Synthesis, Biological Activity and Molecular Modeling of 4-Fluoro-*N*-[ω-(1,2,3,4-tetrahydroacridin-9-ylamino)-alkyl]-benzamide Derivatives as Cholinesterase Inhibitors

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Key words
- Alzheimer's disease
- tacrine
- fluorobenzoic acid
- docking
- Ellman's method

Abstract

The aim of this study was to synthesize and determine the biological activity of new derivatives of 4-fluorobenzoic acid and tetrahydroacridine towards inhibition of cholinesterases. Compounds were synthesized in condensation reaction between 9-aminoalkyl-tetrahydroacridines and the activated 4-fluorobenzoic acid. Properties towards inhibition of acetyl- and butyrylcholinesterase were estimated according to Ellman's spectrophotometric method. Among synthesized compounds the most active were compounds **4a** and **4d**. These compounds, in comparison with tacrine, were characterized by the similar values of IC_{50}. Among all obtained compounds, **4d** presented the highest selectivity towards inhibition of acetylcholinesterase. Molecular modeling studies revealed that all derivatives presented similar extended conformation in the gorge of acetylcholinesterase, however, there were 2 main conformations in the active center of butyrylcholinesterase: bent and extended conformation.

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Introduction

Progressive mental and cognitive deterioration in old age has been identified and described throughout history. At the beginning this pathological state was called 'dementia'. This term is derived from the Latin ('de-' means 'out from' and 'mens' is 'the mind'). Alzheimer's disease (AD), the most common form of dementia, was first identified in 1906 by German neurologist Alois Alzheimer. Some years later Emil Kraepelin named recognized disorder as Alzheimer's disease [1]. Since its discovery more than 100 years ago, there have been many scientific discoveries in AD research. One of them is relation between memory decline and the number of plaques and tangles in the patient's brain.

The diagnostic procedure and therapeutic management of AD have been areas of interest for many scientists. During the last 10 years there has been made enormous progress in understanding of potential risk factors, molecular basis of pathological lesions and brain regions which are affected. However, AD still remains one of incurable diseases. It is believed that there is an urgent need to develop effective disease-modifying treatments to slow or stop progression of AD [2].

At the early stage of AD it is very difficult to differentiate the disease symptoms and memory decline connected with advanced age. [3] AD is frequently diagnosed on the basis of the patient history, accompanied by relatives' history, and clinical observations of cardinal neurological symptoms of the disease. For routine diagnosis of AD conventional structural neuroimaging (computer tomography (CT) and magnetic resonance (MR)) is widely utilized. Nevertheless, these neuroimaging methods are not sufficient for detection of early structural changes in the brain. Therefore, more advanced neuroimaging techniques including positron emission tomography (PET), single photon emission CT (SPECT), and functional magnetic resonance imaging (MRI) give opportunity for identification of the earliest abnormalities during the disease course [4].

Pathogenesis of AD is complex, and is associated with alterations of certain neurotransmitter systems in the central nervous system. The most significant changes concern a deficit of cholinergic transmission which contributes to learning and memory dysfunction. The level of acetylcholine (ACh) depends on activities of 2 ACh-hydrolysing enzymes – acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). It was thought

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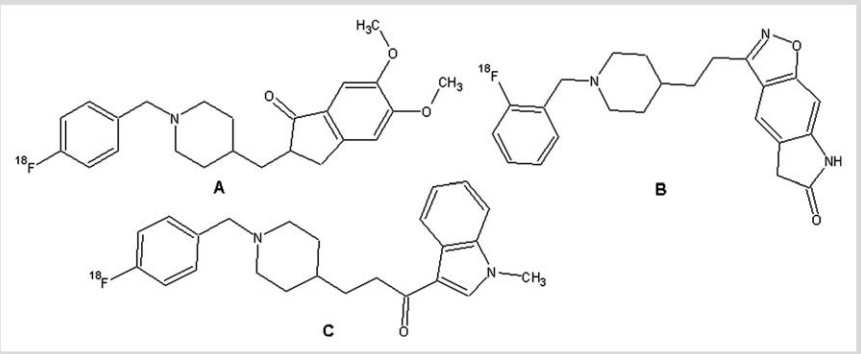

