In Silico, in Vitro and in Vivo Investigation of a Newly Synthesized Ionic Compound As Anti-Alzheimer Multi-Target Dual AChE/BuChE Inhibitor Possessing Neuro-Protective Effects Against Aβ Induced Neurotoxicity in PC12 Cells and Alzheimer Rat Model

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Abstract

Multi-target anti Alzheimer's disease (AD) compounds are promising leads for the development of AD modifying agents. Ionic compounds containing quaternary ammonium moiety were synthesized and their multi-targeted anti-AD effects were examined in the current study. Compound 5g possessed suitable aqueous solubility and cell toxicity. It also showed non-competitive dual hAChE/hBuChE inhibition activity. Compound 5g reversed the Aβ-treated PC12 cells’ morphology alteration and reduced PC12 cells’ death. Compound 5g possessed anti-oxidative stress activity through anti-oxidant, anti-ROS production and anti-lipid peroxidation mechanisms. It also reduced the expression of IL-1β and TNF-α genes. Furthermore, compound 5g LDH inhibition, reduction of neuro-inflammation and prevention of autophagy-apoptosis were approved by the results of in vitro studies. Compound 5g delivery to brain was confirmed by in vivo studies. Administration of compound 5g to Aβ-induced AD rat models improved their cognition function and spatial memory learning behavior. TNF-α and NFKB down-regulated in compound 5g treated AD rats’ hippocamp. Besides, compound 5g reversed the up-regulation of AChE in Aβ treated rats’ hippocamp. Molecular modeling studies confirmed the interaction of compound 5g with both steric and catalytic sites of ChE enzymes. The newly synthesized quaternary ammonium containing derivative (compound 5g) possessed multi-target anti-AD efficacy based on in vitro and in vivo studies and its efficacy in AD rat models were approved by behavioral and molecular investigations.

Highlights

- Multi-target anti-AD compounds were designed synthesized utilizing a modified synthesis method.
- Compound 5g as an ionic derivative containing quaternary ammonium moiety possess better aqueous solubility while its cell toxicity is low.
- Compound 5g reversed Aβ treated PC12 cells’ morphology alteration via its neuro-protective effect.
- Compound 5g on the reduced the expression of IL-1β and TNF-α genes in a dose-dependent manner.
- Compound 5g possessed anti-oxidative stress activity through anti-oxidant, anti-ROS production, anti-lipid peroxidation mechanisms was confirmed.
- Compound 5g possessed LDH inhibition, reduction of neuro-inflammation, prevention of autophagy-apoptosis, and maybe necrosis.
- Compound 5g delivery to AD rats’ brain was confirmed by in vivo studies.
- Administration of compound 5g to Aβ-induced AD rat models improved their cognition function and spatial memory learning behavior. Also, TNF-α and NFKB down-regulated in compound 5g treated AD rats’ hippocamp.
- Compound 5g reversed the up-regulation of AChE in Aβ treated rats’ hippocamp. Molecular modeling studies confirmed the interaction of compound 5g with both steric and catalytic sites ChE enzymes.
- The newly synthesized compound 5g could be a start point for ionic multi-target anti-AD drugs.

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease known mainly by losing of short-term memory[1]. The rapid growth of AD necessitates the acceleration of therapeutics discovery and development[2-5]. Neuronal misfunctions and their gradual death that results in memory loss and cognitive decline are the main characteristics of AD. The dynamic loss of synaptic neurons results in the atrophy in the hippocampus and frontal and tempo-parietal cortex [6,7]. The most specific symptoms of AD are the accumulation of amyloid-beta (Aβ) plaques around neurons, hyperphosphorylation of microtubules associated Tau protein, which in turn leads to the intracellular neurofibrillary tangles (NFT), and the reduction of acetylcholine (ACh) level in the synaptic cleft[8-12]. While the pathogenesis of AD has not been understood well yet, but the interacting cascade of elements presents within a network that its core is the accumulation of Aβ and aggregation of hyperphosphorylated Tau protein. Disintegration of axons, degenerative dysfunction of synapsis, neuroinflammation, dysregulation of membranes, and dysfunction of brain metabolic pathways has been reported[6,7,13]. Oxidative stress and free radical formation, metal dyshomeostasis, excitotoxic processes beyond a large number of AD-associated genes are known as events with the effect on AD progression rate [12].

ACh transmission was studied well in AD. ACh enhancement improves cognitive and behavioral symptoms[14]. The current FDA-approved drugs are anti-cholinesterase (ChE) drugs (donepezil, rivastigmine, and Galantamine) [15] or NMDA antagonists
Compounds 5g and 5i were synthesized in the current study and their synthesizing methods and molecular structures were reported in our previous papers. The details of derivatives synthesizing and molecular structure characterizing were reported in our previous papers.

4.1. Synthesizing of the compounds 5g and 5i

Experimental

The details of derivatives synthesizing and molecular structure characterizing were reported in our previous papers. Compounds 5g and 5i were synthesized in the current study and their synthesizing methods and molecular structures.
characterization are as follows:

**2-[4-(N,N,N-trimethylammonium)benzilidine]-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one (5i):**

Compound 5h (1mmol) was dissolved in acetone (14cc) then CH\textsubscript{3}I (3mmol) was added. The reaction mixture was stirred 48h at 30 °C. The solid was filtered and washed with acetone. Residual solvent was removed under reduced pressure to afford compound 5i as yellow solid; Yield 75%; Mp=282-284 °C; FT-IR (KBr) ν 3000, 3039, 1686, 1632, 1496, 1300, 1088, 836 cm\(^{-1}\); \(^1\)H NMR (400MH\textsubscript{z}, CDCl\textsubscript{3}) δ 3.64(9H, s, \(+N-CH_3\)), 3.84 (3H, s, O-CH\textsubscript{3}), 3.91 (3H, s, O-CH\textsubscript{3}), 4.05(2H, s, inden-CH\textsubscript{2}), 7.23 (1H, s, Ar-H), 7.24 (1H, s, Ar-H), 7.50(1H, s, methine-H), 7.97-7.99 (2H, d, \(J = 8.95 \text{ Hz}, \text{Ar}-H\)), 8.06- 8.08 (2H, d, \(J = 8.98 \text{ Hz}, \text{Ar}-H\)), ppm; \(^{13}\)C NMR (100 MHz, DMSO): δ 30.69, 31.32, 55.70, 56.04, 104.56, 104.61, 108.07, 120.11, 121.12, 128.64, 129.74, 131.54, 136.76, 138.18, 145.36, 147.14, 149.43, 155.58, 191.61 ppm.

IR spectra of 2-[4-(N,N,N-trimethylammonium)benzilidine]-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one (5i)

\(^1\)H NMR spectra of 2-[4-(N,N,N-trimethylammonium)benzilidine]-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one (5i)

**2-[4-(N,N-dimethylpipyrazinoammonium)benzylidine]-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one (5g):**

Compound 5f (1mmol) was dissolved in acetone (14cc) then CH\textsubscript{3}I (3mmol) was added. The reaction mixture was stirred 48h at 30 °C. The solid was filtered and washed with acetone. In conclusion residual solvent was removed under reduced pressure to afford compound 5g as a yellow solid. Yield 82%; mp: 182-184 °C; FT-IR (KBr) ν 2924, 1674, 1302, 1222, 1090, 896 cm\(^{-1}\); \(^1\)H NMR (400MH\textsubscript{z}, DMSO) δ 3.22 (6H, s, \(+N-CH_3\)), 3.56-3.58 (4H, m, piperazino-CH), 3.63-3.67 (4H, t, \(J=8\text{Hz}, \text{piperazino-CH}\)), 3.82 (3H, s, O-CH\textsubscript{3}), 3.89 (3H, s, O-CH\textsubscript{3}), 3.93 (2H, s, inden-CH\textsubscript{2}), 7.10-7.12 (2H, d, \(J = 8.68 \text{ Hz}, \text{Ar}-H\)), 7.18 (1H, s, Ar-H), 7.21 (1H, s, Ar-H), 7.36 (1H, s, methine-H), 7.64-7.67 (2H, d, \(J = 8.85 \text{ Hz}, \text{Ar}-H\)), ppm; \(^{13}\)C NMR (100 MHz, DMSO): δ 31.71, 40.09, 50.36, 55.66, 56.00, 59.95, 104.40, 104.48, 108.11, 115.07, 126.05, 130.30, 131.29, 132.05, 132.58, 149.24, 149.94, 154.96, 191.87 ppm.

