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Conjugates of tacrine with salicylamide as new promising multitarget agents for Alzheimer's disease

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Abstract: New conjugates of tacrine and salicylamide with alkylene spacers were synthesized and evaluated as potential multifunctional agents for Alzheimer's disease (AD). The compounds exhibited high acetylcholinesterase (AChE, IC_{50} to 0.224 μM) and butyrylcholinesterase (BChE, IC_{50} to 0.0104 μM) inhibitory activities. They were also rather poor inhibitors of carboxylesterase, suggesting a low tendency to exert potential unwanted drug-drug interactions in clinical use. The conjugates were mixed-type reversible inhibitors of both cholinesterases and demonstrated dual binding to the catalytic and peripheral anionic sites of AChE in molecular docking that, along with experimental results on propidium iodide displacement, suggest their potential to block AChE-induced β-amyloid aggregation. The new conjugates exhibited high $ABTS^{\bullet+}$-scavenging activity. N-(6-(1,2,3,4-tetrahydroacridin-9-ylamino)hexyl)salicylamide is a lead compound that also demonstrates metal chelating ability toward Cu^{2+}, Fe^{2+} and Zn^{2+}. Thus, the new conjugates have displayed the potential to be multifunctional anti-AD agents for further development.

Alzheimer's disease (AD) is a progressive and fatal degenerative disorder in the central nervous system leading to the most common form of dementia in the aged population.[1–3] AD has a multifactorial causation and results in a wide spectrum of injurious effects, including reduction of acetylcholine levels, accumulation of anomalous β-amyloid (Aβ) and tau proteins, loss of synapses, dysregulation of biometals homeostasis, oxidative stress, neuronal cell death, and inflammation.[4] In response to the multifunctional properties of AD, multitarget directed ligands (MTDLs) that can synergistically affect a number of relevant targets and/or processes has emerged as a widely used strategy in the search for new agents to treat this devastating disease.[5–7]

An especially promising approach to the development of multitarget agents for AD therapy is the synthesis of hybrid structures, where two pharmacophores are linked through a spacer. The known anticholinesterase compounds are often used as one of the pharmacophores.[8] In particular, tacrine is a popular structure for the design of MTDLs.[9–14] It inhibits both acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) with high efficiency. The high affinity of the tacrine fragment for the peripheral anionic site (PAS) of AChE is widely used to develop bifunctional cholinesterase inhibitors capable of blocking AChE-induced aggregation of β-amyloid. For this purpose, various planar aromatic fragments are attached to the tacrine molecule using a spacer of a certain length, which enables binding of the inhibitor to both the catalytic active site (CAS) and PAS of AChE.[15,16]

Additional design features for tacrine-based anti-Alzheimer's MTDLs includes the incorporation of second pharmacophores possessing antioxidant [15,17–22] or chelating properties.[19,23,24] It is worth noting that adding free radical scavengers to the tacrine molecule has alleviated the hepatotoxicity that is seen with tacrine on its own.[18,20]

In our opinion, the aforementioned drug attributes are met by salicylamides, which have an aromatic ring, possess antioxidant properties, and contain a chelating fragment.

It is known that salicylic acid (SA) and its derivatives are widely used to treat various diseases. In addition, salicylates, and mainly amides, have pronounced antioxidant properties.[25,26]

Herein, conjugates of tacrine and salicylamide bound through an alkylene spacer (Fig. 1) were synthesized as potential multifunctional agents for the treatment of AD. Their esterase profile i.e., inhibitory activity against cholinesterases and a structurally related enzyme, carboxylesterase (CES, EC 3.1.1.1) was studied and analyzed based on quantum mechanics (QM)-assisted molecular docking. The ability of the conjugates to displace propidium iodide from the PAS of AChE was studied as an assessment of their potential to block AChE-induced aggregation of β-amyloid. In addition, the primary antioxidant activity of the conjugates and their ability to chelate biometals Fe^{2+}, Cu^{2+}, Zn^{2+} were determined.

