligomerization mixture were applied onto nitrocellulose membranes and allowed to dry. Membranes were blocked for 1 h at room temperature (RT) with 10% skim milk in Tris-buffered saline plus tween 0.05% (TBS-T), washed and incubated with either 6E10 (Covance Princeton, NJ) (1:20,000) which preferentially recognizes the monomeric form of the peptide, or the specific anti-oligomeric Aβ antibody A11 (Invitrogen) (1:500) antibodies diluted in (TBS)-T with 5% milk overnight at 4°C. The following day, membranes were washed and incubated with HRP-conjugated secondary antibody (1: 5000) for 1 h; visualized using enhanced chemi luminescence (Dura ECL, Pharmacia Biotech, Piscataway, NJ, USA), scanned on the Kodak Image Station 440CF and analyzed using NIH Image J software.

Statistical analysis

Differences between groups were analyzed using a two-tailed unpaired Student's *t*-test. The mean values obtained for more than two groups were compared by one-way analysis of variance (ANOVA). In all of the analyses, the null hypotheses were rejected at the 0.05 level. Statistical analyses were performed using the Prism statistical program (GraphPad Software, San Diego, CA, USA). All of the group data are presented as the mean ± SEM.

Results

Cotinine is neuroprotective against Aβ toxicity

In the PI/calcein assay; cotinine/Aβ-treated cultures showed more surviving (calcein-green fluorescent) and less degenerating (PI-stained red nuclei) cells than those treated with the Aβ (5 μM) alone. Cotinine significantly increased cell survival from 63% (Aβ alone) to 83% (Aβ + 10 μM cotinine) of vehicle-treated control cells (Student's *t* test, $p = 0.0007$) (Figure 1A). The effect was specific as no toxicity was induced by the reverse $A\beta_{42-1}$ peptide (5 μM) (data not shown).

To determine whether the neuroprotective activity of cotinine involved nAChR agonism, we studied the effect of cotinine on Aβ toxicity over cortical cells pre-treated with the nonselective nAChR antagonist mecamylamine. Pretreatment with mecamylamine (1 μM), a dose that inhibits nAChRs in cultured brain cells [7], did not affect the neuroprotective activity of cotinine and the increase in neuronal survival ($p > 0.05$) (Figure1B).

The neuroprotective effect of cotinine against Aβ depends on inhibiting the peptide aggregation

The neuroprotective effect of cotinine was dependent on $A\beta_{1-42}$ aggregation. When $A\beta_{1-42}$ was pre-aggregated in the absence of cotinine before being added to the cells, its toxicity was not reduced by the presence of cotinine in the culture media (Figure 2A). However, when Aβ was pre-aggregated in the presence of ascending concentrations of cotinine (1, 5, 10 μM), its toxicity was significantly reduced (Figure 2B). Cultures treated with Aβ plus cotinine showed a significantly higher cell viability than cultures treated with Aβ alone (22% increase) (Figure 2B) (Student's *t* test, $p = 0.019$).

Cotinine prevented $A\beta_{1-42}$ oligomerization *in vitro*

Cotinine treatment reduced $A\beta_{1-42}$ oligomers levels in the brain of AD mice [2]. To determine whether this decrease was due to a decrease in the oligomerization of the peptide, dot-blot immunoassay analysis was performed on Aβ oligomerization solutions incubated for 2 and 6 days at RT. The results show the immunoreactivity for both 6E10 and anti- Aβ oligomers. The results show that cotinine, in the two days analyzed, decreased the level of Aβ oligomers expressed as a reduction in the immunoreactivity for the A11 (Figure 2D, bottom).