IR spectra of 2-[4-(N,N-dimethylpipyrazinoammonium)benzylidine]-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one (5g)

\(^1\)H NMR spectra of 2-[4-(N,N-dimethylpipyrazinoammonium)benzylidine]-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one (5g)

**4.1 hAChE and hBuChE inhibition assay**

**4.1.1 Materials and equipment**

5,5′-Ditiobis(2-nitrobenzoic acid (DTNB) and Acetyltiocholin iodide (ATCI) were purchased from Acros. Butyrylthiocholin iodide (BuTCI) was purchased from TCI Europe. Di-sodium hydrogen phosphate and Hydrochloridric acid were obtained from Merck. Spectrophotometric measurements performed by Shimadzu UV/2550 Spectrophotometer equipped with a thermocycler bath.

**4.1.2. Extraction of hAChE and hBuChE from human blood**

The obtaining of the blood samples from a healthy volunteer was approved by local ethics (IR.TBZMED.REC.868). The blood sample (2.0 mL) was collected in a heparinized tube and mixed with 8.0 mL of sodium phosphate buffer (0.1 M, pH 7.4)). The mixture was centrifuged (5 minutes at 3000 g) and washed with 2-3 volumes of normal saline. After three times of centrifugation and washing, 0.1 mL of the packed RBC was transferred to another tube. To obtain the hemolysate, 6.0 mL of distilled water was added to RBC, and the mixture was centrifuged (1000 g) after 5 minutes of incubation in ice. Aliquots of the erythrocyte membranes were stored at -20˚C until use [64,65]. ChE activity was measured before analysis.

**4.1.3. hAChE and hBuChE inhibition assay**

Modified Ellman's method was performed for the measurement of ChE activity [66]. All synthesized compounds, donepezil and galantamine, were weighed and dissolved in DMSO (to prepare 10\(^{-3}\) M stock solutions). For the preparation of the working standards (10\(^{-4}-10^{-3}\) M), stock solutions diluted in 100 mM phosphate buffer (pH 8.0). The final concentration of DMSO in the working solutions was less than 1.3 (V/V %).
To investigate the anti-cholinesterase activity of compounds, 550 μl phosphate buffer solution (100 mM, pH 8.0), 150 μl DTNB solution (0.5 mM), 150 μl of the studied compound solution and 150 μl ATCl solution (1.0 mM) (for hAChE) were added to test cuvette (1 mL). The mixture was gently shaken and incubated at 37˚C for 5 min. The reaction was begun by the addition of 50 μl of the hemolysate to the test cuvette (total volume of the mixture was 1050 μL). Absorbance was monitored at the wavelength of 412 nm over 10 min. The same method described was applied for hBuChE Inhibition assay, while instead of ATCl, BuTCI was used.

Double reciprocal plots (log [inhibitor concentration] vs. the percentage of inhibition) were used to calculate the IC$_{50}$ values.

4.1.4. Kinetic study

The inhibition assay was done at 4 different concentrations of ACTI (0.06-1.0 mM). The velocity of enzymatic reaction was measured with two different inhibitor concentrations (that showed 30-70% inhibition) and without inhibitor. To obtain inhibition constants ($k_i$) of hAChE, double reciprocal plots (Lineweaver–Burk plot) of 1/V vs. 1/[S] were applied. Also, we calculated non-competitive inhibition constants ($K_i'$) using the plot of 1/V$_{app}$ vs. inhibitor concentration [67].

4.2. Molecular modeling study

Docking studies were done using the Gold software. The crystal structures of hAChE (PDB code: 4EY7) and hBuChE (PDB code: 4BDS) were downloaded from the protein data bank (http://www.rcsb.org).

Before docking, co-crystallized inhibitors and unnecessary water molecules were extracted from the crystal structure, and hydrogens were added using the protein preparation module of gold software. The binding mode of donepezil was studied using Ligandscout software, and the binding pocket, as well as pharmacophores, were calculated. The binding cavity was designed according to the binding mode of these compounds to the enzymes and the obtained information from literature about the important amino acids for interaction in the binding gorge. Studied molecules were docked against the desired enzymes using the gold-score method which followed by a chemscore re-docking procedure. The best conformer was visualized using ligandscout software.

To validate the developed docking method, the co-crystallized compounds i.e. donepezil, and buthyrilethiocholine, respectively, for 4EY7 and 4BDS, were removed, and the drawn structures were docked against the designed binding cavity. The resulted conformers were superimposed onto the original co-crystallized compound, and the orientation and mode of interaction were investigated. The obtained RMSD values were regarded as error criteria of the docking procedure. The results showed that the developed docking method was done by RMSD values of less than 2.0 Å.

4.3. Cell toxicity assay (MTT assay)

Toxicity evaluation of synthetic compounds that showed acceptable inhibitory effects was carried out on cell lines B$_{16}$ and MCF$_7$ using the MTT assay. MCF7 is a breast cancer cell line obtained from the Pasteur Institute of Iran, and the results could be regarded as the ability of the tested compounds cytotoxic activity against cancerous cells. B16 is a murine tumor cell line which used for research as a model for human skin cancer cell line obtained from Pasteur Institute of Iran.

MTT assay is a colorimetric method to evaluate the viability of cells. The reduction of MTT in the mitochondria of living cells leads to the production of a formazan product with purple or violet color, which is not soluble in water [36].

For the MTT method, the cells were passaged in RPMI 1640 with 10% FBS (Gibco, Grand Island, NY, USA). After cell culture, cells were separated with trypsin-EDTA of the flask. (Gibco, Grand Island, NY, USA). 200 microliters of a cell suspension containing 10$^4$ cells per well of 96-well plates load, and for 24 hours at 37 °C, the concentration of 5% CO2 and appropriate humidity were pre-incubated. After this time, cells were exposed to different concentrations of compounds and MTT (Sigma-Aldrich) solution with 5mg/ml concentration, for 48 and 4 hours were placed respectively. Then 200 microliters of DMSO (Merck- Germany) and 25 ml Sorensen's buffer as reaction stopper were added to each plate. The plate was located on a plate stirring device after 40 minutes. Absorption at 570 nm was measured by Elisa plate reader. The data were analyzed by Graphpad prism7 software. For validation of this method, positive control (culture medium and cells) and negative control (culture medium, cells, and DMSO) were placed.

4.3.1. PC12 Cell culture
Rat pheochromocytoma cells PC12 were used to investigate the neuroprotective effect of compound 5g on neurotoxicity. The culture medium was RPMI 1640 medium that contained 10% horse serum, 5% fetal calf serum, and gentamicin 50 mg/ml. Plastic flasks were coated with 0.03% poly-L-ornithine and were used for the incubation of monolayer cultures (density = 0.1 to 0.3 × 10⁶ cells/cm²) in a 95% air, 5% CO₂ and humidified atmosphere at 37°C. The culture medium was refreshed every 48 h. PC12 cells that were grown on 10-cm dishes were exposed to 5 µM Aβ (1-42) (rat/mouse, ab120959) in the presence or the absence of compound 5g at two concentrations (0.1 and 1.0 µM).

4.3.2 DCFH-DA assay

2′,7′-dichlorofluorescein-diacetate (DCFH-DA) was utilized to measure the ROS level. To perform DCFH-DA assay, 10 µM of it was added to the cells of studied groups. To be sure that the DCFH-DA was incorporated into all membrane-bound vesicles the DCFH-DA treated cells were incubated for 15 min at room temperature. This incubation time is enough for esterase to cleave di-acetate group. Then the DAPI counterstained nuclei of the cells were observed under a fluorescent microscope. The excitation wavelength was 358 nm and the emission intensity was recorded at 461 nm. The emission of DCF at 530 nm was measured after excitation at 485 nm using a plate reader. Thermoscientific fluorescence Multiscan spectrophotometer (Finland) was used for all fluorimetric studies.