Fig. 1 Structures of fluorinated ligands: (4-[^{18}F]FDP) – **a**, (2-[^{18}F]fluoro-CP-118,954) – **b**, 3-[1-(4-[^{18}F]fluorobenzyl)piperidin-4-yl]-1-(1-methyl-1 H-indol-3-yl)propan-1-one – **c** [6, 9, 10].

that the main enzyme responsible for ACh hydrolysis is AChE, however, significant evidence pointing to the role of BChE in physiological cholinergic function has appeared [5]. Apart from ACh hydrolysis these both enzymes exhibit noncholinergic functions which include participation in many cellular processes, neuron development and differentiation, regional cerebral blood flow, and in the amyloid cascade [2, 3].

It has been reported that the ratio BChE/AChE gradually elevates in AD brain, partially because of the progressive loss of the cholinergic synapses where AChE activity is located. Thus, the use of BChE inhibitors in treating moderate to severe forms of AD might be favorable, and, on the other hand, AChE has become an attractive target for the diagnosis of AD due to the significant reduction in its activity. Two radioligand approaches: radiolabeled substrates and inhibitors of AChE might be used in *in vivo* studies of AChE. [6] Radiolabeled AChE inhibitors have been developed as a means of visualizing the AChE density, however, some radioligands (for example [^{11}C]physostigmine, N-[^{11}C] methyltacrine) allow only nonspecific binding in the brain regions because of their low selectivity of AChE over BChE and mild binding properties to AChE [6–8]. There are also available ^{18}F-labeled radioligands, such as 1-(4-[^{18}F]fluorobenzyl)-4-[(5,6-dimethoxy-1-oxoindan-2-yl)methyl]piperidine (4-[^{18}F] FDP), [9] 3-[1-(4-[^{18}F]fluorobenzyl)piperidin-4-yl]-1-(1-methyl-1 H-indol-3-yl)propan-1-one, its 3-[^{18}F]fluoromethylbenzyl derivative [10]. A novel radioligand 5,7-dihydro-3-[2-[1-(2-fluorobenzyl)-4-piperidinyl]ethyl]-6 H-pyrrolo[3,2,f]-1,2-benzisoxazol-6-one (2-[^{18}F]fluoro-CP-118,954) in *in vivo* distribution studies demonstrated a high level of radioligand accumulation both in the striatum and the olfactory tubercle, which are AChE-rich regions [6] (**Fig. 1**).

Effective treatment is as much important as early diagnosis of AD. Nowadays the only class of drugs used in the treatment of the cognitive and functional symptoms of AD is acetylcholinesterase inhibitors (AChEIs), which contribute to the retardation of AD progression. There are 4 AChEIs that are widely approved for mild to moderate AD. These are: tacrine (now tacrine is withdrawn), donepezil, rivastigmine and galantamine [11, 12].

Due to the fact that there has been made deep insight into the pathological background of Alzheimer's and understanding of the disease, researchers have contributed to the development of several new treatment strategies that might have the potential to change the course of AD. There are currently in development neuroprotective, and neuron-restorative drugs, such as agonists of the nicotinic acetylcholine receptors (nAChRs), serotonin antagonists, γ-aminobutyric acid$_B$ (GABA$_B$) antagonists, *N*-methyl-D-aspartate (NMDA) receptor antagonists and NMDA ion channel modulators, histamine antagonists, calcium channel blockers, nootropics, and other [13]. Furthermore, there have been studies conducted on novel compounds that present cholinesterase inhibition activity. Among them there are for example: phenserine, metrifonate and ambemonium [12].