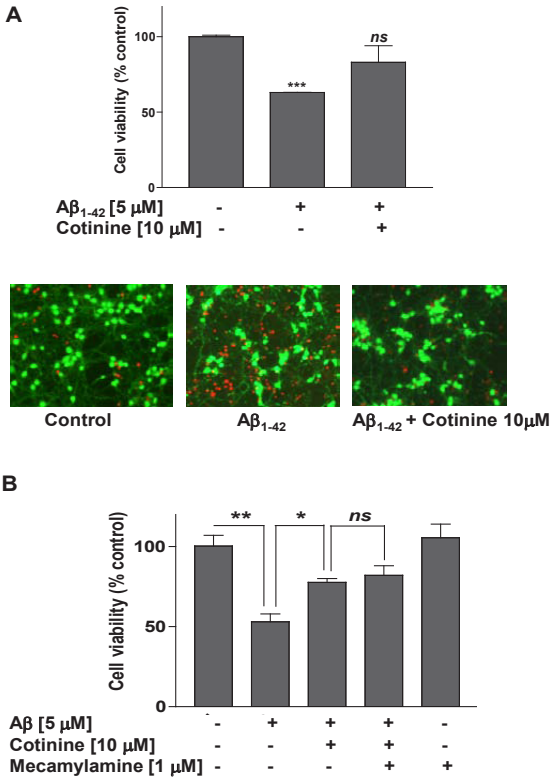

Figure 1: Cotinine protects neurons against Aβ toxicity by a mechanism independent of the nicotinic acetylcholine receptors. Embryonic cortical neurons after 7 DIV were treated with 5μM Aβ either alone or with cotinine (0.1, 1, 10 μM). After 24 h, cell viability was assessed using double calcein-AM and PI staining (Scale bar = 20 μm) (A). MTT analysis of cell viability of cells exposed to pre-aggregated $A\beta_{1-42}$ solutions for 24 h at 37°C in absence or presence of cotinine alone or plus mecamylamine (B). The values were normalized against control values considered 100% and represent the mean ± S.E.M.. The differences of the mean were considered significant with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). ns (not significant).

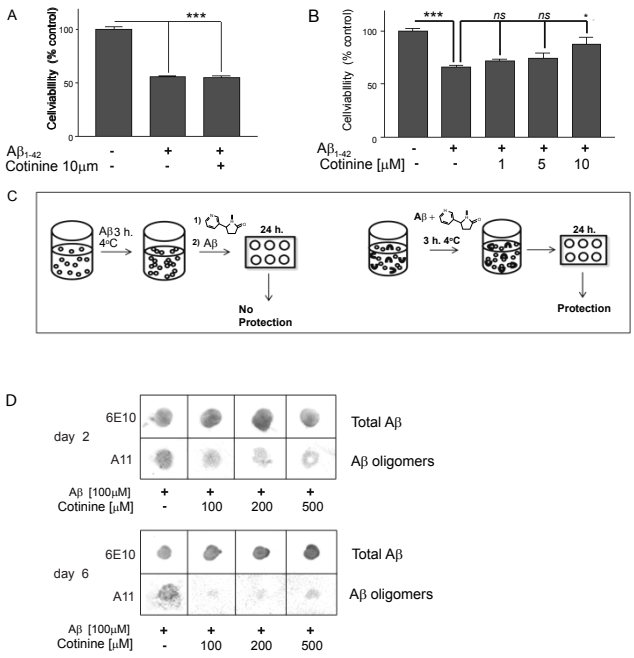

Figure 2: Cotinine is neuroprotective by blocking the aggregation of Aβ. 7 DIV embryonic rat cortical cells were exposed to different forms of $A\beta_{1-42}$ in absence or presence of cotinine 10μM for 24 h at 37°C. A) MTT analyses of cell viability of cells treated with pre-aggregated $A\beta_{1-42}$ in absence or presence of 10 μM cotinine. B) MTT analysis of the cell viability of cortical cells treated with $A\beta_{1-42}$ freshly dissolved in the culture media. Plots represent cell viability as percentage of vehicle-treated controls. C) Diagram representing the experimental design used. D) 100 μM $A\beta_{1-42}$ was aggregated under conditions that favor oligomerization in the presence or absence of cotinine (100, 200, 500 μM). After 2 and 6 days of incubation at RT, aliquots of the oligomerization mix were analyzed by dot-blot immunoassays using the 6E10 and the highly specific anti- oligomeric Aβ antibody A11, (B). The differences were considered significant with $p < 0.05$ (*), and highly significant $p < 0.001$ (***), ns (not significant).

Discussion

The oligomeric forms of Aβ are considered the main neurotoxic forms of the peptide and its accumulation in the brain induces synaptic and cognitive deficits *in vivo* [1,8,9].

In this study, we assessed the effect of cotinine on $A\beta_{1-42}$ oligomerization and neurotoxicity. Interestingly we found that cotinine inhibited Aβ neurotoxicity only when it was included in the pre-aggregation solutions but not when added later to the conditioned media. This result suggests that cotinine is neuroprotective mainly by inhibiting Aβ aggregation *in vitro*. It has been shown that cotinine binds to Aβ with high affinity (K_a=10 nM) [10] inhibiting its aggregation into fibrils *in vitro* [11]. A previous study also showed that cotinine reduced oligomeric Aβ levels *in vivo* [2]. However, whether the reduction in $A\beta_{1-42}$ oligomers was induced by a decrease in the peptide oligomerization it was not defined. In here, we demonstrated for first time that cotinine inhibited $A\beta_{1-42}$ oligomerization *in vitro*.