4.3.3 Tunnel assay

For performing the TUNEL assay, control and treated cells were transferred to slides and exposed to PBS buffer (containing 4% paraformaldehyde, pH 7.4) for 1 h at room temperature. The obtained slides were rinsed with PBS and incubated in blocking solution (methanol 3% in H₂O₂) for 10 minutes. Then they incubated with 0.1% Triton X-100 in 0.1% sodium Citra for 2 minutes in ice after washing with PBS the slides were placed in a wet pan and 50 µl of the reaction mixture was added to each sample and then incubated at 37 °C for 60 minutes. The nuclei of cells were stained with DAPI and were further studied according to the procedure explained in previous section.

4.3.4 TAOC assay

An Assay Kit for measuring the Total Antioxidant Capacity (T-AOC) (Elabscience Biotechnology Inc.) was used to measure the TAOC of the compound 5g. The applied method is based on the inhibition of ABTS oxidation to the ABTS+ by the studied compound. The concentration of the ABTS+ can be obtained by measuring the absorbance of ABTS+ at 414 nm. The studied cells were collected, and after the addition of cold PBS, homogenized using an ultrasonic bath. The supernatant was transferred to a proper tube, and the reagent was added to the solutions. The absorbance intensities and the total protein concentration were recorded for all samples. The calculations were performed based on the kit provider guideline.

4.3.5 MDA assay

Biocore Diagnostik (ZellBio) MDA assay Kit was used for the assessment of lipid peroxidation. The method is based on the formation of MDA (Malondialdehyde) – TBA (thiobarbituric acid) adduct from the MDA-TBA reaction at high temperature. Then MDA is measured in hot (90-100°C) acidic media using colorimetric measurements at 532 (530-540 nm). In summary, after the preparation of the cell homogenate, the reagent was added to the samples, and the results were recorded and manipulated, as noted by the kit provider.

4.3.6 LDH assay

LDH release was measured using a CytoSelect LDH Assay Kit (Cell BioLabs, San Diego, CA) based on the standard protocol. PC12 cells were placed in the 96-well plates (1×10⁴ cells/well) and cultivated 24 h prior to experiment performing. The cells were treated with Aβ1–42 (20 µM), with or without various concentrations donepezil and compound 5g for 48 h. Then 90 µl of the supernatant was transferred to a well containing 10 µl LDH reagent and incubated for 30 min. The optical density was recorded at 450 nm using Epoch™ spectrophotometer (BioTek Instrument).

5. IL-1β and TNF-α genes expression analysis:

SYBR Green method based real-time PCR was used to the measurement of mRNA level of the IL-1β and TNF-α genes in PC12 cells. Total RNA (SinaClon, Tehran, Iran) was isolated using RNX-PLUS solution. Revert Aid Reverse Transcriptase Kit (Thermo Fisher,
Waltham, MA, USA) was used to the synthesis of the complementary DNA (cDNA). β-actin was utilized as an endogenous control for mRNA expression. The relative amount of gene expressions was calculated using $2^{-\Delta\Delta CT}$ method. PC12 cells were treated with Aβ (5µM) and the level of the IL-1β and TNF-α genes were investigated in the presence and absence of the donepezil and compound 5g.

6. in vivo studies

6.1. Animals

60 adult male Wistar rat with the age of eight weeks and weight of 250-280 g were used for animal studied. Standard cages that were exposed to 12/12 h light/dark cycle at 23 ± 2 °C temperature were used to keeping the animals. the access to the food was ad libitum. All experiments were done based on the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH; Publication No. 85–23, revised 1985). This study was ethically approved by Health Ministry of the Islamic Republic of Iran under the grant number of (NO: 97015588) and the obtaining of the blood samples from a healthy volunteer was approved by local ethics (IR.TBZMED.REC.868).

6.2 Intracerebroventricular (i.c.v.) injection of Aβ1–42 peptides

Aβ1–42 was dissolved in PBS (10 µM) and incubated 4 days at 37°C for peptide aggregation. Bilateral i.c.v. injection of obtained Aβ1–42 solution was done by a stereotaxic apparatus. To implanting the sterile stainless steel guide cannula (26-gauge) in the injection sites the rats were anesthetized by i.p. administrating of a ketamine – xylazine (90/10 mg/kg) solution. The injection site coordination was selected based on the rat brain in stereotaxic coordinates [68]. The position was located in the anteroposterior from bregma (AP) = -0.96, mediolateral from the midline (ML) = ±2 and dorsoventral from the skull (DV) = 3.5.

An infusion pump was used to infuse Aβ1–42 (50 pmol per animal) (10.3390/molecules22112007). The same protocol was utilized for Sham-operated animals, except for the injection of vehicle instead of all rats were individually housed following 1 day of surgery then returned to the initial care state.

The rats were divided in 6 different groups (n=10). Animals in sham surgery and AD groups received 1 ml/kg/day normal salin via oral route. Other animals in AD groups received donepezil (2 mg/kg/day) or compound 5g (1, 2 or 4 mg/kg/day) via similar route for 21 days.

6.3 Morris water maze (MWM) protocol

To perform MWM studies we used an apparatus in which the black circular pool (120 cm diameter and 60 cm height) of it filled with water (24–25 °C, depth 30 cm). the pool was equipped with a 10 cm diameter submerged transparent escape platform that was placed 1 cm above (visible platform) or 1 cm below (hidden platform) the water level. Then, the pool was divided into four hypothetical quadrants for test start positions. Data were recorded using a video camera that was connected to a computerized tracking system (EthoVision XT). The camera was placed above the pool center.

6.4 Visible platform

The hidden platform was begun 1 day before the visual version of the water maze. To perform this experiment the platform was marked by beacon and the rat was allowed to find the platform spontaneously within 60 s and left on the platform for 10 s. in the case that platform was not found by rat, it was firmly directed to the platform by investigator. The animals’ performance on the visual platform task confirmed their sufficient vision and the motor and mental competence to do the task.

Hidden platform

The working memory could be evaluated using hidden platform. The hidden platform was 1 cm below the water surface. For the spatial learning (acquisition and probe trials) task, in each trial, the time spent by the rat to find the platform was recorded as an escape latency time.

6.5 Probe test
Reference memory was evaluated via a probe test. To do this on the next day after the last learning trial, a single probe trial was performed in which animals were allowed to freely swim without a platform for 60 s. The analysis of probe trial shows the index of memory, in which the time spent in the platform quadrant was noted.

### 6.6 Novel object recognition (NOR) Test

A NOR test was done in 3 sessions (habituation, training, and retention) with moderate modifications. The Plexiglass open-field box (50 ×50 ×30 cm) and regular objects with various shapes and textures were utilized. The rat's nose direction to the object (distance of ≤2 cm) and rearing up against the object was noted as exploration. The total locomotor activity (in habituation session) and the time spent with each object was recorded using video camera and scored using EthoVision XT video tracking software.

After the placing of the rats in the box in the absence of objects for 10 min for habituation. They were trained using 2 identical objects (A and A'). The total time spent to explore objects was recorded. In the retention session, the animals was returned to the same task by replacing the objects with new objects named B.

Discrimination index (DI) was calculated and utilized as an index of the recognition memory through the following equation.

\[
DI = \frac{(N-F)}{(N+F)}
\]

where N stands for the time that was spent to explore the new object and F is the time that was spent to explore the familiar object in retention sessions.

### 6.7 Rat brain tissue sampling

After the finalization of the behavioral test brain tissues were removed for enzyme activity and cytokine level investigations. Prior to the removing of brain tissue, deeply anesthetized animals following i.p. injection of ketamine/xylazine (90/10 mg/kg) and decapitated. As soon as the brain tissue was removed it transferred on ice the hippocampal tissue was isolated. The isolated hippocampal tissue was frozen in liquid nitrogen and stored at −70 °C for further analysis.