Incorporation of fluorine atoms into drug candidate structure is becoming a commonplace, and enables to obtain various improved properties, such as enhanced binding interactions, metabolic stability, changes in physical properties, and selective reactivity [14]. Therefore, it is of vital importance to search for highly selective and potent compounds with fluorine atoms in their structure with AChE inhibitory properties. Bis-(6-fluoro)-tacrines, synthesized by Hu et al. were found to be more potent in inhibiting rat AChE than tacrine and the unsubstituted bis-tacrine [15].

Development of new therapies for AD is extremely important, thus in continuation of our previous study [16], we present synthesis of a new series of amino derivatives of tetrahydroacridine coupled with fluorobenzoic acid as potential multifunctional drugs. Fluorobenzoic acid, for the first time coupled with tetrahydroacridine derivatives, possesses dual course of action. Firstly, it could improve anticholinesterase activity and, after radiolabeling, it could be regard as a potential marker for diagnostic imaging. Within this study all synthesized compounds were evaluated towards inhibition of both cholinesterases, and studies of molecular modeling were performed to estimate the binding mode with both enzymes.

Materials and Methods

Chemistry

Accomplished synthesis of all compounds comprised 3 stages. The first part involved preparation of the heterocycle and, afterwards, connection of it with aliphatic chain varying in number of carbon atoms. Then, we combined obtained moiety with 4-fluorobenzoic acid which was activated by 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT). As previously reported [15] we prepared 9-chloro-1,2,3,4-tetrahydroacridine by the cyclization of anthranilic acid with cyclohexanone in $POCl_3$. The next step of synthesis involved reaction between 9-chloro-1,2,3,4-tetrahydroacridine and the appropriate ω-diamine (2 equivalents) in presence of phenol and sodium iodide [17, 18].

Intermediates (**2a–2e)** were obtained according to previously published protocols [16, 19–24]. The last step of the synthesis was to couple activated 4-fluorobenzoic acid with compounds (**2a–2e**) in 2 independent steps in a one-pot synthesis. In the first phase, the carboxylic group of fluorobenzoic acid was acti-

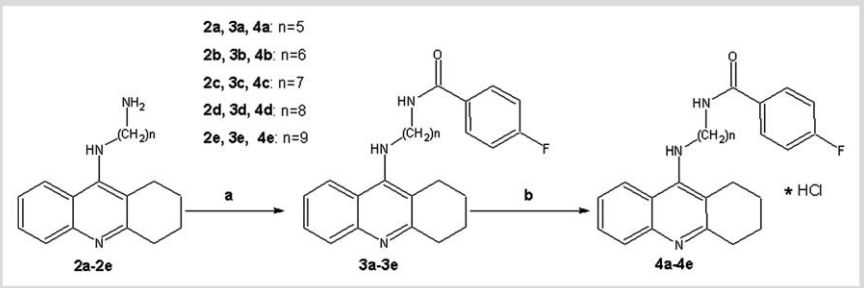

Fig. 2 Synthesis of compounds **3a–3e** and **4a–4e**. Reagents: **a** 4-fluorobenzoic acid, CDMT, methylmorpholine, THF; **b** HCl/ether. Compounds **2a–2e** were obtained according to previous papers [16, 19–24].

vated by CDMT in tetrahydrofuran (THF) and *N*-methylmorpholine at −5 ° C, the intermediate product could hardly be isolated. [25,26] After 4 h to the reaction mixture compounds (**2a–2e**) in small volume of THF were added dropwise and mixed for 24 h The resulting compounds (**3a–3e**) were then converted into hydrochlorides (**4a–4e**) by crystallization from HCl in ether (**Fig. 2**).

Accurate description of the synthesis of all compounds, and the descriptions of spectra confirming their structures are available in Supplementary material section.

Biochemical studies

Materials: Acetylthiocholine iodide (ATChI) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louise, MO). Sodium hydrogen phosphate anhydride and sodium dihydrogen phosphate were from J.T. Baker (Europe). All other chemicals used were of analytical grade and the highest chromatographic purity available.

