Focused design of polypharmacophoric neuroprotective compounds: Conjugates of \(\gamma\)-carbolines with carbazole derivatives and tetrahydrocarbazole

Abstract: Alzheimer's disease has a complex multifactorial nature; therefore, a promising approach for the development of efficient therapeutic agents is the concept of multitarget drugs, which affect several biological targets involved in the pathogenesis of the disease. We developed a synthetic algorithm for conjugating several pharmacophoric ligands acting on the key stages of pathogenesis of several neurodegenerative diseases and synthesized hybrid structures combining the \(\gamma\)-carboline fragment of Dimebon with carbazole and tetrahydrocarbazole moieties. Using the complex primary screening system the structures have been revealed that combine the high inhibitory activity and selectivity towards butyrylcholinesterase with the radical-scavenging activity and the ability to potentiate tubulin polymerization to microtubules with a normal structure and/or prevent mitochondrial permeability transition. The lead compound was identified for future optimization and development of new multi-target drugs against neurodegenerative diseases combining the cognitive-stimulating and neuroprotective potentials.

Keywords: Alzheimer’s disease; butyrylcholinesterase; carbazole; \(\gamma\)-carboline; conjugates; Mendeleev XX; microtubules; MPT; multitarget drugs; neuroprotection; radical-scavenging.

Introduction

The search for efficient agents for the treatment of neurodegenerative diseases is an important trend of modern medicinal chemistry. This refers, first of all, to Alzheimer’s disease (AD), the most widely encoun-
tered type of dementia in elder people, which is characterized by a persistent decline in the cognitive function and memory until complete degradation of personality. According to the forecast of Alzheimer’s Disease International experts [1], the number of dementia patients will increase from 36 million in 2010 to 66 million in 2030 and to 115 million by 2050.

The drugs used today are mainly symptomatic; they partly compensate for the lost cognitive functions by activating some neurotransmitter systems. Despite the enormous financial expenditures, not a single new drug for the treatment of this disease has entered the market in the last 13 years [2, 3].

It is known that the key neurotransmitter systems affected during the development of Alzheimer’s disease are the cholinergic and glutamatergic systems [4, 5]. The search for the drugs that can restore the lost functions of these neurotransmitter systems was initially regarded as the most obvious drug design strategy for the therapy of AD. The cholinesterase inhibitors – donepezil, rivastigmine, galantamine and a low-affinity noncompetitive NMDA receptor antagonist memantine are still the main therapeutic agents for treating this disease [6–8].

The low efficiency of the applied mono-target drugs is due to the multifactorial nature of AD. In this connection, the concept of multi-target drugs affecting simultaneously a number of biological targets involved in the pathogenesis of the disease appears to be a highly promising approach to the design of new-generation drugs for treating AD [2, 9–11]. It can be expected that such drugs would be able not only to compensate for the lost cognitive functions, but also to suppress the further development of the neurodegenerative process [12–15].

Oxidative stress, characterized by unbalance between the formation of reactive oxygen species (ROS) and removal of ROS by various antioxidant system mechanisms, is known to be a key factor adversely influencing the vital activity of brain neurons [16, 17]. The efficiency of brain antioxidant system gradually declines with age, being this decline more pronounced in AD patients. In turn, this substantiates the use of antioxidants in the AD therapy, and the development of cholinesterase inhibitors with additional antioxidant properties is a modern trend in the research towards efficient therapy of AD [15, 18, 19].

Neurodegenerative impairments in AD are a complex combination of neurochemical processes, among which the disturbances of mitochondrial functions and the state of microtubule system are the most important [20, 21]. Mitochondria play the crucial role in a number of processes, such as apoptosis, necrosis, inflammation, stress response, and in a wide range of pathologies (blood strokes, infarctions, diabetes, neurodegeneration, etc.). Furthermore, mitochondria are very important for the age-related degradation and dysfunctions of organs and systems determining normal or premature aging. The increasing sensitivity of mitochondria to agents inducing a specific process of mitochondrial permeability transition (MPT) is not only a typical feature inherent in aging, but also an important component of the neurodegenerative process [22]. Indeed, the synaptic activity and axonogenesis largely depend on the preserved functional activity of mitochondria and active tubulin assembling into microtubules, which provide both the polarization during axon formation and transport of mitochondria into axon terminals [23]. Neurodegenerative diseases, in particular, Alzheimer’s disease, are associated with impairments of the microtubule system, destabilization of microtubules in axons being especially significant [24]. One of the reasons is pathological hyperphosphorylation of a microtubule-associated protein, namely, τ-protein; this process reduces the microtubule-stabilizing role of τ-protein and leads to its aggregation with the formation of neurofibrillary tangles, which represent a typical pathomorphological marker of AD [25].