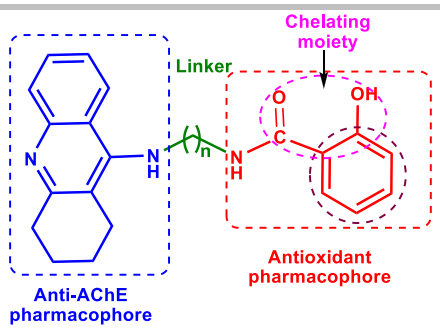
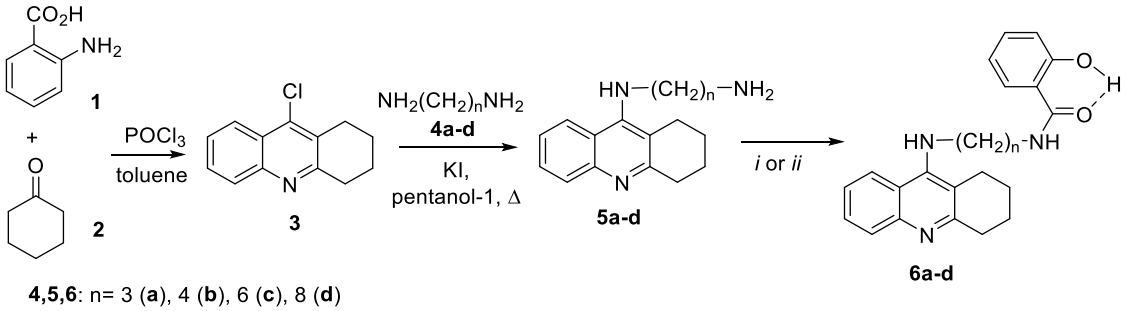


Fig. 1. Designed conjugates of tacrine and salicylamide.

For the synthesis of conjugates of tacrine and salicylamide, 9-chloro-1,2,3,4-tetrahydroacridine **3** was first obtained according to the previously described procedure[18,24] by cyclization of commercially available anthranilic acid **1** and cyclohexanone **2** under the action of $POCl_3$ (Scheme 1). Then, an alkylene spacer was introduced into compound **3** by substitution of the chlorine atom with the amino group of diaminoalkane **4a-d**, containing 3, 4, 6, or 8 methylene units, according to a known procedure.[24] As a result, aminopolymethylene-containing derivatives of tacrine **5a-d** were obtained and used for the synthesis of target conjugates **6a-d.** We have tested two approaches for introducing the salicylic acid moiety into compounds **5a-d**. The first method consisted of acylation of the amino group of heterocycles **5a-d** with a step-by-step addition of salicyloyl chloride (obtained *in situ* from salicylic acid and thionyl chloride, see Experimental) in anhydrous methylene chloride in the presence of pyridine at –20 °C and the subsequent stirring of a reaction mixture at room temperature for 4 h. According to the second method, the fragment of salicylic acid was introduced by condensation of compounds **5a-d** with salicylic acid in anhydrous methylene chloride in the presence of HATU/DIPEA at room temperature. Using the second method, it was possible not only to increase the yield of conjugates **6a–6d** from 48–60% to 59–71%, but also to significantly simplify the reaction procedure and reduce the number of synthesis steps due to the absence of the need to synthesize salicyloyl chloride.

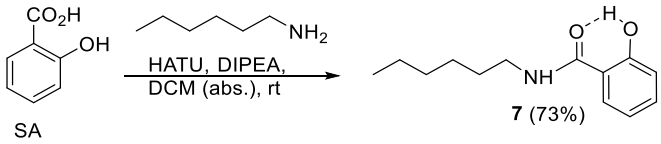


4,5,6: n= 3 (**a**), 4 (**b**), 6 (**c**), 8 (**d**)

Conditions and reagents:
i: Salicyloyl chloride, Py, DCM (abs.), –20 ºC → rt, 4 h. **6a** (60%), **6b** (54%), **6c** (59%), **6d** (48%);
ii: Salicylic acid, DCM (abs.), HATU, DIPEA, rt, 4 h. **6a** (64%), **6b** (71%), **6c** (61%), **6d** (59%).

