Biochimie 138 (2017) 82–89

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Effects of phenothiazine-structured compounds on APP processing in Alzheimer's disease cellular model

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ARTICLE INFO

Article history:
Received 28 February 2017
Accepted 25 April 2017
Available online 27 April 2017

Keywords:
Alzheimer's disease
Amyloid-β peptide
Cholinesterase inhibitor
Phenothiazine

ABSTRACT

The excess accumulation of amyloid-β (Aβ) peptides derived from the sequential cleavage of amyloid precursor protein (APP) by secretases, is one of the toxic key events leading to neuronal loss in Alzheimer's disease (AD). Studies have shown that cholinergic activity may also be involved in the regulation of APP metabolism. In the current study, we have investigated the roles of toluidine blue O (TBO) and thionine (TH), newly recognized phenothiazine-derived cholinesterase inhibitors, on the metabolism of APP in Chinese hamster ovary cells stably expressing human APP751 and presenilin 1 (PS70 cells). We assessed the effects of both compounds on the levels of Aβ, soluble APP-α (sAPPα), intracellular APP and β-site APP-cleaving enzyme 1 (BACE1). After treatment of PS70 cells with TBO or TH without any side effect on cell viability, the levels of secreted Aβ40, Aβ42 and sAPPα were assayed by specific sandwich ELISAs while APP and BACE1 in cell lysates were analyzed using Western blot. The secreted Aβ40, Aβ42 and sAPPα in TBO- and TH-treated cells were found to be reduced in a dose-dependent manner compared to vehicle-treated cells. Results suggest that TH mitigated the Aβ pathology by lowering APP levels whereas reduced Aβ caused by TBO treatment seems to be the outcome of both less substrate availability and amyloidogenic APP processing. Taken together, our results represent the first report demonstrating that TBO and TH can affect amyloid metabolism *in vitro*.

1. Introduction

Alzheimer's disease (AD), the most prevalent neurodegenerative disorder in the elderly, results in progressive loss of memory and cognition. The aggregation and deposition of amyloid-β (Aβ) peptides as well as neurofibrillary tangles composed of hyperphosphorylated tau are believed to be the major events in the appearance and progression of AD [1]. Although Aβ peptides are normally produced throughout life, their extracellular aggregation as amyloid plaques represents an abnormal pathological lesion [2].

Aβ peptides of 37–43 amino acid lengths are formed from amyloid precursor protein (APP) by consecutive action of β- and γ-secretases via the amyloidogenic pathway. β-secretase, also called β-site APP-cleaving enzyme 1 (BACE1), initiates Aβ generation by releasing a soluble amino-terminal fragment of APP (sAPPβ) while APP carboxy-terminal fragment (β-CTF or C99) remains membrane-bound. A further cleavage of C99 peptide by γ-secretase results in the liberation of Aβ into the extracellular fluid [3,4]. In the other principal processing pathway, named anti-amyloidogenic pathway, APP is initially cleaved by α-secretase, generating the soluble ectodomain of APP (sAPPα) and a carboxyl terminal fragment (α-CTF or C83). C83 peptide lacks the amino terminal portion of the Aβ domain and is subsequently cleaved by γ-secretase, releasing a truncated Aβ peptide [3,5]. Among Aβ peptides, Aβ40 is the major secreted form whereas Aβ42 is the main pathological form with a higher propensity to aggregate [6]. In recent years, several studies have demonstrated that soluble forms of Aβ forming oligomers rather than amyloid plaque burden correlate with neuronal loss severity [7–9].

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; APP, amyloid precursor protein; ACh, acetylcholine; AChE, acetylcholinesterase; BACE1, β-site APP-cleaving enzyme 1; BChE, butyrylcholinesterase; ChE, cholinesterase; ChEI, cholinesterase inhibitor; sAPPα, soluble APP-α; MethB, methylene blue; TBO, toluidine blue O; TH, thionine.

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http://dx.doi.org/10.1016/j.biochi.2017.04.012
0300-9084/

Deficits in cholinergic neurotransmission accompanied by decreased levels of neurotransmitter acetylcholine (ACh) and ACh degrading enzyme, choline acetyltransferase, are also observed in AD [10]. The treatment strategy of majority of current AD drugs aims to inhibit the cholinesterases (ChEs) that are responsible for the hydrolysis of ACh, so that the cholinergic function may be enhanced by prolonging the availability of ACh [11]. Acetylcholinesterase (AChE, EC 3.1.1.7) is the major ChE in the healthy human brain whereas its activity is highly decreased in AD [12]. In contrast, butyrylcholinesterase (BChE, EC 3.1.1.8), has the ability to atone for the lack of ChE activity [12,13]. This brings out the possibility that dual inhibition of both ChEs might be more efficient in the treatment of AD [11,14]. Although some of AD drugs on market such as donepezil, galantamine and rivastigmine, which inhibit human AChE and BChE to different degrees [15], have also been shown to have actions on APP processing by lowering Aβ in cellular [16–18] and animal models [19,20], they remain to be symptomatic drugs with no success in preventing, treating or curing the disease [11].