In this connection, it appears promising to carry out targeted search for compounds able to stabilize the mitochondrial function and to promote ordered polymerization of tubulin, the major protein of microtubules.

The strategy of our research was based on focused synthesis and investigation of compounds that exhibit multitarget action on cholinesterases, polymerization of microtubules, the opening of mitochondrial non-specific pores, and are capable of scavenging reactive free radicals. These structures could have synergistic action on a set of targets, providing cognitive-stimulating, neuroprotective, and disease-modifying effects, resulting in enhancement of the overall pharmacological effect. One of such approaches is the design of hybrid molecules combining two or more pharmacophores [10, 11, 15, 26–28]. It is believed that the design of a common chemical molecule affecting simultaneously numerous pathogenetic mechanisms
of the disease may have additional advantages over combinations of drugs regarding both the optimal ADMET profile and reduction of the risk of adverse effects caused by the interaction of particular drug components [29, 30].

For this purpose, we synthesized several groups of novel compounds, which combined pharmacophoric groups of specific ligands for the biological targets known to be involved in AD pathogenesis. In this paper, we consider one class of the synthesized compounds, namely, conjugates of tetrahydro-γ-carboline with carbazole derivatives and tetrahydrocarbazole. γ-Carboline derivatives have attracted close attention in recent years as new-generation agents for the treatment of various neurodegenerative diseases, including AD [31–35]. A well-known representative of this series of compounds is the antihistamine drug Dimebon (Latrepirdine) (Fig. 1), possessing a broad spectrum of pharmacological activity [36, 37], in particular, promoting the improvement of cognitive function [32, 38–40] and exhibiting neuroprotective, antidepressant, and geroprotective actions [37, 39, 41]. Recent studies showed that Dimebon is able to protect neurons in different neurodegeneration models [37, 42–44].

The structurally related to γ-carbolines class of carbazole derivatives possess a very broad spectrum of biological activity [45] including antioxidant and neuroprotective properties [46, 47]. In recent years, promising compounds for the development of disease-modifying drugs for the therapy of AD have been found among carbazole derivatives. In particular, these are aminotetrahydrocarbazoles [48], which are able to stimulate neurogenesis and stabilize the endoplasmic reticulum (ER) calcium homeostasis by attenuating the FAD-PS1 mediated exaggerated ER calcium release. Furthermore, they can improve mitochondrial function measured by increased mitochondrial membrane potential and lower Aβ peptide production by decreasing the cleavage of amyloid precursor protein (APP) by β-secretase, without notably affecting α- and γ-secretase cleavage activities [49–51].

Results and discussion

Synthesis

Tetrahydro-γ-carbolines were conjugated with car bazoles or tetrahydrocarbazole via alkylation of tetrahydro-γ-carbolines 1a–h by 9-oxiranylmethylcarbazoles and 9-oxiranylmethyltetrahydrocarbazole in the superbasic DMFA/KOH medium by the reported method [52, 53].

Heating of equimolar amounts of tetrahydro-γ-carbolines 1a–d,g,h and 9-oxiranylmethylcarbazoles or 9-oxiranylmethyltetrahydrocarbazole in DMF for 2 h at 50–60 °C in the presence of a catalytic amount of KOH afforded conjugates 2a–o [52, 53] and 4a–f in 73–78 % yields. The 1H NMR spectra showed a superposition of indole moities, characteristic multiplet signals in the 3.79–4.63 ppm range for the 2-hydroxypropylene spacer, and a broadened singlet at 4.52–4.75 ppm for the OH group. Conjugates 2a–o and 4a–f were converted

Fig. 1: Structures of basic pharmacophores: Dime bon, 2,8-dimethyltetrahydro-γ-carboline, carbazole, and tetrahydrocarbazole.
to the corresponding hydrochlorides $3a-o$ and $5a-f$. Physical properties, elemental analysis, and spectral data of synthesized compounds were described in previous articles [52, 53].