Scheme 1. Synthesis of conjugates **6a-d.**

In addition, *N*-hexylsalicylamide **7** was obtained by condensation of **SA** with hexylamine under the action of the HATU/DIPEA catalytic system (Scheme 2) to assess the radical-scavenging activity of the salicylamide fragment.



Scheme 2. Synthesis of compound **7.**

The structure of the obtained compounds was confirmed by IR, ^{1}H and ^{13}C NMR spectroscopy, elemental analysis, and mass spectrometry. In the IR spectra of compounds **6a–d, 7**, the band corresponding to vibrations of the amide carbonyl group is observed at 1636–1638 cm^{-1}. Such a decrease in the vibration frequency compared to the characteristic value for the free amide carbonyl group is due to its participation in an intramolecular hydrogen bond with the hydroxyl substituent. The presence of this interaction indicates the potential chelating ability of the obtained conjugates **6a-d** and amide **7**.

The esterase profile of the conjugates **6** as new potential anti-AD molecules was assessed. This procedure enables the estimation of both their primary pharmacological effects — inhibition of AChE and BChE — and their possible unwanted side effects — inhibition of CES — an enzyme that hydrolyzes numerous ester-containing drugs.[27–29]

The esterase profile study showed (**Table 1**) that hybrids **6a-d** inhibited AChE and BChE at a level equal to or above that of the parent compound tacrine, and being like tacrine, more selective against BChE. The inhibitory activity toward AChE increased with elongation of the spacer: inhibition power increased 5-fold upon lengthening the spacer from –$(CH_2)_3$– to –$(CH_2)_8$–. Regarding BChE inhibition, the compounds **6b-d** with a spacer greater than –$(CH_2)_4$– were three times more active than tacrine. Compounds **6a-d** weakly inhibited CES, an enzyme that is responsible for the hydrolysis of numerous ester-containing drugs.[30] The inhibition of CES by anticholinesterase compounds used by a patient may lead to unwanted drug-drug interactions.[31,32] Salicylamide **7** demonstrated negligible activity against cholinesterases and CES. Salicylic acid weakly inhibited BChE.

Table 1. Esterase profile of hybrids **6a-d,** their ability to displace propidium iodide from the peripheral anionic site of *Ee*AChE, and antioxidant activities.

Compound	Inhibitory Activity Against AChE, BChE, and CES			Propidium iodide displacement (%)[b]	ABTS$^{+\bullet}$-scavenging activity	
	Human RBC AChE, IC_{50} (μM)	Equine Serum BChE, IC_{50} (μM)	Porcine Liver CES, (%)[a]		TEAC[c]	IC_{50}, μM
6a: n= 3	1.020±0.040	0.1290±0.0050	3.4±0.9	10.1±0.8	0.70±0.07	27.7±0.9
6b: n= 4	1.170±0.050	0.0107±0.0005	1.6±1.1	10.6 ±0.9	0.72±0.04	25.3±1.1
6c: n= 6	0.294±0.008	0.0119±0.0001	19.5±0.9	9.8 ±0.7	0.90±0.05	21.3±1.5
6d: n= 8	0.224±0.017	0.0104±0.0013	29.5±2.5	8.2 ±0.7	0.73±0.05	25.9±1.1
7	(2.7±0.4)[a]	(10.6±1.1)[a]	4.9±0.5	n.d.	0.39±0.04	46.5±2.3
SA	(1.3±0.2)[a]	47.6±3.2	8.9±0.6	n.d.	n.a.[d]	n.d.
Tacrine	0.601±0.047	0.0295±0.0002	n.d.	3.1±0.2	n.a.[d]	n.d.
Donepezil	0.040±0.004	19.2±3.0	n.a.	11.2±0.7	n.d.	n.d.
Trolox	n.d.	n.d.	n.d.	n.d.	1.0	20.1±1.2

[a] % inhibition at 20 μM compound concentration; [b] % displacement at 20 μM compound concentration; [c] TEAC (trolox equivalent antioxidant capacity) was determined from the ratio of the slopes of the concentration-response curves test compound/Trolox; [d] - n.a. (not active, TEAC < 0.01); n.d. – not determined; Data are expressed as mean ± SEM, n=3.