### 6.8 Cholinesterase activity assay in the rat brain

The activity of the cholinesterase enzyme in rats treated with compound 5g was studied using the cholinesterase quantitative assay kit (HITACHI 917 / MODULAR P). To do this the proper dilution of the tissue lysates supernatant were subjected to the reagent which contains Butyrylthiocholin. Cholinesterase enzymes hydrolyze the butyrylthiocholin to thiocholin which reduces the yellow \((\text{Fe(CN)}_6)^{3-}\) to the colorless \((\text{Fe(CN)}_6)^{4-}\). The results could be analyzed using colorimetric assays.

### 6.9 Measurement of the expression of pro-inflammatory cytokines (TNFα and NFκB) and AChE in rat brain using western blot analysis

The expression of proinflammatory cytokines (TNFa and NFkB), AChE and GADH in rat brain in the presence of compound 5g and donepezil was studied using western blot. The tissue samples were lysed and the lysates were centrifuged at 4°C 12000 rpm for 10 minutes. Protein concentration of supernatants were determined by Bradford method. Bovine serum albumin was used as standard protein. After mixing the samples with sample buffer (containing Tris, glycerol, -mercapto ethanol, Bromo phenol blue and SDS), they were heated for 10 minutes in boiling water. Proper amount of protein extract were subjected to 12% SDS-PAGE gels and the resulted spots were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane were shaken 5 times (15 min) with the blocking agent (5% defatted milk powder in TBST (Tris buffered saline-Tween 20)) at room temperature before the incubation with primary antibody. After the blocking the membrane were incubated primary antibodies for 18 hours. The primary antibody was diluted with blocking buffer. The primary antibodies were β-actin (sc-47778, 1:300) as internal standard, AChE (E-AB-70014, 1:1000), NFkB-p65 (E-AB-22066, 1:1000), TNFa (SC-130349, 1:1000). The membrane were washed three times (15 min) with TBST, and were incubated (75 min) with secondary antibodies (m-IgGkBP-HRP (sc-516102, 1:1000) and mouse anti-rabbit IgG-HRP (sc-2357, 1:1000)). Enhanced chemiluminescence (ECL) (Millipore, USA) was used for visualization. Optical density analysis was performed for semi quantitative measurements.

### 7. Statistical analysis:
The obtained data for all cell based and animal studies were statistically analyzed using One-Way ANOVA, Two-Way ANOVA or Post-hoc analysis. GraphPad Prism software (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) and Excel software were used for visualization of graphs. p<0.05 was considered to be statistically significant.

Results And Discussion

Synthesize of the quaternary ammonium derivatives

The synthetic route of the key intermediate is shown in Figure 2. 4-flourobenzaldehyde (1a) reacted with various secondary amines (2a-f) by nucleophilic substitution reaction [35] and compounds 3a-f were produced. Then 1b-d, 3a-f and 3h compounds reacted with 5, 6-dimethoxy-2, 3-dihydro-1H-inden-1-one. More details of the synthesis procedure optimization and characteristic of synthesized compounds could be found in our previous papers[34,35].

We synthesized the designed compounds in the current study with a minor revision comparing to our previous papers[34,35], to avoid superfluous byproducts produced. We conducted the reaction at room temperature, using EtOH as the solvent and aqueous NaOH (10 %) as the base. The quaternary ammonium compounds (5g-i) were synthesized from (5f-h) reacting with methyl iodide. The details of the chemical characterization of two newly synthesized compounds (5g and 5i) using 13C NMR are presented in figure 3 and detailed characterization and IR and 1H NMR results are presented and methods section.

hAChE and hBuChE inhibitory activity of synthetized molecules

The anti-cholinesterase activity of the synthesized compounds was studied against hAChE and hBuChE. Compounds 5h, 5j, 5k, and 5l were practically insoluble in the applied solvents, and consequently, their inhibitory activity was not further evaluated.

The IC50 values (Table 1) of the studied compounds toward hAChE were in the range of 0.1-75 μM. Compound 5g (IC50 = 0.1 μM) possesses the highest hAChE inhibitory activity lower than donepezil (IC50=0.014 μM). In comparison with Galantamine (IC50=3 μM), which binds to the anionic site of the hAChE, compounds 5g (IC50 = 0.1 μM) and 5f (IC50 = 0.9 μM) possessed higher inhibitory activity.

hBuChE inhibitory evaluations showed that compound 5g inhibitory activity (IC50=4 μm) is higher than donepezil (IC50=5.25 μm) and galantamine (IC50=31.6 μM). The selectivity index for hAChE to hBuChE inhibition was calculated for studied compounds, and the results (Table 1) showed that compound 5i possessed much higher selectivity toward hAChE in comparison with donepezil and other studied compounds. Compound 5g is almost inactive against hBuChE. Also, compound 5f was more selective toward hAChE in comparison with 5g.

Kinetic studies results (Table 1) showed that the synthesized compounds possessed mixed non-competitive inhibition, except for compound 5d that showed competitive inhibition. According to the results, inhibition constants (ki) of compounds 5f and 5d were smaller than their non-competitive inhibition constant (ki’) values. This finding showed that the tendency of these compounds to bind to the active site is more than the steric site. In addition, ki values of compounds 5f and 5g were smaller than ki value of compound 5d, which shows the higher affinity of compounds 5f and 5g to the steric site. In conclusion, the better inhibitory activity of compounds 5f and 5g in comparison with compound 5d can be a result of their tendency to bind to both steric and active sites of enzymes. Double reciprocal plots and secondary plots of compound 5g inhibitory activity are shown in Figure 4.

SAR analysis of the studied compounds’ cholinesterase inhibitory activity

Incorporating the quaternary ammonium to the para position of phenyl ring (compounds 5g and 5i) resulted in increased ChE inhibitory activity significantly. Comparing compounds 5i and 5g shows that when this quaternary ammonium is a part of piperazine ring (5g) the inhibitory effect against both enzymes increased significantly. Corresponding non-quaternary amine substituted compounds (i.e. 5f and 5h), showed much less ChE inhibitory activity (Table 1). Comparison of compounds 5f and 5h confirmed the importance of piperazine ring. Substitution of piperazine with morpholine (compound 5c) leads to a significant reduction in inhibition efficacy in both enzymes, which shows the importance of a positively charged moiety rather than a functional group with hydrogen bonding capability.
A similar range of activities for compounds 5a-e against both enzymes revealed that the presence of positive charge in the exact distance from the aromatic linker is important for ChE inhibitory activity in which replacing piperazine with piperidine moiety (compound 5a) leads to significant activity reduction (Table 1). This finding confirms the importance of the presence of a positively charged moiety in this position [37]. This parameter also is much more important than hydrophobic moieties or the size of the group, as it’s obvious from compound 5i, in which the ring is substituted with quaternary ammonium, while hAChE inhibitory activity is about 10 times more than compounds 5a-e which have a different ring in this position. Simultaneously, a significant reduction in hBuChE inhibitory activity of compound 5i shows a significant impact of hydrophobic interactions on the interaction of studied compounds with hBuChE binding pocket. The same finding was approved by a significant reduction of compound 5f activity against hBuChE compared with 5g, which has one more methyl group. Also, loss of action of compounds 5h-5l against hBuChE confirms the findings above of the importance of positive charge and hydrophobic groups for both enzymes.

Toxicity profiling of compounds 5g and 5f

The cytotoxicity of the compounds with higher hAChE inhibitory activity (5f and 5g) was studied using MTT assay on MCF7, B16 cell lines, and the resulted LD50 values are represented in Table 2. It is obvious that compounds 5g and 5f possessed lower cell growth inhibitory activity against studied cells (MCF7, B16). Comparing the LD50 values of these compounds with donepezil revealed that they show less cell toxicity. Galantamine LD50 values toward both cell-lines are higher than the studied compounds. The obtained LD50 values for both studied compounds were significantly higher than their IC50 values toward hAChE and hBuChE.