We investigated the effects of conjugates of $\gamma$-carboline with carbazole derivatives ($3a-o$) and tetrahydrocarbazole ($5a-f$) on the key enzymes of the cholinergic nervous system, the ability of compounds to scavenge free radicals, characteristics of mitochondria, and tubulin polymerization to the microtubules.

**Study of the inhibitory activities of $\gamma$-carboline conjugates with carbazoles and tetrahydrocarbazole towards acetylcholinesterase (EC 3.1.1.7, AChE), butyrylcholinesterase (EC 3.1.1.8, BChE), and carboxylesterase (EC 3.1.1.1, CaE)**

Anticholinesterase drugs are symptomatic and are aimed at compensating for the neurotransmitter acetylcholine deficiency [54]. They replenish the acetylcholine deficiency in the brain by inhibiting cholinesterases and increase the duration of acetylcholine action on postsynaptic receptors, thus enhancing the cholinergic transmission. Initially, cholinergic therapy in AD was directed to act on acetylcholinesterase (AChE, EC 3.1.1.7), as this is the main enzyme for acetylcholine breakdown in brain. Recent studies demonstrated that butyrylcholinesterase (BChE, EC 3.1.1.8) also participates in cholinergic transmission and can compensate for some functions of AChE by optimizing the cholinergic neurotransmission [55–58].

In a normal brain, acetylcholine is predominantly (80%) hydrolyzed by AChE, whereas BChE plays a supplementary role. However, with the progression of AD, the AChE activity decreases, whereas the activity of BChE gradually increases [57, 59]. This increases the significance of BChE as a therapeutic target for reducing the cholinergic deficiency inherent in AD [56, 60–63]. According to recent rodent tests, selective BChE inhibitors increase the acetylcholine level in brain and improve the cognitive function without the well-known side effects inherent in AChE inhibitors [56, 60, 64]. Therefore, development of selective BChE inhibitors is a new strategy for improving the quality of life of Alzheimer’s disease patients, especially in severe cases where a decrease in the acetylcholine level in the brain is accompanied by depletion of AChE activity, whereas BChE activity increases [57, 59, 63, 65–67].

The enzyme carboxylesterase (CaE, EC 3.1.1.1), structurally related to cholinesterases, hydrolyzes many therapeutically important agents containing ester groups [68, 69]; therefore, the ability of anticholinesterase compounds used for the therapy of AD to inhibit CaE may induce undesirable drug-drug interactions [70]. The approach involving determination of the esterase profiles of compounds, that is, comparative evaluation of their inhibitory activity towards several serine esterases [10, 71–76], including AChE, BChE and CaE, developed at the Institute of Physiologically Active Compounds, Russian Academy of Sciences, can serve to elucidate both the potential primary pharmacological effect and the possible adverse effects of a compound.

Using enzyme kinetics and molecular docking, we studied the inhibitory activity of conjugates $3$ and $5$ (Scheme 1) towards AChE, BChE and CaE. The basic pharmacophores, Dimebon, 2,8-dimethyltetrahydro-$\gamma$-carboline, and carbazole, were used as reference compounds (Fig. 1). Tacrine, which is an efficient AChE and BChE inhibitor, and bis-4-nitrophenyl phosphate (BNPP), a selective CaE inhibitor, served as positive controls [77].

