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Novel hybrids of oxoisoaporphine–tryptamine as inhibitors of acetylcholinesterase-induced *β*-amyloid aggregation with improved antioxidant properties

HAI-TAO ZHAO, SHU-MING ZHONG, JIANG-KE QIN and HUANG TANG*

Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry and Pharmaceutical Sciences of Guangxi Normal University, Guilin 541004, China

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Abstract: A series of dual binding site acetylcholinesterase (AChE) inhibitors was designed, synthesized, and tested for their antioxidant ability and inhibitory potency on AChE and AChE-induced *β*-amyloid (A*β*) aggregation. The new hybrids consisted of a unit of 1-azabenzanthrone and tryptamine or its derivative, connected through an *α*,*ω*-alka(e)nediamide bridge. These hybrids exhibited moderate AChE inhibitory activity with IC_{50} values in the micromolar range and significant *in vitro* inhibitory activity towards AChE-induced A*β* aggregation. Moreover, six of the nine hybrids of this series exhibited a higher oxygen radical absorbance capacity than trolox, which makes them promising anti-Alzheimer drug candidates.

Keywords: Alzheimer's disease; acetylcholinesterase inhibitor; A*β* anti-aggregating activity; oxoisoaporphine–tryptamine.

INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by progressive cognitive impairment, a variety of neuropsychiatric and behavioral disturbances, and restrictions in activities of daily life. A century has passed since AD was first described by the German psychiatrist and neuropathologist Alois Alzheimer in 1906. The treatment of Alzheimer's disease remains a challenge for the pharmaceutical community. Although many factors have been implicated in AD, its etiology and pathogenesis remain unclear.

The "cholinergic hypothesis" represents one of the most useful approaches involved in the design of new agents for the treatment of AD.[1] This hypothesis asserts that most of the cognitive impairments suffered by AD patients are the consequence of a deficit in acetylcholine (ACh) and thus in cholinergic neuro-

* Corresponding author. E-mail: hyhth@163.com
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transmission. Therefore, inhibition of AChE appears to be a useful therapeutic path to reduce, at least temporarily, the cognitive deficit in AD. To date, most of the drugs available on the market for the treatment of AD are acetylcholinesterase inhibitors (AChEI). However, the practical effectiveness of these drugs remains controversial. Recent AD trials concluded that AChEI therapies were not cost effective and the AD patients treated with cholinesterase inhibitors did not show widespread cortical atrophic changes.

Recently, most approaches to explain the pathogenesis of AD focus on two proteins: the *β*-amyloid peptide (A*β*)[2,3] and the filament protein tau.[4,5]

According to the "amyloid hypothesis", one of the major neuropathological hallmarks of AD is the altered production, aggregation, and deposition of A*β*, which results in amyloid plaque formation. Moreover, oxidative stress and increased intracellular Ca^{2+} generated in response to A*β* were reported to enhance glutamate-mediated neurotoxicity *in vitro*, with additional experiments suggesting that A*β* could increase *N*-methyl-D-aspartic acid (NMDA) responses and therefore excitotoxicity.[6,7] Many *in vivo* and *in vitro* findings showed that amyloidosis could be inhibited by antioxidants and the free radical scavengers, vitamin E and propyl gallate, protected neuronal cells against A*β* toxicity. It is becoming evident that a close relationship may exist between glutamate excitetoxicity, oxidative stress, and A*β* formation. Actually, AChE could also bind to the A*β* non-amyloidogenic form, inducing a conformational transition to the amyloidogenic conformation with subsequent amyloid fibril aggregation.[8] This action involves the peripheral anionic binding site (PAS) of AChE.[8,9] These effects, together with the fact that AD pathogenesis appears to be multifactorial, have led to the current opinion that drugs that interact with the PAS and possess antioxidant activity might exert a multi-pharmacological effect, which combine enhancement of cholinergic neurotransmission, effective antioxidant neuroprotection and the reduction in the pro-aggregating action of AChE, thus opening the way to a new promising therapeutic approach to Alzheimer's disease (AD).[3,10,11]