The mechanism of AChE and BChE inhibition by the conjugates is demonstrated for compound **6d** as an example. The graphical analysis using double reciprocal Lineweaver–Burk plots is shown in Fig.2. The plots demonstrate that the binding of conjugate **6d** to either AChE or BChE resulted in changes in both V_{max} and K_m. This suggests a mixed-type inhibition. The values of inhibition constants (K_i – competitive component and αK_i – non-competitive component) were K_i = 0.152± 0.012 μM and αK_i = 0.263± 0.018 μM for AChE, and K_i = 0.0106± 0.0007 μM and αK_i = 0.0171± 0.0013 μM for BChE.

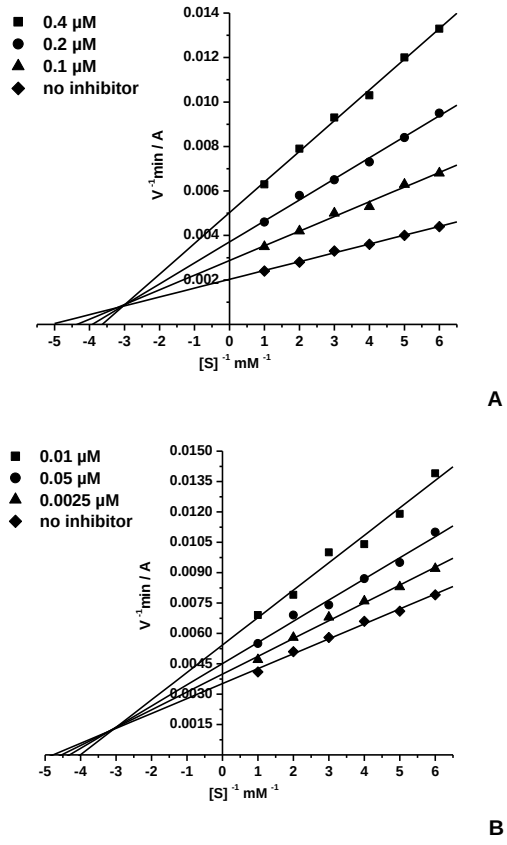


Figure 2. Lineweaver-Burk double-reciprocal plots of steady state inhibition of AChE (**A**) and BChE (**B**) by compound **6d**. Each plot indicates mixed-type inhibition.

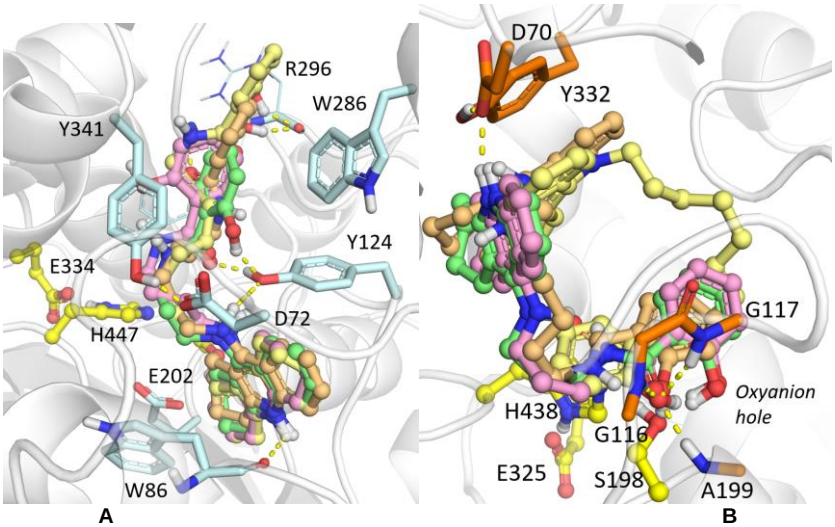


Figure 3. Molecular docking of conjugates **6a-d** to AChE (A) and BChE (B) active sites. Carbon atoms of compound **6a** are colored green, **6b** – pink, **6c** – orange, **6d** – yellow.