Inhibition of Aβ-induced death of PC12 cells by compound 5g

The cytotoxic effect of Aβ aggregation in neuronal cells is one of the main hallmarks of AD. Attenuation of Aβ aggregates is an understudy strategy for developing anti-AD drugs[38,39]. One of the frequently applied in vitro models for studying the neuroprotective effect of drug candidates is PC12 cell death induced by Aβ1-40 [40]. The underlying mechanism of cell death due to the accumulation of Aβ aggregates is not clearly known yet, but some studies suggested the crosstalk between autophagy, apoptosis, ROS production, and inflammation as probable mechanisms[41,39]. Autophagy modulation has been suggested as a target for AD drugs. The autophagy activation mediates neuroprotection against induced cytotoxicity by Aβ, oxidative stress and apoptosis[42,43,40]. ROS role in the neurological disorders development have been described in different studies [44]. Rising number of studies are indicating the transaction between ROS and autophagy as a determinant factor in the neuronal homeostasis modulation [44]. Drugs that modulate autophagy and/or apoptosis attracted much attention in recent years[45,39].

To examine the multi-targeted capability of the compound 5g, we studied its neuroprotective effect against Aβ-induced PC12 cell death. PC12 cells were incubated with 5 μM Aβ (1-42) for 24h in the presence and absence of the compound 5g (0.1 and 1.0 μM) [46]. Fig 5 shows the transmission electron microscopy (TEM) images of the normal, Aβ treated, and Aβ+5g treated PC12 cells. The normal PC12 cells (figure 5a) shapes are round, clear, and spindle, dispersed normally (Figure 5). Figure 5b shows the decreased viability of PC12 cells’ after incubation with Aβ. Apoptotic bodies were seen easily; cells grew more slowly than normal cells. The morphology of the Aβ treated cells changed in a way that they are less clear, and they kept their spindle shape [47]. The addition of compound 5g at both concentrations (0.1 and 1.0 μM) was reversed the Aβ induced cell death (figure 5c and 5d). The protection was increased at a higher concentration of compound 5g.

The prevention of Aβ-induced neurotoxicity may occur via different mechanisms such as acting as an anti-oxidant agent, attenuating neuroinflammation, reduction of Aβ fibril formation, inhibition of cell membrane damage, and some other mechanisms [39]. The probable mechanism of the Aβ-induced cell death reduction by compound 5g was studied, and the results are reported in the following sections.

Compound 5g effect on oxidative stress

Oxidative stress is one of the AD hallmarks, and drugs with antioxidant activity are candidates for MTD anti-AD agents. The antioxidant activity of drugs attributed to their capacity for biological system protection against the ROS and reactive nitrogen species (RNS) damage. Total antioxidant activity (TAC) is the general ROS and/or RNS scavenging capacity. Acute oxidative stress can lead to the rapid up-regulation of autophagy via post-translational modifications of key autophagy regulators. ROS can induce single-strand DNA breaks, specifically in telomeric regions, resulting in collapsed replication forks, un-replicated single-stranded DNA, and telomeric loss. Also, ROS can target lipid double bonds that will lead to the production of reactive aldehydes like
malondialdehyde (MDA) via lipid-peroxidation procedure [48]. Reactive aldehydes can disrupt the cell membrane structure and function by interaction with membrane proteins, which consequently leads to the neuro-toxicity in the AD brain [49,50]. To examine the effect of compound 5g on Aβ induced death in PC12 cells via oxidative stress-related mechanisms, we studied compound 5g effect on TAC, ROS production, and lipid peroxidation (MDA level).

**Total anti-oxidant, anti-ROS production and autophagy modulation capacity of compound 5g**

We used 20, 70-dichlorofluorescein di-acetate (DCFH-DA) assay to the measurement of the ROS level. Cellular esterase’s can converted nonpolar DCFH-DA (an oxidant-sensing fluorescent probe) to its polar derivative (DCFH). DCFH is a non-fluorescent moiety which can be oxidized by intracellular ROS to highly fluorescent DCF. Fig 6 A shows the images obtained using the fluorescence microscope for Aβ treated cells in the presence and absence of the compound 5g.

The counter staining of the nuclei of the cells was done using 4′,6′-diamidino-2-phenylindole (DAPI). Using the DAPI staining, the cell nuclei along with the apoptotic bodies that include condensed and fragmented nuclei) can be illustrated (Figure 6A). The nuclei of living or dead cells can emit blue fluorescence upon binding of DAPI to adenine–thymine regions of DNA. An increased number of the cells with small, condensed nuclei after incubation with Aβ shows the enhancement of the apoptotic cells.

The morphological evaluation of DAPI stained cells (Figure 6A) shows the capability of the compound 5g on the prevention of Aβ induced apoptosis. The apoptotic cells tend to lose cell structure. They also showed altered nuclear condensation. The control cells keep their intact and evenly shaped structures along with nuclear condensation.

Fluorescence intensities (Figure 6B) indicate the ROS level in the studied samples. Much intense green fluorescence of Aβ-treated cells in comparison with normal cells (figure 6A) shows enhanced production of ROS. The presence of the compound 5g leads to a reduction in ROS production. The DCF fluorescent intensity of the normal cells (20%) increased in Aβ treated cells (Figure 6B). In contrast, compound 5g leads to less enhancement of the DCF fluorescent intensity. ROS reduction in the presence of the compound 5g could occur both via radical scavenging property of compound 5g and autophagy modulation.

The total antioxidant activity of compound 5g was investigated using the fluorescein based assay for oxygen radical absorbance capacity. As seen in Fig. 6C, the lowest absorbance of fluorescein observed in Aβ-treated cells (69%), while in the presence of compound 5g (0.1 and 1 µM) the fluorescein absorbance was increased from to 74% and 87%, respectively. These results indicate the capability of the compound 5g for the cell protection against Aβ-induced cell death is partially via anti-oxidant activity.

**Lipid peroxidation inhibitory effect of compound 5g and Preventive effect of compound 5g on membrane damage**

The capability of compound 5g for the inhibition of lipid peroxidation was studied by the measurement of the MDA level in Aβ treated PC12 cells. Figure 7A shows the MDA concentration in the studied cells. MDA levels was increased significantly in the Aβ1-42 treated PC12 cells in comparison with control cells. The increased level of the MDA due to the Aβ1-42, was reduced by the compound 5g. The results showed that the inhibition of lipid peroxidation plays a role in compound 5g prevention against Aβ induced cell death.

Plasma membrane damage is one of the key features of different forms of cellular damage, such as apoptosis and necrosis. Lactate dehydrogenase (LDH) is rapidly released into the cell culture supematant when the plasma membrane is damaged [51]. Also, the intracellular LDH level is a measure of cell viability. LDH activity is significantly increased from about 20% in normal cells, to73.6% in Aβ treated cells (Fig. 7B). This finding suggests that the oxidative modification by Aβ (1-42) contributes to the impaired function of the PC12 cells. The compound 5g resulted in a reduction in LDH levels at both studied concentrations (68% for 0.1 µM and 55% for 1.0 µM), respectively. It could be concluded that the compound 5g contributes to the prevention of cellular membrane damage mechanisms. As mentioned, cellular membrane damage could occur via different mechanisms due to different pathways activation such as oxidative stress, apoptosis, autophagy, and other mechanisms.

**The effect of compound 5g on DNA fragmentation and morphology of the apoptotic cells**

The degree of the DNA fragmentation and morphology of the apoptotic cells were studied using a terminal deoxynucleotidyl transferase nickend labeling (TUNEL) assay. Apoptosis index for the normal cells and Aβ treated cells in the presence, and the absence of the compound 5g are shown in figure 8A. The counterstained nuclei by DAPI and merged images are presented as well. The much intense green color in the Aβ treated cells compared with control cells shows that the Aβ cell toxicity happens in part via an
apoptotic mechanism. The apoptotic index (percentage of the apoptotic cells, Figure 8B) increased from about 18% in control cells to 60% in the Aβ treated cells. The addition of the compound 5g to the incubation medium resulted in the reduction of the apoptotic index from 60% to 35% and 25% for the compound 5g concentration of 0.1 and 1.0 µM, respectively.