**Kinetic measurements**

The results summarized in Table 1 demonstrate that all $\gamma$-carboline conjugates with carbazoles ($3a-o$) and tetrahydrocarbazole ($5a-f$) selectively inhibit BChE, while the activity towards AChE and CaE is low. Most of these compounds are more potent BChE inhibitors than the basic pharmacophores – 2,8-dimethyltetrahydro-$\gamma$-carboline, carbazole, and Dimebon. All conjugates with unsubstituted carbazoles ($3a-f$) and tetrahydrocarbazole ($5a-f$) show high inhibitory activity towards BChE with the $IC_{50}$ values of about 1–6 μM, the replacement of carbazole by tetrahydrocarbazole having no effect on the anti-BChE activity.
For the conjugates of unsubstituted carbazoles (3a–f) (R₂ = H), a change in the structures of R and R₁ groups in the γ-carboline moiety has virtually no effect on the anti-BChE activities: all of compounds (3a–f) are potent inhibitors of the enzyme. Meanwhile, in the case of γ-carboline conjugates with dichloro- or dibromo-substituted carbazoles, (R₂ = Cl: 3g–k) and (R₂ = Br: 3l–o), the degree of BChE inhibition depends on the structure of R and R₁. Among compounds with dichloro-substituted carbazoles, the highest activity was found for 3g (R = H, R₁ = CH₃) and 3j (R = F, R₁ = CH₃) characterized by IC₅₀ = 3.48 ± 0.69 and IC₅₀ = 1.93 ± 0.03, respectively. Out of the dibromo-substituted carbazoles, the activity was maximum for 3l (R = H, R₁ = CH₃), 3n (R = CH₃, R₁ = C₂H₅), and 3o (R = F, R₁ = CH₃) with IC₅₀ of 2.69 ± 0.25, 3.16 ± 2.1, and 1.18 ± 0.06, respectively.

Thus, the lead compounds in this series (Table 1) are all conjugates with unsubstituted carbazoles 3a–f and tetrahydrocarbazole 5a–f and dichlor derivatives 3g (R = H, R₁ = CH₃) and 3j (R = F, R₁ = CH₃) and dibromo derivatives 3l (R = H, R₁ = CH₃), 3n (R = CH₃, R₁ = C₂H₅), and 3o (R = F, R₁ = CH₃). One more conclusion that can be drawn from the data of Table 1 is that the conjugates formed by γ-carboline derivatives with R = F and R₁ = CH₃, that is, 3e (R₂ = H), 3j (R₂ = Cl), and 3o (R₂ = Br), show high anti-BChE activity, irrespective of the structure of the carbazole moiety.

For the series of active compounds 3a, 3c, 3k, 3n, 5a, 5c, and 5e, the kinetics and mechanism of BChE inhibition were studied in detail. The kinetic data were analyzed using the Lineweaver–Burk double reciprocal plot (Fig. 2). The resulting values of BChE inhibition constants (Kᵢ is the competitive component and αKᵢ is non-competitive component) are summarized in Table 2. As shown in Table 2, all the γ-carboline conjugates with carbazoles and tetrahydrocarbazole are mixed-type BChE inhibitors.
The mechanism of inhibitory action of γ-carbolines conjugates with carbazoles and tetrahydrocarbazole is illustrated in relation to two of the active compounds 3c and 5e. The graphical analysis of kinetic data on the BChE inhibition by compounds 3c and 5e in the Lineweaver–Burk plot is presented in Fig. 2.

The tested compounds are combinations of various pharmacophores: 3c contains the γ-carboline and carbazole moieties, while 5e comprises the γ-carboline and tetrahydrocarbazole units. As observed in Figs. 2a and b, binding of either 3c or 5e to BChE induces changes in both $V_{\text{max}}$ and $K_{\text{m}}$, which is typical of mixed inhibition. Variation of the pharmacophore combination does not affect the mechanism of BChE inhibition by these compounds. The inhibition constants were determined: for compound 3c, $K_i = 2.55 \pm 0.18 \mu\text{M}$ (competitive component) and $\alpha K_i = 12.5 \pm 0.7 \mu\text{M}$ (non-competitive component), and for compound 5e, $K_i = 3.89 \pm 0.15 \mu\text{M}$ (competitive component) and $\alpha K_i = 10.2 \pm 1.1 \mu\text{M}$ (non-competitive component).