Recently, it was reported that synthetic derivatives of oxoisoaporphine alkaloids (Fig. 1) exhibited high acetylcholinesterase inhibitory activity and high selectivity for AChE over butyrylcholinesterase (BChE).[12,13] Molecular docking simulations on the oxoisoaporphine derivatives with AChE from *Torpedo californica* demonstrated that the 1-azabenzanthrone moiety of the ligands can interact with the PAS of acetylcholinesterase, especially with Trp 279 of the PAS.[13]

Tryptamine and its derivatives are known as natural antioxidant. Melatonin, one of tryptamine family, was shown to prevent the hyperphosphorylation of the tau protein in rats[14] and to possess neuroprotective properties against A*β* toxicity *in vivo*.[15] Several studies suggested that melatonin might be effective for the treatment of Alzheimer's disease.[15–18]

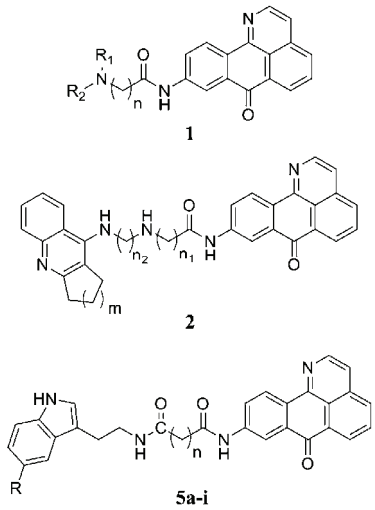


Fig. 1. Chemical structure of synthetic derivatives of oxoisoaporphine alkaloids **1**, hybrids of oxoisoaporphine–tacrine congeners **2** and hybrids of oxoisoaporphine–tryptamine derivatives **5a**–**i**.

It was thus predicted that compounds that combined inhibition of AChE and neuroprotective properties in a single small molecule would exert greater biological activity than tryptamine or oxoisoaporphine themselves and may represent an important pharmacological advance in the management of AD.

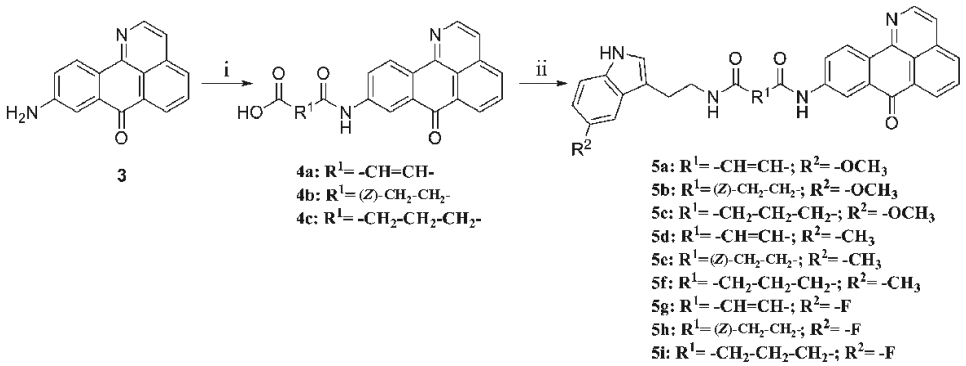
Recently, the synthesis of multifunctional compounds that combined 1-azabenzanthrone and tacrine congeners in a single small molecule was reported.[19] These hybrids exhibited high AChE inhibitory activity and significant *in vitro* inhibitory activity towards AChE-induced and self-induced Aβ aggregation. Ongoing research is focused on the design of new multi-activity compounds in which different active units could be anchored to a biocompatible scaffold.