For all compounds, the ability to competitively displace propidium iodide, a selective ligand of the PAS of AChE, was evaluated by a fluorescence method.[33] Propidium iodide demonstrated a significant decrease in AChE-induced Aβ aggregation (82% at 100 μM).[33,34] This observation has served as the basis for the fluorescent evaluation of competitive propidium iodide displacement from the PAS of AChE as a primary screening method to assess the potential ability of compounds to bind to the PAS and thus block the pro-aggregation activity of AChE.[35] The method has been described in detail.[12]

It can be seen in Table 1 that all compounds displaced propidium iodide (8-11%), at a level comparable to that of the reference compound donepezil, which was shown to be effective in inhibiting AChE-induced aggregation of beta-amyloid.[33,34]

Molecular docking was used to explore the mode of binding of the conjugates to AChE and BChE.

Molecular docking of compounds **6a**-**6d** to AChE revealed binding typical for tacrine based conjugates: the tacrine fragment was bound in the active site, with its protonated endocyclic nitrogen atom hydrogen-bonded to the Trp86 main chain oxygen atom. The salicylamide group was bound in the PAS, increasingly advancing into it with elongation of the linker growth. However, due to the amide-containing spacer and small aromatic fragment size, its occupation of the PAS was incomplete. The salicylate fragment was mostly located near the acyl-binding loop, interacting with the main chain peptide groups of Phe295 and Arg296, which undermined its ability to displace propidium iodide.

In contrast, in BChE, compounds **6a**-**6d** were bound with the salicylate fragment in the active site, with the phenolic or amide fragment oxygen bound to the oxyanion hole, while the positively charged tacrine fragment was directed to Asp70. However, the binding mode of compound **6d** differed from the others in that it retained the same interaction pattern with increasing linker length. Compounds **6b-d** with the spacer ≥ –$(CH_2)_4$– were three times more active as BChE inhibitors than tacrine, apparently due to interactions with two sites of the enzyme: the oxyanion hole in the active site and Asp70 in the PAS.

The primary antioxidant activity of conjugates **6a-d**, **7** was determined spectrophotometrically by the ABTS assay,[36] as previously described in detail.[37] The results are shown in Table 1.

As can be seen (Table 1), the new conjugates **6a-d** exhibited rather high $ABTS^{\bullet+}$ scavenging activity (TEAC = 0.70 - 0.90) near the level of the standard antioxidant Trolox. Changes in the spacer length did not significantly alter the radical-scavenging activity. Compound **6c** with a spacer length n = 6 was the most active ABTS-radical scavenger, having an activity very close to that of Trolox (TEAC = 0.90±0.05). Salicylamide **7** also had an ability to scavenge free radicals, but to a lesser degree (TEAC = 0.39±0.04) than conjugates **6**. On the contrary, the parent compound, salicylic acid, had no antiradical activity at the studied concentration range (1-100 μM), which is in good agreement with literature data.[25,26]

Reducing excess concentrations of metals in the brain by chelating agents is one of the approaches to AD treatment.[38,39] Among the hybrids, compound **6c** was chosen as an example for the analysis of chelating capacity. The complexation abilities of compound **6c** for biometals such as Cu^{2+}, Fe^{2+} and Zn^{2+} were studied in ethanol (95% v/v) by UV–VIS spectrometry as described.[24] The results are shown in Figure 4. In the spectrum of **6c**, the broad band from 270 to 375 nm was ascribed to π-π* transitions and the peaks around 240 nm were attributed to intramolecular charge transfer. All of the electronic spectra of **6c** exhibited a red shift after the addition of biometals Fe^{2+}, Zn^{2+}, and Cu^{2+}, indicating the formation of a ligand-ion complex.[24]