Thus, unlike the reference compounds, 2,8-dimethyltetrahydro-γ-carboline and carbazole, their conjugates are potent and selective BChE inhibitors efficient in the micromolar range. The esterase profile of the main reference compound, Dimebon, also changes. The BChE inhibition is reversible and corresponds to the mixed type.

### Molecular modeling

According to the molecular docking of conjugates into BChE, they were found to bind both to PAS in the middle of the gorge and at the gorge bottom where they interact with the active site amino acid residues (Fig. 3), which accounts for the mixed type of BChE inhibition observed for these compounds.

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**Table 1:** Inhibitory activity of conjugates of γ-carbolines with carbazole derivatives and tetrahydrocarbazole towards AChE, BChE, and CaE.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (μM) ± SEM or inhibition % at 20 μM</th>
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<tbody>
<tr>
<td></td>
<td>AChE</td>
</tr>
<tr>
<td>3a H</td>
<td>15.2 ± 1.1%</td>
</tr>
<tr>
<td>3b H</td>
<td>19.5 ± 1.5%</td>
</tr>
<tr>
<td>3c CH$_3$</td>
<td>13.6 ± 1.1%</td>
</tr>
<tr>
<td>3d CH$_3$</td>
<td>n.a.</td>
</tr>
<tr>
<td>3e F</td>
<td>29.2 ± 2.6%</td>
</tr>
<tr>
<td>3f F</td>
<td>17.0 ± 1.8%</td>
</tr>
<tr>
<td>3g H</td>
<td>14.6 ± 1.4%</td>
</tr>
<tr>
<td>3h CH$_3$</td>
<td>14.8 ± 0.8%</td>
</tr>
<tr>
<td>3i CH$_3$</td>
<td>8.3 ± 0.9%</td>
</tr>
<tr>
<td>3j F</td>
<td>20.3 ± 1.8%</td>
</tr>
<tr>
<td>3k F</td>
<td>14.2 ± 1.5%</td>
</tr>
<tr>
<td>3l H</td>
<td>10.9 ± 0.4%</td>
</tr>
<tr>
<td>3m H</td>
<td>10.4 ± 0.7%</td>
</tr>
<tr>
<td>3n CH$_3$</td>
<td>12.3 ± 1.6%</td>
</tr>
<tr>
<td>3o F</td>
<td>11.6 ± 1.1%</td>
</tr>
<tr>
<td>3p H</td>
<td>13.4 ± 1.7%</td>
</tr>
<tr>
<td>3q H</td>
<td>15.1 ± 1.4%</td>
</tr>
<tr>
<td>5a CH$_3$</td>
<td>20.7 ± 1.8%</td>
</tr>
<tr>
<td>5b CH$_3$</td>
<td>7.7 ± 0.5%</td>
</tr>
<tr>
<td>5c F</td>
<td>22.7 ± 1.9%</td>
</tr>
<tr>
<td>5d F</td>
<td>14.6 ± 1.1%</td>
</tr>
<tr>
<td>5e CH$_3$</td>
<td>36.3 ± 5.8</td>
</tr>
<tr>
<td>5f CH$_3$</td>
<td>18.9 ± 2.9</td>
</tr>
<tr>
<td>5g CH$_3$</td>
<td>0.60 ± 0.05</td>
</tr>
<tr>
<td>5h CH$_3$</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

n.a., means no activity.
The bold values correspond to the most active compounds.
The positions in the PAS are energetically preferred, but at the same time, they correspond to less specific interactions of the carbazole and tetrahydrocarbazole moieties. The replacement of carbazole in the conjugate molecule by tetrahydrocarbazole, for example, for compounds 3a/5a does not affect the anti-BChE activity (Fig. 4a).

Similarly, in some cases, the substituent in the γ-carboline moiety located outside the contact area with active site amino acids (Fig. 4b) may also have no effect on the position of molecule in the active site and, hence, on the inhibitory activity, as indicated by comparison of the conjugates formed by tetrahydrocarbazoles 5b and 5e.