In this paper, a series of hybrids of oxoisoaporphine–tryptamine derivatives were designed and synthesized. Their ability to inhibit AChE, AChE-induced Aβ aggregation and antioxidant activity was tested. These compounds (Fig. 1) consist of a unit of tryptamine, or a derivative that possesses neuroprotective properties, and the 1-azabenzanthrone moiety the position of which along the enzyme gorge and the peripheral site could be modulated by a suitable tether that connects tryptamine and 1-azabenzanthrone.

RESULTS AND DISCUSSION

Chemistry

The synthetic routes to compounds **5a**–**i** are outlined in Scheme 1. Compound **3**[20] was treated with the required anhydride in toluene to afford the corresponding aromatic carboxylic acid **4a**–**c**. Then compounds **4a**–**c** was reacted with the desired tryptamine derivative in dimethylacetamide (DMA) in the presence of BOP reagent, benzotriazol-l-yloxytris(dimethylamino)phosphonium hexafluorophosphate, to afford the target compounds **5a**–**i**.



Scheme 1. The synthetic route to the hybrids of oxoisoaporphine–tryptamine. Reagents and conditions: i) the required anhydride, toluene, reflux, 7 h; ii) the corresponding tryptamine derivatives, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), DMA, 65 °C, 6 h.

Pharmacology

The inhibitory activities of the newly synthesized compounds **5a–i** against AChE were investigated by determining the rate of hydrolysis of acetylthiocholine (ATCh) in comparison with reference compound tacrine, using a modified Ellman method.[21] The IC_{50} values of all the investigated compounds are summarized in Table I.

All the synthesized compounds showed moderate inhibitory potency against AChE with inhibitory activity IC_{50} values in the micromolar range. The compounds **5b**, **5e** and **5h**, which had a succinamide linker, showed higher inhibitory effects on AChE compared with the compounds other linkers. This suggested that rigid and long linkers were not favorable for fitting into the enzyme cavity. In the screening results, the structure of the tryptamine derivatives had little effect on the AChE inhibitory potency, showing only a slightly increased effect in the compounds bearing fluorine-substituted tryptamine units (compound **5g–i**).

In fact, AChE directly promotes *in vitro* the assembly of the Aβ peptide into amyloid fibrils, forming stable AChE–Aβ complexes.[8] Structural analysis by X-ray crystallography[22] and competition assays with AChEIs clearly identified the PAS of the enzyme as the locus of protein interaction with Aβ.[23] It was previously demonstrated that 1-azabenzanthrone could interact with the PAS of acetylcholinesterase, especially with Trp 279 of the PAS.[13] To further explore the dual action of these compounds, the AChE-induced Aβ(1–40) aggregation inhibitory activity was examined employing the thioflavin T (ThT)-based fluorometric assay.[24] The Aβ-anti-aggregation activity of the novel hybrids and reference compounds are summarized in Table I. The nine tested oxoisoaporphine–tryptamine derivatives exhibited, at a 100 μM concentration, a significant Aβ--anti-aggregation effect with inhibition ranging from 32.5 to 60.5 %, being 8- to

13-fold more potent than tacrine. There was a clear correlation between the AChE-induced Aβ aggregation inhibitory activity and the AChE inhibitory potency. Compound **5h** bearing fluorine-substituted tryptamine units and a succinamide linker had the highest Aβ-aggregation inhibitory ratio that corresponded to the lowest IC_{50} value for AChE inhibition. Compound **5g** possessing the lowest Aβ-aggregating inhibitory ratio showed the highest IC_{50} for AChE inhibition. A scatter plot of the *in vitro* inhibitory percent of AChE-induced Aβ aggregation *versus* the IC_{50} value for AChE inhibition is shown in Fig. 2. A statistically significant linear fit of the data was obtained, which clearly showed that the inhibitory effects for Aβ aggregation and acetylcholinesterase were positively correlated. These results appear to validate the theory[23] that a compound that binds to the PAS of AChE could strongly inhibit enzyme-mediated Aβ aggregation.