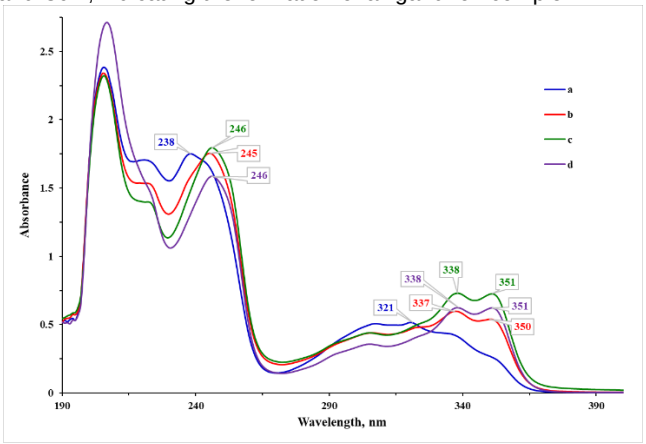


Figure 4. (a) UV spectrum of compound **6c**. (b) Spectrum of a mixture of **6c** and $CuCl_2$. (c) Spectrum of a mixture of **6c** and $FeCl_2 \cdot 4H_2O$. (d) Spectrum of a mixture of **6c** and $Zn(NO_3)_2 \cdot 6H_2O$.

In conclusion, new conjugates of tacrine and salicylamide with different lengths of alkylene spacers were designed and synthesized The compounds exhibited high dual anticholinesterase activity with selectivity toward BChE. They also had rather poor anti-CES activity, suggesting a low tendency to exert potential unwanted drug-drug interactions in clinical use. Conjugates **6c-d** inhibited AChE and BChE 2–3 times more effectively than tacrine. They were mixed-type reversible inhibitors of both cholinesterases. Molecular docking indicated dual binding sites of the conjugates in AChE and the possibility of binding to the AChE PAS that, along with results on propidium iodide displacement, suggest their potential to block AChE-induced Aβ aggregation, thereby exerting a disease-modifying effect. Effective BChE inhibition of conjugates **6b-d** was due to interactions with two sites of the enzyme: the oxyanion hole in the active site and Asp70 in the PAS. The new conjugates exhibited high $ABTS^{\bullet+}$-scavenging activity. Conjugate **6c** was a lead compound for which metal-chelating activity was also shown. Thus, the synthesized group of new tacrine-salicylamide conjugates is promising for further development as potential multifunctional agents for AD therapy. An extended series of conjugates with variation of structure in the salicylamide fragment is under investigation.

Experimental Section

See the Supporting Information for synthesis and characterization of new compounds, biological assay protocols, molecular modeling method.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Tacrine • salicylamide • cholinesterase inhibitors • molecular docking • anti-AD multifunctional agents

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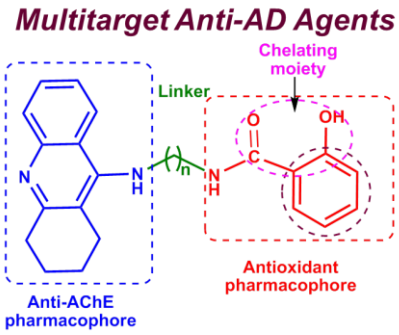

Conjugates of tacrine and salicylamide were synthesized as potential multifunctional anti-AD agents. The compounds have high anticholinesterase (IC_{50} AChE to 0.22 μM, IC_{50} BChE to 0.01 μM) and low anti-CES activity, displaced propidium iodide from the AChE PAS, being in agreement with the results of molecular docking. The conjugates exhibited $ABTS^{\bullet+}$-scavenging (TEAC to 0.9) and metal-chelating activity.