Meanwhile, comparison of the positions of compounds with different substituents in γ-carboline for the series of dichloro- and dibromocarbazoles demonstrated that, although the radii and properties of these two halogens differ little, in some cases, this can dictate the preferred position of the dihalogenated conjugate in the BChE active site and, hence, affect the inhibitory efficiency to some extent. Indeed, the positions are very similar for compounds 3g, 3l, and 3n, whereas the position of less active compound 3l is different (Fig. 5), although the difference is not very large.
Fig. 3: Docked poses of compound 5a at the gorge bottom (the carbon atoms of 5a are shown in pink) and the same compound in the middle of BChE gorge (the carbon atoms are shown in blue). The yellow dashed line indicates the hydrogen bond with a catalytic serine residue, the orange dashed line shows the π-cationic interaction, and the blue one stands for the salt bridge with the PAS amino acid residues. Presented poses correspond to the most populated clusters after 256 AutoDock runs (see more parameters in the Methods section).

Fig. 4: (a) Docked positions of conjugates with the carbazole 3a (carbon atoms are shown in orange) and tetrahydrocarbazole moieties 5a (carbon atoms are shown in pink) with identical substituents in the γ-carboline moiety in the BChE active site. (b) Docked positions of tetrahydrocarbazole 5b (carbon atoms are shown in white) and 5e (carbon atoms are shown in red) conjugates with various substituents in the γ-carboline moiety in the BChE active site. Presented poses correspond to the most populated clusters after 256 AutoDock runs (see more parameters in the Methods section).
As regarding the weak interaction of the $\gamma$-carboline–carbazole and $\gamma$-carboline–tetrahydrocarbazole conjugates with AChE, it is known that the major difficulty involved in binding of compounds with bulky groups to AChE is to overcome the so-called bottleneck, a narrow part of the gorge formed by the Tyr341, Tyr124, and Tyr337 side chains [78]. Furthermore, compounds without a fixed positive charge (quaternary nitrogen atom), like the conjugates in question, can form non-specific contacts in the AChE peripheral anionic site and on the surface, thus partly inhibiting the enzyme [79]; this accounts for the low inhibitory activity of the studied conjugates towards AChE.

The low inhibitory activity of $\gamma$-carboline conjugates with carbazoles and tetrahydrocarbazole towards AChE indicates that these compounds would not induce undesirable adverse effects inherent in AChE inhibitors. The lack of inhibitory activity towards CaE in these compounds implies the absence of drug-drug interactions typical of CaE inhibitors.

**Radical-scavenging activity of $\gamma$-carboline conjugates with carbazoles and tetrahydrocarbazole**

The antiradical activity of the new synthesized compounds was determined by ABTS assay [80], as a preliminary screening assessment of total antioxidant capacity of the compounds. Trolox and ascorbic acid were used as standard antioxidants.

Antioxidant activity was reported as Trolox equivalent antioxidant capacity (TEAC values) by comparing $(A_0 - A_{\text{test}})$ of a test compound with $(A_0 - A_{\text{Trolox}})$ of the Trolox standard after reaction time of 1 h, where $A_0$ is the absorbance of a control lacking any radical scavenger, $A_{\text{test}}$ and $A_{\text{Trolox}}$ are the absorbances of the remaining ABTS$^+$ in the presence of a test compound or Trolox. For the most active compounds, we also determined IC$_{50}$ values (compound concentration required for 50 % reduction of ABTS radical).
Lower IC<sub>50</sub> values are indicative of higher ABTS free radical scavenging ability. The results are presented in Table 3.

The results demonstrate that all conjugates of γ-carbolines with carbazoles (3a–o) are markedly less effective scavenging ABTS<sup>•+</sup> than Trolox (TEAC < 1). The γ-carboline conjugates with unsubstituted carbazoles (R<sub>2</sub> = H) bind the ABTS<sup>•+</sup> more efficiently than the conjugates formed by dichloro- and dibromo-substituted carbazoles (R<sub>2</sub> = Cl, Br): 3a and 3g; 3b and 3m; 3c and 3h. The difference is leveled out with increasing hydrophobicity of the substituents R and R<sub>1</sub> in the γ-carboline moiety: 3c and 3h; 3d, i and 3n.