The TUNEL assay also revealed apoptotic characteristics in Aβ treated cells. The untreated cells showed blurred coloration, while apoptotic cells were stained by TUNEL reagent (Figure 8A). These results indicate the apparent DNA fragmentation and apoptotic nuclei. Also, the morphological variation and losing cell integrity of apoptotic cells are obvious compared to the normal cells.

Compound 5g could prevent Aβ induced apoptosis by preventing both loss of cell integrity and DNA fragmentation. Cell membrane integrity loss is a hallmark of necrotic cell death, and necrotic cell death has been suggested as a player in neurodegenerative disease. According to the results, it could be concluded that compound 5g prevents PC12 cell death by contributing to oxidative stress, autophagy, apoptosis, and maybe necrosis. It also reduces radicals, prevents lipid peroxidation, and inhibits cell membrane damage.

**Effect of compound 5g on neuro-inflammation**

Activated microglia mediates the Aβ peptide based neuroinflammation. interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) are pro-inflammatory cytokines which can be increased following the microglia activation and could influences the brain tissue and play a role in neurodegeneration as well as in neuroprotection [52]. Anti-inflammatory compounds that could reduce the expression of TNF-α and IL-1β have been investigated for their anti-AD potencies. We studied the expression of mRNA levels of TNF-α and IL-1β in the presence and absence of compound 5g and donepezil. The mRNA level of IL-1β and TNF-α in Aβ treated PC12 cells increased in comparison to the normal cells (Figure 9). Also, compound 5g prevented the enhancement of the IL-1β and TNF-α mRNA levels due to the Aβ induced cell toxicity at concentrations of 0.1 µM (p=0.0272 for IL-1β and p=0.01 for TNF-α) and 1 µM (p=0.0002 for IL-1β and p=0.0002 for TNF-α). Furthermore, decreased expression levels of these genes were observed in compound 5g (1 µM) treated cells compared to control cells (p=0.0397 for IL-1β and p=0.0478 for TNF-α). The results were illustrated in figure 9 and Table 3. According to the results compound 5g is capable for decreasing of inflammatory genes expression in which its potency increases at higher doses. These findings suggested an anti-inflammatory mechanism for the compound 5g in addition to other pharmacological effects.

**In vivo anti-Alzheimer efficacy of compound 5g**

Effect of the compound 5g on the recognition function of Aβ induced AD rats

The capability of the compound 5g on the recognition function of rats was studied using the novel object recognition (NOR) task. NOR measures a specific form of recognition memory, and it has been increasingly used as an experimental tool in assessing drug effect on memory. The results of NOR test (Figure 10) revealed that there is no significant difference among studied groups in the locomotor activity (p = 0.39) and total observation time (p = 0.77) (Fig 10A and 10B). This approves the reliability of the task results for episodic memory. It also shows that the task results are independent from locomotor or observation biases. Discrimination index (DI) factor is an episodic-like memory index that was calculated for the studied groups and the results indicated that the DI between groups are significantly different (p < 0.001). DI reduced in the normal saline group (p < 0.001), which indicates the impairment of episodic-like memory. Furthermore, a significant increase of DI in donepezil (p < 0.01) and compound 5g (1 µM) treated groups (p < 0.001) (Fig 10 C) was observed, which could be an indicator of protective effect of donepezil and compound 5g against episodic-like memory impairment.

According to the results, it could be concluded that although compound 5g includes a permanently charged amin group, it reaches the brain of AD rats. As mentioned in the introduction choline transporter has been suggested as a root for transportation of the permanently charged molecules to the brain. As can be seen from the results (Figure 10C), by increasing the compound 5g dose from 0.5 to 1 µM, an obvious improvement was observed in the recognition function of rats. This could be a result of the role of a transporter in compound 5g transportation to the brain. As we used the Aβ model of AD, and Aβ has been suggested as an inhibitor of choline transporter, the significantly lower efficacy at lower concentrations of compound 5g could be a result of their disability to reach the brain due to the inhibition of choline transporter. Moreover, the efficacy of compound 5g in the improvement of cognition function of AD rats is comparable with donepezil (0.5 µM) as a positive control. Due to the probable dependence of compound 5g transportation to the brain to choline transporter and its inhibition by Aβ, its application of albumin may lead to different results.
Effect of the compound 5g on the spatial learning and memory function of Aβ induced AD rats

In addition, we conducted a Moris water maze (MWM) test to investigate the spatial learning and memory function of Aβ-induced AD in rats. The escape latency time was studied during the training days in control and treated groups. The comparison of the results showed that there is a significant effects of day and group (p < 0.001), as well as group×day (p = 0.04). Such results indicated that despite the fact that the task was learned by animals across training days, different learning outcomes were observed among groups. Furthermore, posthoc analysis of the obtained results revealed a longer escape latency time for the normal saline-treated group in the 2-4 days of the training session (p < 0.001, p < 0.01, and p < 0.05, respectively), which shows the lower target-platform finding ability of animals in this group. Furthermore, escape latency time on the 3rd and 4th days were significantly decreased in the presence of donepezil and compound 5g (p < 0.05 or p < 0.01) (Fig. 11A). Also the time that spent to find the target quadrant was significantly different between groups in the probe session (p < 0.001). The time spent in the target quadrant was different between sham and normal-saline-treated groups (p < 0.01), that confirms the impairment of spatial memory. Also, a significant difference was observed between the normal-saline-treated group with donepezil and compound 5g (1 µM) treated groups (p < 0.05) (Fig. 11B).

Effect of compound 5g on the expression of pro-inflammatory cytokines (TNFα and NFκB) in Aβ-induced AD rat brain

We used the western blot analysis of the hippocamp tissue biopsy obtained from AD rats’ brain to investigate the effect of compound 5g on the pro-inflammatory cytokines (TNFα and NFκB) level. Minor downregulation of TNFα in hippocamp tissue was observed in compound 5g treated AD rats’ brain (Figure 12). We showed that a significant reduction occurs in TNFα mRNA expression in Aβ+ compound 5g treated PC12 cells. NFκB downregulated due to the compound 5g administration significantly in a dose-dependent manner (Figure 12). Activated NFκB regulates the expression of COX-2 enzyme, TNF, IL-1, IL-6, IL-8 cytokines, adhesion, and cell cycle regulatory molecules [39]. The compound 5g mediated reduction of NFκB may results in neuroinflammation modulation of neuronal cells. Downregulation of NFκB and TNFα was observed in donepezil treated AD rats’ hippocamp (Figure 12).

Effect of compound 5g on total ChE activity and AChE level in rat brain

It is well known that the ChE activity and expression show a diverse correlation with inhibitors and physiologic conditions such as variations in Aβ precursor’s level. It is reported previously that the Aβ treated neuronal cells show downregulation of AChE expression. Also some studies reported that Aβ could enhance the ChE hydrolyzing activity, which could be a result of the formation of soluble ultra-reactive acetylcholine-hydrolyzing Aβ-ChE complexes [53]. Our results showed that the Aβ treatment leads to reducing the ChE activity and downregulation of AChE in rat brain. The reduction of ChE activity can be a result of its downregulation or complex formation with Aβ.

For the evaluation of the compound 5g effects on the ChE activity in the Aβ-treated rat’s brain, the butrylthiocholine concentration was measured using a colorimetric method. Moreover, the AChE level was quantitatively measured using the western blot method. The results are shown in figure 13.

According to the results compound 5g (0.5 µM) prevented the downregulation of AChE in Aβ-received rats’ brains. Compound 5g also decreased ChE activity in comparison with the control group (figure 13B) while the activity reduction was less than Aβ treated group and more than donepezil treated group.

Donepezil could not prevent the Aβ induced downregulation of AChE while it inhibited the ChE activity reduction due to Aβ. Compound 5g (0.1) almost completely reversed the regulation profile of AChE, while its effect on ChE activity was not significant comparing with Aβ treated group at all studied doses. ChE activity reduction due to compound 5g could be a result of ChE inhibition by it. While the prevention of downregulation of AChE could be a result of non ChE inhibitory anti-AD effects of compound 5g which was investigated and reported in this study.