Mecamylamine, at a concentration that inhibited nAChRs in cultured brain cells [7], and block nicotine's neuroprotective activity [12], did not affect cotinine neuroprotective activity. This evidence strongly suggests that cotinine triggers neuroprotective mechanisms distinctive from nicotine. In fact cotinine has a 100-fold lower affinity than nicotine for these receptors [13,14] and is 100-fold more toxic and 10-fold more rapid at inducing respiratory arrest than cotinine [13].

Overall, this study provides evidence that cotinine inhibits Aβ oligomerization and Aβ-induced cortical cell death. Previous evidence obtained *in vivo* [2] and this *in vitro* evidence suggests that cotinine has the potential to be a new pharmacological therapy against AD.

Acknowledgements

This material is the result of work supported with resources and the use of facilities at the Bay Pines VA Healthcare System and the James A. Haley Veterans' Hospital. The contents do not represent the views of the Department of Veterans Affairs or the United States Government. It was also supported by grants from the James and Esther King Biomedical Research Program 1KG03 (to VE). We are also grateful to the Bay Pines Foundation, Inc.

References

1. Echeverria V, Cuello AC (2002) Intracellular A-beta amyloid, a sign for worse things to come? Mol Neurobiol 26: 299-316.
2. Echeverria V, Zeitlin R, Burgess S, Patel S, Barman A, et al. (2011) Cotinine reduces amyloid-β aggregation and improves memory in Alzheimer's disease mice. J Alzheimers Dis 24: 817-835.
3. Liu Q, Zhang J, Zhu H, Qin C, Chen Q, et al. (2007) Dissecting the signaling pathway of nicotine-mediated neuroprotection in a mouse Alzheimer disease model. FASEB J 21: 61-73.
4. Echeverria V, Clerman A, Doré S (2005) Stimulation of PGE receptors EP2 and EP4 protects cultured neurons against oxidative stress and cell death following beta-amyloid exposure. Eur J Neurosci 22: 2199-2206.
5. Necula M, Kayed R, Milton S, Glabe CG (2007) Small molecule inhibitors of aggregation indicate that amyloid beta oligomerization and fibrillization pathways are independent and distinct. J Biol Chem 282: 10311-10324.
6. Echeverria V, Burgess S, Gamble-George J, Arendash GW, Citron BA (2008) Raf inhibition protects cortical cells against beta-amyloid toxicity. Neurosci Lett 444: 92-96.
7. Fu W, Jhamandas JH (2003) Beta-amyloid peptide activates non-alpha7 nicotinic acetylcholine receptors in rat basal forebrain neurons. J Neurophysiol 90: 3130-3136.
8. Walsh DM, Hartley DM, Condron MM, Selkoe DJ, Teplow DB (2001) In vitro studies of amyloid beta-protein fibril assembly and toxicity provide clues to the aetiology of Flemish variant (Ala692-->Gly) Alzheimer's disease. Biochem J 355: 869-877.
9. Resende R, Ferreiro E, Pereira C, Resende de Oliveira C (2008) Neurotoxic effect of oligomeric and fibrillar species of amyloid-beta peptide 1-42: involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. Neuroscience 155: 725-737.
10. Szymańska I, Radecka H, Radecki J, Kaliszan R (2007) Electrochemical impedance spectroscopy for study of amyloid beta-peptide interactions with (-) nicotine ditartrate and (-) cotinine. Biosens Bioelectron 22: 1955-1960.
11. Salomon AR, Marcinowski KJ, Friedland RP, Zagorski MG (1996) Nicotine inhibits amyloid formation by the beta-peptide. Biochemistry 35: 13568-13578.
12. Liu Q, Zhao B (2004) Nicotine attenuates beta-amyloid peptide-induced neurotoxicity, free radical and calcium accumulation in hippocampal neuronal cultures. Br J Pharmacol 141: 746-754.
13. Riah O, Dousset JC, Courriere P, Stigliani JL, Baziard-Mouysset G, et al. (1999) Evidence that nicotine acetylcholine receptors are not the main targets of cotinine toxicity. Toxicol Lett 109: 21-29.
14. Riah O, Dousset JC, Bofill-Cardona E, Courrière P (2000) Isolation and microsequencing of a novel cotinine receptor. Cell Mol Neurobiol 20: 653-664.