Enzymes: Acetylcholinesterase from *Electrophorus electricus* (electric eel) – Type III and butyrylcholinesterase from equine serum were obtained from Sigma Chemical Co. (St. Louise, MO).

Enzyme assays: The activity of synthesized compounds towards inhibition AChE and BChE was measured according to the method of Ellman et al., [27] using acetylthiocholine as substrate. The compounds **4a–4d** activities of the cholinoesterases were assayed as described earlier [28,29].

Acetylthiocholine iodide at 7 concentrations in phosphatate-buffered solution (0.1 M, pH 8.0) and solution of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.05 ml, 0.5 M) were mixed in the absence and presence of compounds **4a–4d**. Then AChE from *Electrophorus electricus* at a concentration 5 units/ml was added to the samples to a final volume 3 ml and placed at 37 °C in the cuvette holder of a PerkinElmer fluorescence spectrophotometer. The emission spectra were recorded with wavelength set at 412 nm after 1 min. Butyrylcholinesterase (BChE) inhibitory activity studies were carried out similarly using 5 units/ml of BChE instead of AChE in a final volume 3 ml.

Statistical analysis: The drug concentration producing the 50 % AChE and BChE activity inhibition (IC_{50}) was calculated by non-linear and linear regression.

Molecular modeling

The 3-dimentional structure of ligands was created by Corina on-line (Molecular Networks) and then prepared with Sybyl 8.0 (Tripos). Atom types were checked, hydrogen atoms were added and then Gasteiger-Marsili charges were assigned. Ligands were docked to acetylcholinesterase from 2CKM and butyrylcholinesterase from 1P0I crystal complex. Before docking with GoldSuite 4.1 (CCDC) the protein was prepared. All histidine residues were protonated at Nε, the hydrogen atoms were added, ligand and water molecules were removed and the binding site was defined as all amino acid residues within 10 Å from bis-(7)-tacrine for AChE and 20 Å from the glycerol molecule, present in the active center of BChE. A standard set of genetic algorithm with population size 100, number of operations 100 000 and clustering with a tolerance of 1Å were applied. As a result 10 ligand poses, sorted by GoldScore (AChE) and ChemScore (BChE) function value were obtained. The results were visualized by PyMOL 0.99rc6 (DeLano Scientific LLC).

Results

The biological testing was conducted according to spectrophotometric Ellman's method [27–29]. We estimated acetylcholinesterase and butyrylcholinesterase activity of all synthesized compounds. Among the group of synthesized compounds the most active towards AChE inhibition were molecules **4a,** and **4d** which possess 5 and 8 carbon atoms in the aliphatic chain between fluorobenzoic acid and tetrahydroacridine, respectively. However, these compounds exhibited IC_{50} value similar to tacrine. In our previous study [16], we described synthesis and biological evaluation of 3 derivatives of fluorobenzoic acid and 1,2,3,4-tetrahydroacridin-9-ylamine with 2, 3 and 4 carbon atoms in the aliphatic chain. These 3 compounds were characterized, in comparison with tacrine, by 4-fold higher inhibitory activity towards AChE, suggesting that introduction of aliphatic chain was a good alteration. However, according to the results of our current study incorporation of longer than 4 carbon atoms aliphatic chain between fluorobenzoic acid and tetrahydroacridine did not contribute to the higher activity towards AChE inhibition. Unfortunately, on the basis of the result of the study, we cannot observe a straightforward relationship between the length of aliphatic chain and the activity of the synthesized compounds.