These results could be due to the differences in ChE inhibition mechanism of donepezil and compound 5g in a way that donepezil possess competitive inhibition while compound 5g possess mixed non-competitive inhibition based on the kinetic studies. Furthermore, compound 5g is inactive against BuChE while donepezil inhibits both enzymes that could be a reason for their different efficacy on the reversion of Aβ induced ChE activity and AChE expression variation in part.

Molecular docking analysis and pharmacophore mapping
To optimize and validate the docking procedure, donepezil was docked to the binding cavity and the results compared with co-crystallized donepezil with hAChE (pdb code 4EY7). Figure 5 shows the superimposition of docked donepezil on co-crystallized donepezil with hAChE. The developed docking procedure was able to predict the binding mode well and resulted in high geometric similarity to the ligand conformation observed in the crystal structure.

Active compounds were docked in the binding pocket of hAChE (pdb code 4EY7) and hBuChE (pdb code: 4BDS). The best conformer (highest score and best orientation) of each compound was further studied using MOE (add reference: Molecular operation environment v2019.0102, Chemical Computing Group, Montreal, Canada) and LigandScout [54] software to investigate ligand–protein interactions and pharmacophore mapping of them.

Figure 14 shows the 3D illustration of the positioning of the compounds 5f and 5g in the binding cavity of hAChE and hBuChE. Three main common structural characteristics of the binding pocket of both hAChE and hBuChE (Figure1) are the availability of a deep narrow gorge (20 Å), the catalytic active (anionic) subsite (CAS) in conjunction with a choline-binding pocket (esteratic subsite) makes the catalytic machinery located in the bottom of the gorge beyond a peripheral acyl pocket [55] and an anionic site in the entrance of the binding gorge which is known as peripheral anionic site (PAS).

The structural features of AChE and BuChE binding sites are shown in figure 1. CAS of both enzymes consists of conserved amino acids known as a catalytic triad; i.e., Ser203, His447, Glu334 in hAChE and Ser198, His438, Glu325 in hBuChE. The choline-binding pocket is consisting of Trp86, Phe330, and Tyr337 amino acids in hAChE and Trp82, Phe329, and Ala328 in hBuChE. The aliphatic moiety of inhibitors is interacting with Trp86/82. There is an oxyanion domain near to choline-binding pocket, which is responsible for a hydrogen-bond network between substrate and enzyme. Conserved water molecules in this domain are taking part in this network. Also, there is a peripheral acyl binding pocket in both enzymes, consisting of different amino acids. Peripheral acyl binding pocket of hAChE includes Phe295 and Phe297 replaced by Leu286 and Val288 in hBuChE. PAS takes part in complex formation with Aβ and initiates the fibril formation. PAS of hAChE includes hydrophobic residues, i.e., Tyr72, Asp74, Tyr124, Trp286, Tyr337, which enables interactions with charged moieties of inhibitor compounds [56]. Blockers of PAS would inhibit the interaction of hAChE with Aβ and decrease consequent neurotoxicity [57,58]. PAS of hBuChE consists of Asp70, Phe298, and Try332, which like hAChE PAS interacts with positively charged substrates and guides them down the gorge to the catalytic triad [59]. The main difference between PAS in hAChE and hBuChE is the less acidic nature of the hBuChE PAS. Lack of aromatic residues in three subsites of the binding gorge; i.e., PAS, choline binding pocket, and acyl binding pocket of hBuChE leads to the significantly larger gorge of hBuChE in comparison with hAChE [60,61], which enables the catalysis of larger substrates. The difference between acyl pockets of these enzymes is one of the most valuable features for developing selective inhibitors against hAChE and hBuChE, while the similarities at the same time facilitate dual inhibitor development.

The results show that the availability of a positive charge in an appropriate position facilitates interaction with Glu197 in hBuChE, and a p-cation interaction with Trp86 and Trp82 of hAChE and hBuChE, respectively. Also, a hydrophobic interaction of one of the methyl groups of compound 5g piperazine ring with Trp86 and Trp82 of hAChE and hBuChE have been observed (Figure 14), which could be a partial replacement of Donepezil phenyl ring interaction with those amino acids. In comparison with donepezil, compound 5g lacks one of the main interactions with Trp86 (i.e. p-p stacking with phenyl ring of donepezil) in hAChE, which could be a reason for lower potency. At the same time, a strong contact (which could be a hydrogen bond rather than an ionic interaction) with Glu197 of hBuChE leads to a bit higher efficacy of compound 5g in comparison with donepezil. Recent studies suggested that Glu197, which is a conserved amino acid within cholinesterase enzymes, has a significant role in the binding of substrate/inhibitors with hBuChE binding sites [62]. Molecular docking studies of the interaction between hBuChE and the studied compounds compared with donepezil revealed that the compound 5g possess similar interactions with the binding pocket while compound 5f lacks interaction with Trp82, which could be resulting from one methyl group removing. Quaternary ammonium itself does not lead to this variation in interaction, as the results showed that the consequent amino moiety of piperazine ring converted to the quaternary amino moiety due to ionization in physiologic pH.

The carbonyl moiety of the indanone ring in compound 5f interacts with catalytic triad amino acids of hAChE via hydrogen bonds [63]. Also, the indanone carbonyl group establishes a hydrogen bond with Phe295 backbone NH. The additional interaction with the acyl binding pocket of hBuChE is obvious for both compounds. Benzilidine group of compounds 5g and 5f possess interaction with Tyr341 and Tyr332 of hAChE and hBuChE, respectively.
The flexibility of CAS interacting fragment of the molecule is important to enable its movement through the bottleneck of the gorge, and the introduction of one double bond to the linker leads to the reduction of the possibility of compounds to reach the CAS. This rigidity leads to the obvious confirmation orientation variation in comparison with donepezil for compounds 5g and 5f, which is presented in figure 15.

This miss-orientation can be observed in both enzymes. Molecular dynamics simulation of donepezil interaction with hAChE revealed that placement of the donepezil in the active site is facilitated by a conformational change (90° bending), which leads to suitable orientation. At the same time, this miss-orientation leads to an extra interaction with both enzymes’ acyl binding pocket, which is not available in donepezil.

Exploring the water molecules in the active site of both enzymes showed that there are some conserved water molecules in hAChE binding gorge, which are necessary for the interaction. The results of molecular docking without removing those water molecules showed that there is the possibility of hydrogen bonding between methoxy moieties and at least one water molecule in PAS. Investigation of the hAChE-donepezil complex shows one water molecule in the suitable distance for such an interaction in PAS. The results are consistent with the previous studies [37], which suggested conserved water molecules in similar situations. Also, according to the reported B-factors for those water molecules, they could be regarded as conserved molecules. Our studies suggested no similar hydrogen bonding in PAS of hBuChE.

Interaction with the peripheral site inhibits the interaction of the hAChE with Aβ, and accordingly, the developed compounds can be regarded both as dual inhibitors [37] and hAChE-hBuChE inhibitors.

Conclusion

Newly synthesized indanone ionic analogs’ structures were characterized using spectroscopic methods. The synthesized compounds cholinesterase inhibitory activity were examined against hAChE and hBuChE. The studied compounds possessed IC\textsubscript{50} values in the range 6 - >75 μM and 4 - >1000 μM respectively against hAChE and hBuChE, except for compounds 5f and 5g, which exhibited IC\textsubscript{50} values of 0.9 and 0.1 μM for hAChE and 126 and 4 μM for hBuChE.