TABLE I. Inhibition of AChE activities, AChE-induced Aβ aggregation and oxygen radical absorbance capacity (*ORAC*) by the synthesized compounds; IC_{50}: 50 % inhibition concentration (means±*SEM* of three experiments) of AChE; inhibition of co-aggregation of Aβ(1–40) and AChE 0.06 U was detected by the ThT assay; the data showed that the test compounds inhibited the co-aggregation at 100 μM. The values are expressed as means±*SEM* of three experiments; the *ORAC* data are expressed as μmol of trolox equivalent per μmol of tested compound and are the mean (n = 3)±*SD*

Compound	IC_{50} / μM, for AChE	Inhibition, %, of Aβ aggregation	*ORAC* trolox-equivalents
5a	14.94±0. 23	35.2±1.2	0.9±0.03
5b	6.10±0.25	50.4±1.5	1.2±0.01
5c	16.77±0.34	41.6±2.3	0.9±0.04
5d	10.85±0.15	38.5±1.7	1.7±0.05
5e	5.10±0.31	57.7±0.9	1.3±0.03
5f	14.0±0.73	49.7±1.9	1.4±0.02
5g	14.95±0.31	32.5±2.1	1.1±0.02
5h	2.37±0.40	60.5±1.5	1.0±0.01
5i	7.12±0.32	59.3±2.2	1.3±0.05
Tacrine	0.13±0.01	4.5±0.7	<0.01
Curcumin	–	32.8±0.5	–
Propidium iodide	–	83.7±1.7	–
Melatonin	–	–	1.9±0.09

The oxygen radical absorbance capacity assay (ORAC) at 37 °C using fluorescein (ORAC–FL) as a fluorescence probe measures the scavenging capacity against peroxyl radicals induced by AAPH. The antioxidant activity of the new oxoisoaporphine–tryptamine hybrids **5a**–**i** was determined by their competition with fluorescein in the radical capture, using a fluorescence microplate reader. The vitamin E analogue trolox was used as a standard, and the results were expressed as trolox equivalents (μmol of trolox equivalents per μmol of tested compound, Table I). Tacrine and melatonin were also checked for comparison.

Tacrine showed rather weak radical capture, whereas melatonin had an ORAC–FL value 1.9-fold higher than that of trolox.

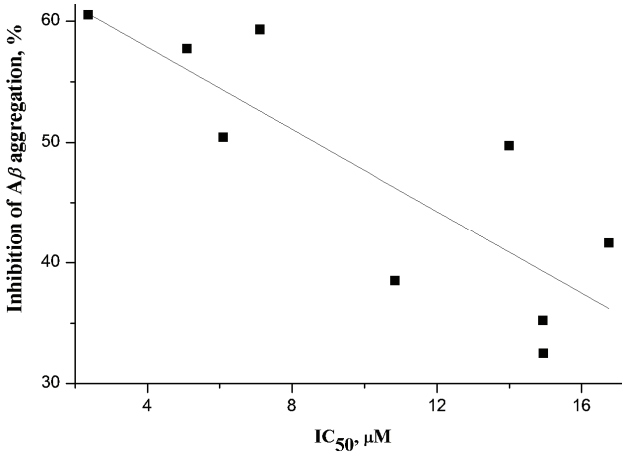


Fig. 2. Scatter plot of the *in vitro* percent inhibition of AChE-induced Aβ aggregation *vs*. the IC_{50} value for AChE inhibition for the series of oxoisoaporphine–tryptamine derivatives **5a**–**i**.

Hybrids **5a**–**i** were tested in 1–10 μM concentrations and they exhibited moderate peroxyl radical absorbance capacities ranging from 0.9- to 1.7-fold the value of trolox. Tryptamine was the main element responsible for increasing the antioxidant activity. Its structure had a direct effect on the antioxidant activity. The derivatives obtained from conjugation with the 5-methyltryptamine (**5d**–**f**) showed the higher radical-scavenger activity with oxygen radical absorbance capacity at 1.7, 1.3 and 1.4 trolox-equivs, respectively. The structure and length of the linker had no effect on their antioxidant capacity.