The conjugate of unsubstituted carbazole 3b (R = H, R<sub>1</sub> = C<sub>2</sub>H<sub>5</sub>) is most active: TEAC = 0.75, IC<sub>50</sub> = 28.3 μM. It is worth noting that the radical-scavenging activity of all conjugates of carbazole derivatives (including both unsubstituted (3a–f) and dichloro- (R<sub>2</sub> = Cl: 3g–k) or dibromo-substituted (R<sub>2</sub> = Br: 3l–o) carbazoles) markedly decreases as electron-withdrawing fluorine atom is introduced in the γ-carboline moiety (R = F: compounds 3e, f; 3j, k; 3o).

The conjugates of γ-carbolines with tetrahydrocarbazole (5a–f) scavenge the ABTS<sup>•+</sup> more efficiently than carbazole derivatives (3a–o). The increase in the antiradical activity is promoted by the presence of ethyl group as R<sub>1</sub> in the γ-carboline moiety. Compounds 5b (R = H, R<sub>1</sub> = C<sub>2</sub>H<sub>5</sub>) and 5d (R = CH<sub>3</sub>, R<sub>1</sub> = C<sub>2</sub>H<sub>5</sub>) are lead compounds for this group, with their radical-scavenging activity being roughly equal to that of Trolox. It is noteworthy that the combination of R = H and R<sub>1</sub> = C<sub>2</sub>H<sub>5</sub> in the γ-carboline moiety of the conjugates results in the highest antiradical activity, as in the case of unsubstituted carbazole derivatives (3b). The presence

<table>
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<th>Compound</th>
<th>ABTS&lt;sup&gt;•+&lt;/sup&gt; scavenging</th>
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<td></td>
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<td>3a H CH&lt;sub&gt;3&lt;/sub&gt; H</td>
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<td>0.75 28.3 ± 1.8</td>
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<tr>
<td>3c CH&lt;sub&gt;3&lt;/sub&gt; CH&lt;sub&gt;3&lt;/sub&gt; H</td>
<td>0.59 36.3 ± 2.2</td>
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<tr>
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<td>0.62 35.9 ± 2.1</td>
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<tr>
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<td>0.11 n.d.</td>
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<td>0.76 28.1 ± 2.1</td>
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<td>0.92 23.04 ± 1.9</td>
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<tr>
<td>5c CH&lt;sub&gt;3&lt;/sub&gt; CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.73 30.3 ± 1.8</td>
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<td>0.80 26.2 ± 2.5</td>
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<td>5e F CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.68 32.5 ± 1.4</td>
</tr>
<tr>
<td>5f F C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0.68 31.7 ± 1.9</td>
</tr>
<tr>
<td>Dimebon</td>
<td>0.02 n.d.</td>
</tr>
<tr>
<td>2,8-dimethyltetrahydro-γ-carboline</td>
<td>1.08 18.2 ± 1.1</td>
</tr>
<tr>
<td>Carbazole</td>
<td>0.025 n.d.</td>
</tr>
<tr>
<td>Trolox</td>
<td>1 20.4 ± 1.7 (n = 6)</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.99 21.8 ± 2.3 (n = 8)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM, n = 3.

n.d. stands for not determined.

<sup>a</sup> - TEAC (Trolox equivalent antioxidant capacity) determined at 20 μM.

The bold values correspond to the most active compounds.
of the R = F substituent in γ-carboline ring entails some decrease in the antiradical activity of compounds (5e, 5f).

According to the results, all γ-carboline conjugates with carbazoles, except for compound 3b, are rather weak antioxidants, whereas most conjugates of γ-carboline with tetrahydrocarbazole are comparable with Trolox in the activity. Furthermore, analysis of the antiradical activities of the conjugates and reference compounds (Table 3) suggests that the antiradical activity of conjugates is largely caused by the γ-carboline moiety.