In our latest study [30] we presented synthesis and biological evaluation of derivatives of 4-fluorobenzoic acid and 2,3-dihydro-1*H*-cyclopenta[b]quinoline. We reported that incorporation of a 5 membered ring significantly influenced the activity of synthesized compounds towards inhibition of AChE. However, similarly to the current study, we could not come to conclusion that together with the length of aliphatic chain between fluorobenzoic acid and 2,3-dihydro-1*H*-cyclopenta[b]quinolone the activity of synthesized compounds was increased. In that study the most active were compounds with those with 2 and 4 carbon atoms in aliphatic chain. These compounds were 5- and 11-fold more active than tacrine, respectively. However, unlikely to our previous study [16], the compound with 3 carbon atoms in aliphatic chain between fluorobenzoic acid and 2,3-dihydro-

1*H*-cyclopenta[b]quinolone moiety is 2-fold less active than tacrine, and, as a consequence, we cannot observe the increased activity for all compounds with the shortest aliphatic chain. On the other hand, unlikely to the current study, among the compounds based on, 3-dihydro-1*H*-cyclopenta[b]quinolone with longer aliphatic chain the one with 7 carbon atoms in the chain was 2-fold more active than tacrine.

In case of inhibition of BChE all synthesized constituents, apart from **4a** (similar IC_{50} value), were characterized by lower BChE inhibitory activity in comparison with tacrine.

Table 1 contains also values of relative inhibitory effects towards acetylcholinesterase (ratio IC_{50} BChE/AChE) and butyrylcholinesterase (ratio IC_{50} AChE/BChE).

All synthesized compounds are characterized simultaneously by higher selectivity for AChE and lower selectivity for BChE in comparison with tacrine. Also in case of compounds presented in our previous paper [16] we observed higher selectivity for AChE and lower selectivity for BChE when compared with tacrine. The most selective towards BChE among synthesized constituents was compound **4a**, however, it was still 4-fold less selective in comparison to tacrine. It could be a promising result, as Greig et al. reported that selective BChE inhibition may ameliorate a cholinergic deficit, which likely worsens in AD due to increased activity of BChE [5].

Molecular modeling studies revealed the binding mode of obtained compounds. All derivatives presented similar extended conformation in the gorge of acetylcholinesterase whereas there were 2 main conformations in the active center of butyrylcholinesterase: bent conformation and extended one. The new series of compounds acted as dual binding site acetylcholinesterase inhibitors i.e. all inhibitors interacted with catalytic and peripheral active center. The most potent compound **4a** created the most advantageous interactions in the active gorge due to the 5 carbon linker which provided the best fit of the outermost fragments of molecule to both active sites (**Fig. 3**).

The tacrine fragment formed a characteristic sandwich with Trp84 and Phe330 by π-π stacking. The protonated form of tacrine gave hydrogen bond with carbonyl group of His440. The linker was located in the middle of gorge where it created hydrophobic interactions, mainly with Tyr334. The phenyl ring of benzamide formed the second characteristic sandwich by π-π stacking with Trp279 and Tyr70. It also created CH-π interactions with Tyr121. In case of longer linkers (8 or 9 methylene groups) the phenyl ring was slightly shifted and it resulted in a slightly lower activity (compounds **4d**, **4e**). The carbonyl group of benzamide moiety created H-bond with hydroxyl substituent from Tyr121. In case of compounds **4b** and **4c** the carbonyl group could be oriented at opposite direction and in that position it wasn't able to form hydrogen bond which could result in slightly decreased activity. The fluorine substituent created hydrophobic interactions mainly with side chain of Ile175 and also with Trp279 and Tyr70. Obtained compounds showed 2 different binding modes with butyrylcholinesterase. For the most active

Compounds	AChE Inhibition IC_{50}, (nM) ± SEM[a]	BChE Inhibition IC_{50}, (nM) ± SEM[a]	Selectivity for AChE[b]	Selectivity for BChE[c]
4a	5.30 ± 0.15	0.05 ± 0.006	0.01	106
4b	24.30 ± 0.8	3.96 ± 0.17	0.16	6.14
4c	22.80 ± 1.4	4.87 ± 0.6	0.21	4.68
4d	5.60 ± 0.4	50.2 ± 4.4	8.96	0.11
4e	13.40 ± 4.0	3.12 ± 0.23	0.23	4.29
THA	5.46 ± 1.00	0.02 ± 0.006	5.49	273

[a] Means ± SEM of 3 experiments

[b] Selectivity for AChE is defined as IC_{50}(BChE)/IC_{50}(AChE)

[c] Selectivity for BChE is defined as IC_{50}(AChE)/IC_{50}(BChE)

Table 1 IC_{50} values of activities towards acetylcholinesterase and butyrylcholinesterase.