EXPERIMENTAL

The reactions were monitored by TLC using aluminum plates pre-coated with silica gel containing a fluorescent indicator. Detection was performed with UV (254 nm) irradiation followed by charring with 0.5 % phosphomolybdic acid in 95 % EtOH. Anhydrous $MgSO_4$ was used to dry the organic solutions during work-up and the solvents were removed under vacuum using a rotary evaporator. The ^{1}H-NMR spectra were recorded on a Varian Mercury-Plus 300 and Bruker Avance AV500 NMR Spectrometer with tetramethylsilane (TMS) as an internal standard. Elemental analysis was realized on a PE2400II Elemental Analyzer. Mass spectral analysis was performed on a Bruker HCT mass spectrometer and recorded on an electrospray ionization mass spectrometer as the m/z value.

Characterization data for the synthesized compounds are given in Supplementary material to this paper.

*General procedure for the synthesis of compounds **4a**–**c***

Compound **3**[20] (3.0 mmol) and the required anhydride (30 mmol) were mixed in toluene (40 mL). The mixture was refluxed under stirring for 7 h and then cooled to room tempe-

rature. The resulting precipitate was collected by filtration, washed with toluene, and dried. Recrystallization from toluene afforded the products **4a**–**c** as yellow solids in 43–55 % yields.

*General procedure for the synthesis of compounds **5a**–**i***

BOP (0.9 g, 2 mmol) in DMA (3 mL) was added over 30 min to a solution of compound **4a**–**c** (2 mmol) and the appropriate tryptamine derivatives (20 mmol) in DMA (4 mL) at 65 °C. The mixture was stirred at 65 ° C for 5 h and concentrated under vacuum. The residue was taken up with dichloromethane and the solution washed with a solution of sodium carbonate, dried (Na_2SO_4), and concentrated under vacuum. Column chromatography of the residue, eluting with 5% methanol in dichloromethane, afforded compounds **5a**–**i** in 43–55 % yields.

In vitro *inhibition studies on AChE*

All the assays[21] were performed in 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 8.0, using a PerkinElmer LAMBDA 45 Spectrophotometer. AChE from *Electrophorus electricus* (Sigma) were prepared to give 2.0 units mL^{-1} in 2 mL aliquots. The assay medium contained phosphate buffer, pH 8.0 (1 mL), 50 μL of 0.01 M 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 10 μL of enzyme, and 50 μL of 0.01 M substrate (acetylthiocholine chloride). The substrate was added to the assay medium containing enzyme, buffer, and DTNB with inhibitor after 15 min of incubation time. The activity was determined by measuring the increase in absorbance at 412 nm in 1 min intervals at 37 °C. The calculations were performed using the Ellman equation.[21]

Inhibition of AChE-induced Aβ(1–40) peptide aggregation assay

To analyze the inhibition of co-aggregation, the ThT fluorescence method was employed. The fluorescence emission was measured at 490 nm for excitation at 446 nm. For the co-incubation experiments,[24,25] mixtures of A*β*(1–40) peptide (GL Biochem) and AChE from *E. electricus*, were incubated for 8 h at 37 °C in the presence or absence of the test inhibitors. The final concentrations of A*β* (2.3 mM A*β* DMSO solution added to 0.215 M sodium phosphate buffer, pH 8.0) and AChE (dissolved in 0.1 M sodium phosphate buffer, pH 8.0) were 23 μM and 0.06 U, respectively. After co-incubation, 180 μL of 1.5 μM ThT was added to 20 μL of the solution mixtures. The percent inhibition (*I*) of AChE-induced aggregation due to the presence of the tested compound was calculated by the following expression:

$$I\ /\ \% = 100 - \left(\frac{IF_\mathrm{i}}{IF_0} \times 100 \right)$$

where IF_i and IF_0 are the fluorescence intensities obtained for A*β* plus AChE in the presence and in the absence of inhibitor, respectively, minus the fluorescence intensities due to the respective blanks.