A potential therapeutic agent, namely methylene blue (methylthioninium chloride; MethB), was found to inhibit both ChEs, to remodel toxic mature soluble Aβ oligomers into benign conformers [21,22], to enhance the clearance of Aβ peptides by increasing proteasome activity [23], and to inhibit β secretase activity [24]. However, the functionality problems related to absorption, metabolism and distribution of MethB during phase II clinical trials resulted in the use of a reduced form of it called leucomethylthioninium for further clinical trials [25]. Similar to MethB, toluidine blue O (tolonium chloride; TBO) and thionine (TH), are also phenothiazine-derived compounds with a chemical core $S(C_6H_4)_2NH$ (Fig. 1) [26]. In a previous research, MethB as well as TBO and TH were found to inhibit the formation of *in vitro* aggregates made of truncated tau protein [27]. TBO and MethB were also proposed to provide neuroprotective effects for the treatment of various neurological disorders, as assessed by their antioxidant properties [28]. Moreover, phenothiazine derivatives with substitutions were proposed as good candidates to be used as therapeutic agents in cerebral pathologies due to increased ability to pass through blood-brain barrier [29].

In a latest study performed in our laboratory, we have investigated the inhibitory effects of various phenothiazine-derived compounds on human cholinesterases [30]. Our results showed that TBO and TH caused nonlinear inhibition of human BChE, compatible to double occupancy, with respective K_i values of 0.008 ± 0.003 μM and 2.1 ± 0.42 μM. TBO acted as a linear mixed type inhibitor of human erythrocyte AChE with $K_i = 0.041 \pm 0.005$ μM. Besides, site-directed mutagenesis studies showed that peripheral anionic site of human BChE is responsible for the binding of TBO to BChE [30].

Many studies have focused on the synthesis of new phenothiazine derivatives with robust BChE inhibition since the above-mentioned importance of BChE in AD was reported [31–33]. As being potent inhibitors of ChEs, especially BChE, we thought it was worth to study the multifunctional anti-AD properties of TBO and TH. Therefore, in the present study, we questioned whether they may modulate amyloid metabolism. Both phenothiazines were found to inhibit Aβ40 and Aβ42 secretion into the conditioned media of a cellular model of amyloid pathology (PS70 cells). We also analyzed whether these compounds change the levels of secreted sAPPα, intracellular APP and BACE1 *in vitro*. The observed effects of TBO and TH on amyloid metabolism were obtained at subtoxic conditions as shown by cell viability assay using flow cytometry.

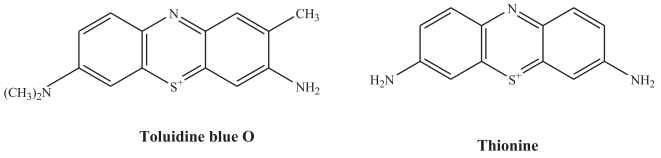

Fig. 1. Chemical structures of the studied ligands.

2. Materials and methods

2.1. Materials

Toluidine blue O (TBO; dye content approximately 85%) and thionin acetate salt (TH; dye content approximately 90%) were bought from Sigma-Aldrich (St. Louis, USA). The purities of the dyes have been taken into consideration while preparing phenothiazine solutions. The rest of the chemicals were of the highest grade available from Sigma-Aldrich (USA), Thermo-Scientific (USA) or Biological Industries (Israel) unless otherwise indicated.

2.2. Cells and cell culture conditions

Chinese hamster ovary cells stably overexpressing wild-type human APP751 and wild-type human presenilin 1 (PS70 cells) were kindly provided from Prof. Sascha Weggen (University of Heinrich Heine, Düsseldorf, Germany). PS70 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and 100 U penicillin-0.1 mg/mL streptomycin at 37 °C with 5% CO_2 in a tissue culture incubator.

2.3. Treatment of PS70 cells with TBO or TH

PS70 cells (passage# ≤ 5) were seeded in multiwell plates (6-well format) or 100 mm tissue culture dishes at a density of 2.5–3 x 10^5 cells per well or 1.5–2 x 10^6 cells per dish in modified DMEM until they reached approximately 70–80% confluence before treatment. Stock TBO or TH solutions (0.25–3 mM) were prepared in phosphate buffered saline (PBS) or methanol, respectively and filter-sterilized through 0.2 μm filters. Cultured cells in fresh DMEM (no phenol red) with 1% FBS and 1 mM sodium pyruvate were treated with TBO or TH solutions at a final concentration of 1.25–15 μM and PBS or methanol (as vehicle-treated controls) for 6 or 24 h, respectively, so that the concentration of PBS or methanol in the culture media always remained the same 0.5%. The selected concentrations of phenothiazines were based on a previous study done with MethB using Chinese hamster ovary cells stably expressing wild-type human APP751 [24]. After phenothiazine treatment, cell culture media were collected, treated with protease inhibitor cocktail (Sigma-Aldrich, St. Louis) and stored at −20 °C until further use. Cells were washed with ice-cold PBS twice and removed from the plates or dishes by scraping on ice with cold RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0; Sigma-Aldrich) supplemented with 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail. After centrifugation at 13000*g* for 20 min at 4 °C, the supernatants were frozen at −20 °C as cell lysates until use. The total protein concentration of each sample was calculated by using the BCA protein assay kit (Pierce).

2.4. ELISA analysis of Aβ peptides and sAPPα

The levels of Aβ40, Aβ42 and sAPPα were quantified in cultured PS70 cell supernatants in duplicate by using human Aβ40, ultrasensitive human Aβ42 and human APP sandwich ELISA kits (Invitrogen, Camarillo, CA, USA), respectively. The manufacturer's