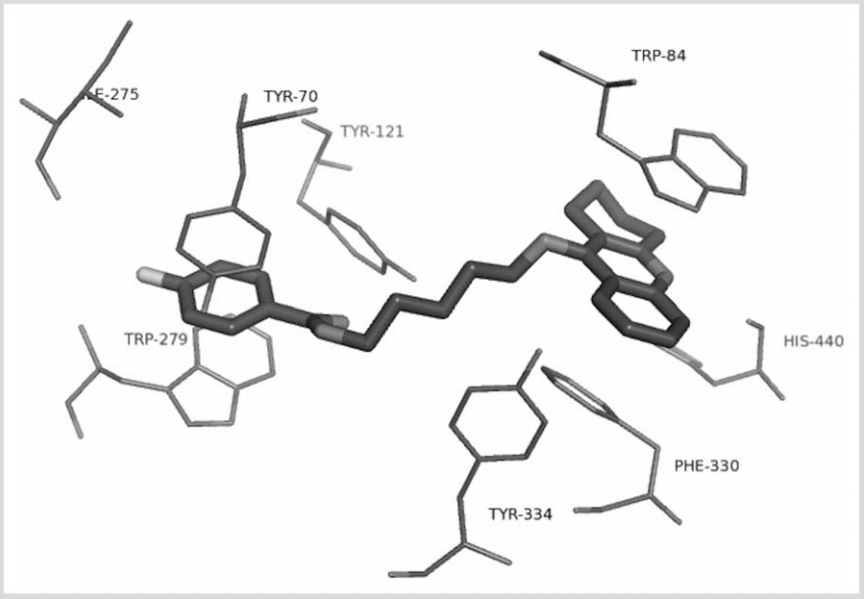

Fig. 3 Binding mode of compound **4a** with acetylcholinesterase.