Determination of antioxidant activity

The antioxidant activity was determined by the oxygen radical absorbance capacity––fluorescein (ORAC–FL) assay of Ou *et al*.[26] and modified by Rodriguez-Franco *et al*.[27] 2,2′--Azobis(2-amidinopropane) dihydrochloride (AAPH) and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) solutions were prepared daily and fluorescein (FL) was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). The reaction was performed in 75 mM phosphate buffer (pH 7.4) and the final reaction mixture was 200 μL. Antioxidant (20 μL) and FL (120 μL; 70 nM, final concentration) solutions were preincubated for 10 min at 37 °C. AAPH solution (60 μL; 12 mM, final concentration) was added and after shaking before the first reading, the fluorescence was recorded every 56 s for 96

min. Trolox was used as standard (1–10 μM, final concentration) and the sample was measured at different concentrations (1–10 μM). An Infinite® M1000 PRO plate reader (Tecan Trading AG, Switzerland) with λ_{ex} = 485 nm and λ_{em} = 520 nm was used. The equipment was controlled by Magellan™ software for fluorescence measurements. Black 96-well microplates were used. All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample. Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (*AUC*) was calculated as:

$$AUC = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0}$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*. The net *AUC* for a sample was calculated as follows:

$$\text{net } AUC = AUC_{\text{antioxidant}} - AUC_{\text{blank}}$$

The regression equation between net *AUC* and antioxidant concentration was calculated. The slope of the equation was used to calculate the ORAC–FL value by using the trolox curve obtained for each assay. Final ORAC–FL values were expressed as μmol of trolox equivalent per μmol of pure compound.

CONCLUSIONS

The present study reports the synthesis and biological evaluation of a series of new hybrids of oxoisoaporphine–tryptamine derivatives. Regarding AChE inhibition, synthesized compounds show less inhibitory potency than that of 9-substituted 1-azabenzanthrone derivatives which were previously reported.[12,19] However, these new synthetic derivatives showed strong AChE-induced Aβ anti-aggregation activity, being more potent than tacrine and curcumin. In addition, these derivatives showed moderate antioxidant activity, though their activities were less than that of melatonin. This could be related to the poor solubility of the hybrids. Overall, these results were encouraging for the further development of Aβ aggregation inhibitors with higher antioxidant activity and AChE inhibitory activity based on oxoisoaporphine in the future.

SUPPLEMENTARY MATERIAL

Characterization data of the prepared compounds and their IR spectra are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

НОВИ ДЕРИВАТИ ОКСОИЗОАПОРФИН–ТРИПТАМИНА КАО ИНХИБИТОРИ АГРЕГАЦИЈЕ β-АМИЛОИДА ИНДУКОВАНЕ АЦЕТИЛХОЛИНЕСТЕРАЗОМ, КОЈИ ПОСЕДУЈУ УНАПРЕЂЕНЕ АНТИОКСИДАТИВНЕ ОСОБИНЕ

HAI-TAO ZHAO, SHU-MING ZHONG, JIANG-KE QIN и HUANG TANG

Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry and Pharmaceutical Sciences of Guangxi Normal University, Guilin 541004, China

Серија инхибитора ацетилхолинестеразе (AChE) са двоструким везивањем у активном месту је дизајнирана и синтетисана, а тестирана су њихова антиоксидативна својства и инхибиторни потенцијал према AChE и AChE-индукованој (Aβ) агрегацији β-амилоида. Нови деривати садрже 1-азабензантрон и триптамин или његове деривате, повезане α,ω-алка(е)ндиамидним мостом. Ови деривати показују умерену инхибиторну активност према AChE са IC_{50} вредностима у микромоларном опсегу, и значајну *in vitro* AChE-индуковану Aβ агрегацију. Осим тога, шест од девет деривата показује већи капацитет апсорпције кисеоничних радикала у поређењу са тролоксом, што их чини перспективним кандидатима за лечење Алцхајмерове болести.

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