Kinetic studies revealed that all compounds have mixed non-competitive inhibition mechanisms except compound 5d, which showed a competitive mechanism. A molecular modeling study of the compounds showed that compound 5g has dual binding site interaction. In addition, the availability of permanent quaternary ammonium facilitates interaction with the oxyanion hole, which enables the inhibition of hAChE driven Aβ aggregation by the developed compounds. In addition to the inhibition of hAChE compound 5g possessed better hBuChE inhibitory activity comparing with donepezil. Furthermore, compound 5g had neuroprotection against Aβ-induced neurotoxicity. Its anti-oxidant, anti-ROS production, anti-lipid peroxidation, LDH inhibition, anti-apoptotic, and anti-neuroinflammation activity was studied and confirmed using Aβ treated PC12 cells. In addition, in vivo administration of compound 5g to Aβ-induced AD rats improved their cognition function and spatial memory, and learning function. Western blot analysis of Aβ-induced AD rats’ brain biopsies confirmed NfKB reduction, AChE elevation, and CHE activity reduction in studied rats. Compound 5g is a promising lead for the development of hAChE and hBuChE dual inhibitors with the capability of multi target anti-AD activity, while its delivery to the brain has been confirmed by in vivo studies.

Declarations

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Conflicts of interest
The authors declare no conflict of interest.

Author contributions

All authors made substantial contributions to conception, design, and acquisition of data and interpretation of data. In addition all authors participate in drafting the article or revising it and give final approval of the version to be submitted.

Availability of data and material

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Compliance with ethical standards

This study was ethically approved by Health Ministry of the Islamic Republic of Iran under the grant number of (NO: 97015588) and the obtaining of the blood samples from a healthy volunteer was approved by local ethics (IR.TBZMED.REC.868).

- Consent to participate

This study was ethically approved by Health Ministry of the Islamic Republic of Iran under the grant number of (NO: 97015588) and the obtaining of the blood samples from a healthy volunteer was approved by local ethics (IR.TBZMED.REC.868) under informed consent.

Consent for Publication

Not applicable

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Tables

Table 1. Inhibitory Activity of studied compounds
Table 2: Inhibition mechanism, KI and Ki constants of the studied compounds
| Compound | IC$_{50}$ (µM) hAChE | IC$_{50}$ (µM) hBuChE | hAChE/hBuChE | Ki µM | KI µM |
|----------|-------------------|-------------------|-------------|------|------|
| 5a       | 26                | 562               | 0.05        | 37   | 2.1  |
| 5b       | 64                | 501               | 0.13        | 170.0| 78   |
| 5c       | 57.5              | 631               | 0.08        | 1000.0| 47   |
| 5d       | 70                | 501               | 0.14        | -    | 14   |
| 5e       | 55                | 398               | 0.14        | 51   | 13   |
| 5f       | 0.9               | 126               | 0.01        | 0.62 | 0.157|
| 5g       | 0.1               | 4.00              | 0.02        | 0.29 | 0.017|
| 5h       | >75               | >1000             | -           |      |      |
| 5i       | 6.0               | >1000             | -           | 15   | 3.2  |
| 5j       | >10               | >1000             | -           |      |      |
| 5k       | >10               | >1000             | -           |      |      |
| 5l       | >10               | >1000             | -           |      |      |
| Galantamine | 3.0               | 31.6              | 0.009       | 0.84 | 0.090|
| Donepezil | 0.014             | 5.25              | 0.002       | 0.0041| 0.0081|

Table 2: Cytotoxicity of compounds 5f, 5g and reference drugs

| Compound | MCF$_7$ (LD$_{50}$ µM) | B$_{16}$ (LD$_{50}$ µM) |
|----------|-----------------------|------------------------|
| 5g       | 2.21                  | 3.17                   |
| 5f       | 24.25                 | 23.97                  |
| Donepezil | 0.06                 | 0.36                   |
| Galantamine | 31.19               | 34.09                  |

LD$_{50}$: The concentration of compound that causes 50% of cell viability.

Table 3. Effect of the compound 5g and donepezil on neuro-inflammatory genes expression in PC12 cells treated with Aβ

| Target Gene | Control Mean±SD (G1) | Aβ Mean±SD (G2) | 0.1 µmol 5g Mean±SD (G3) | 1 µmol 5g Mean±SD (G4) | P Value |
|-------------|----------------------|-----------------|--------------------------|--------------------------|---------|
|             |                      |                 |                          |                          | G1 vs  G2 | G1 vs  G3 | G1 vs  G4 | G2 vs  G3 | G2 vs  G4 | G3 vs  G4 |
| IL-1β       | 1.012±0.0986         | 3.178±1.422     | 0.6117±0.3142            | 0.1917±0.1493            | N       | N       | 0.0397    | 0.0272    | 0.0002   | N       |
| TNF-α       | 1.002±0.0646         | 3.648±0.473     | 0.58±0.2635              | 0.29±0.239               | N       | N       | 0.0478    | 0.0100    | 0.0002   | N       |

| Target Gene | Control Mean±SD (G1) | Aβ Mean±SD (G2) | 1 µmol donepezil Mean±SD (G3) | 10 µmol donepezil Mean±SD (G4) | P Value |
|-------------|----------------------|-----------------|-------------------------------|-------------------------------|---------|
|             |                      |                 |                               |                               | G1 vs  G2 | G1 vs  G3 | G1 vs  G4 | G2 vs  G3 | G2 vs  G4 | G3 vs  G4 |
| IL-1β       | 1.002±0.1103         | 2.895±2.018     | 0.5633±0.2001               | 0.2083±0.1857               | N       | N       | 0.0173    | 0.0225    | 0.0003   | N       |
| TNF-α       | 1.012±0.0716         | 3.038±0.8302    | 0.6333±0.2901               | 0.2267±0.1253               | N       | N       | 0.0423    | 0.0256    | <0.0001  | N       |
Figure 1

Schematic overview of binding gorge of hAChE (left) and hBuChe (right). Catalytic site of enzymes including the anionic site (similar triad aminoacids), acylpocket (larger in hBuChe), oxianionic hole (similar) and preferal binding pocket (including aromatic amino acids in hAChE and non aromatic amino acids in hBuChE) are obvious in schematic view. (residue numbers are for 4EY7 and 4BDS pdb codes).
Figure 2

Synthesis of compounds 5a-l (compounds 5g and 5i designed and synthesized in current study other derivatives were reported in our previous publications\textsuperscript{21-22}).

Figure 3

\textbf{13C NMR spectra of compounds 5i and 5g}

Figure 4

Lineweaver–Burk and secondary plots of compound 5g
Figure 5

TEM images of normal PC12 cells (a), Aβ treated PC12 cells (b), Aβ+0.1 μM 5g and Aβ+1 μM 5g. Apoptotic bodies were shown by arrows.

Figure 6

Inhibition of ROS production by compound 5g in Aβ-induced neurotoxicity on PC12 cells. (A) The image of DCF and DAPI treated normal, Aβ and Aβ+compound 5g treated cells, (B) DCF fluorescent intensity of studied cells and (C) TAC% of studied cells.
Figure 7

The effect of compound 5g on Lipid peroxidation (A) and LDH activity (B) on Aβ-teated PC12 cells.
Figure 8

Compound 5g effect on Aβ induced apoptosis investigated by TUNNEL assay and DAPI counterstaining.
Figure 9

The effect of compound 5g and donepezil on the inflammatory genes expression.
Figure 10

Mean (A) locomotor activity, (B) total observation time, and (C) displacement index (DI) in the NOR task in different groups. Each bar represents the mean ± SEM, (n = 10). ***p < 0.001 compared with the sham. ##p < 0.01 and ###p < 0.001 compared with the AD+NS.
Figure 11

Mean escape latencies during 4 days of training sessions of MWM in different
Figure 12

Effect of compound 5g on the expression of pro-inflammatory cytokines (TNFα and NFκB), and GADH in AD rats’ hippocamp.

Figure 13

Effect of compound 5g on the AChE relative amount and ChE relative activity in AD rats’ hippocamp.
Figure 14

3D illustration of superimposition of docked donepezil on cocrystallized donepezil (Pdb: 4EY7) (left) and cocrystallized butyrylthiocholine (Pdb: 1P0P) (right) along with pharmacophore mapping calculated by Ligandscout.

Figure 15

Binding pose of compounds 5g and 5f docked to the binding cavity of hAChE (4EY7) (A) and hBuChE (4BDS) (B) along with 2D illustration of complexes.