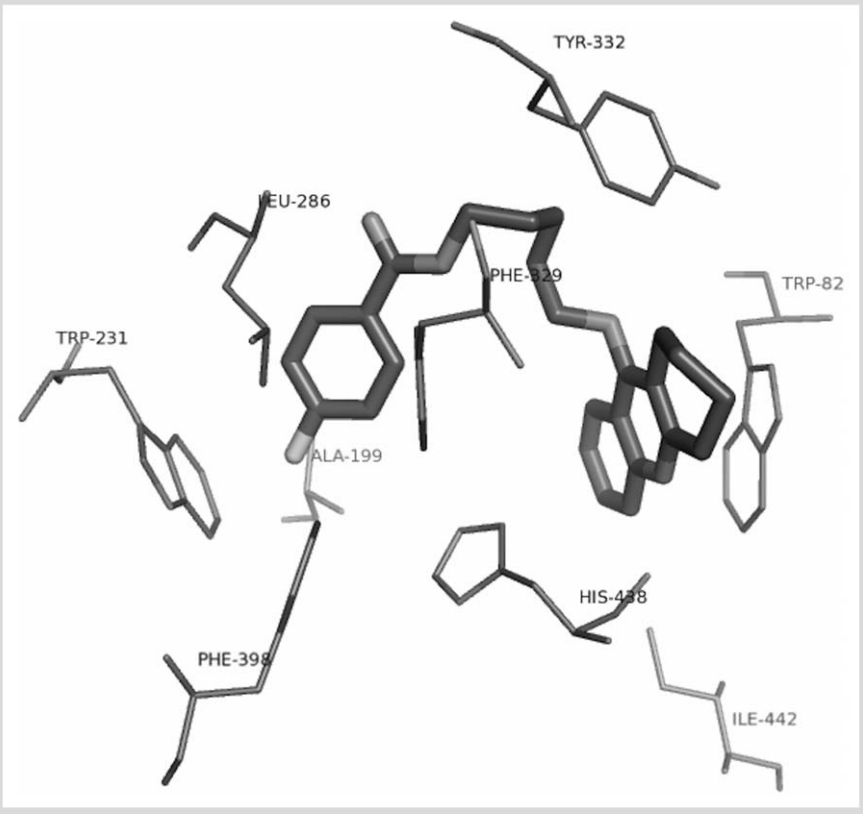

Fig. 4 Binding mode of compound **4a** with butyrylcholinesterase.

compound **4a** the docking runs were more converged and it occurred in bent conformation (▶ **Fig. 4**).

The tacrine moiety interacted by π-π stacking with Trp82 and formed hydrophobic interactions with Ile442. Protonated form of tacrine created hydrogen bond with carbonyl group of His438. Chain was directed to Tyr332. Fluorobenzamide was located in hydrophobic region which was set by Phe329, Phe398, Trp231, Leu286 and Ala199. In case of compounds **4b** and **4c** the extended conformation appeared but still the bent one was dominated. The compounds with longer linker (**4d**, **4e**) presented mainly extended conformation with fluorobenzamide moiety near Phe278 and Tyr282. The diversification of binding modes for **4b** and **4c** or interactions with lower amount of hydrophobic residues for **4d** and **4e** could result in a bit decreased potency. Concluding, the compound **4a** presented the most advantageous binding mode with acetyl- and butyrylcholinesterase which made it the most active inhibitor in the whole series. On the basis of the current stage of knowledge the next step of our study will be labeling of compounds presented in this work with ^{18}F atoms and then evaluation them as radioligands for the *in vivo* mapping of AChE. Replacement of fluorine atom with the radioactive fluorine isotope will enable to use the synthesized compounds for scintigrapic diagnosis by means of positron emission tomography (PET).

Conclusion

Enhancement of cholinergic function in the central nervous system in the course of AD is very important. This goal might be achieved by use of acetylcholinesterase inhibitors such as tacrine, the first AChEI drug approved by FDA for clinical use, rivastigmine, galantamine and donepezil.

However, there is still a great need to search for new compounds with anticholinesterase activity and, simultaneously, high selectivity and specificity. Therefore, we decided to synthesize a novel series of tetrahydroacridine derivatives. We synthesized 5 novel tetrahydroacridine derivatives coupled for the first time with fluorobenzoic acid as a series of acetylcholinesterase inhibitors with high activity. We estimated inhibitory properties towards inhibition of AChE and BChE. Compounds **4a**, and **4d** had similar activity towards AChE in comparison with tacrine.

In case of BChE all compounds were less active towards its inhibition in comparison with tacrine. Only compound **4a** had slightly lower activity towards BChE than tacrine. Compound **4a**, which has high selectivity towards BChE, appear to be promising and encourage us for further optimization, because of detrimental role of BChE in AD.

Acquired data is of extreme importance because we obtained 2 compounds (**4a** and **4d**) with good activities and special selectivity towards enzymes which suggest that these compounds might be utilized in further studies in direction to AD therapy. On the other hand simultaneous radiolabelling with fluorine isotope (^{18}F) and the following estimation of the level of both cholinesterases certainly could be regarded as novelty. It would enable to indicate the progress of neurodegeneration advancement of nerve cells.

In summary, this study describes synthesis and biochemical evaluation towards inhibition of acetylcholinesterase and butyrylcholinesterase. Two novel compounds (**4a** and **4d**) showed similar to tacrine anticholinesterase activity and are characterized by promising selectivity towards enzymes.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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