Effect of γ-carboline conjugates with carbazoles and tetrahydrocarbazole on the characteristics of mitochondria and tubulin polymerization

The neuroprotective potential and the ability to stimulate neurogenesis characteristic of the known pharmaceutical drug Dimebon, a γ-carboline derivative [81], and a number of carbazole derivatives, in particular, P7C3 [50], are largely determined by their ability to increase the neuronal survival by increasing the resistance of mitochondria to the induction of mitochondrial permeability transition (MPT). In addition, in our previous study, compounds that actively stimulated the tubulin assembly into microtubules and possessed mitoprotective and anticholinesterase activity were identified among binary carbazole and amino adamantane derivatives [2].

These data predetermined the use of primary screening tests to study the interaction of new compounds with mitochondria and their influence on microtubules assembly. In this regard, the compounds influence on the mitochondrial potential upon energization with mitochondrial respiratory chain (RC) complex I substrate (glutamate and malate) or complex II substrate (with complex I being inhibited by rotenone) and calcium-induced swelling of mitochondria, which, to a considerable extent, characterize the MPT pores opening, were studied. We also investigated the effect of compounds on the tubulin polymerization with estimation of the structure of the microtubules thus formed.

The results of primary screening on these targets are summarized in Table 4.

In the assessment of the effect of γ-carboline conjugates with carbazoles 3 and tetrahydrocarbazole 5 on the mitochondrial potential upon energization of mitochondria with complex I or complex II substrates, weak depolarization of less than 20% was observed only for compounds 3a–d. The other compounds not only did not induce depolarization of mitochondria, but even reduced the spontaneous depolarization of isolated mitochondria. This effect may be a consequence of antioxidant (radical-scavenging) potential of the compounds and/or suppression of spontaneous MPT pore opening. It would be reasonable to suggest that the observed decrease in the spontaneous depolarization of mitochondria is attributable to the radical-scavenging activity of compounds found in this study. Indeed, all tetrahydrocarbazole-containing compounds possess this combination of activities. However, the range of compounds reducing the spontaneous depolarization of mitochondria is much wider and includes a series of carbazole conjugates.

The potential inhibition of calcium-dependent MPT induction was studied. Some of the γ-carboline conjugates with carbazoles 3 and tetrahydrocarbazole 5 demonstrated the ability to reduce the calcium-induced swelling of mitochondria: carbazoles 3g,j, and 3k and tetrahydrocarbazoles 5a,b, and 5d–f in 30 μM concentration reduce the rate of the decrease in absorbance of a mitochondrial suspension by 40% and more, i.e. the activities of these compounds as potential inhibitors of MPT pore opening are comparable with the Dimebon activity.

The possible properties of conjugates 3 and 5 as microtubule stabilizers were evaluated by measuring the effect of γ-carboline conjugates with carbazoles 3 and tetrahydrocarbazole 5 on the tubulin polymerization with microtubules assembly. In the presence of 0.1 mM of some of the compounds (3a–f, 3l–n, 5c, and 5e), the polymerization rate was 1.5-fold or more higher than that for control samples. Tetrahydrocarbazole-containing analogues are generally less active than carbazole-containing ones. It is noteworthy that the initial compounds, Dimebon and unsubstituted carbazole, possess virtually the same activity towards tubulin polymerization, i.e. no considerable increase in potentiation of the assembly of microtubules was achieved upon
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We were unable to identify structural correlations upon the introduction of various substituents that would determine the effect of γ-carboline conjugates with carbazoles and tetrahydrocarbazole on mitochondria or polymerization of tubulin.

### Conclusion

It was found that all conjugates with unsubstituted carbazoles and tetrahydrocarbazole and some dichlorine and dibromine derivatives show high inhibitory activity towards BChE with the IC50 values of 1–6 μM and high selectivity towards BChE relative to AChE or CaE. Furthermore, it can be expected that these compounds would exhibit cognitive-stimulating properties without undesirable cholinergic side effects or adverse drug-drug interactions. The compound with unsubstituted carbazole and all tetrahydrocarbazole conjugates also fairly possess a high radical-scavenging activity. Simultaneously, compounds are microtubule stabilizers, while compounds are MPT inhibitors.

Among the studied conjugates, a lead compound was found, which was active towards all of the targets, in particular, it selectively inhibited BChE, scavenged free radicals, suppressed calcium-induced MPT pores opening, and enhanced tubulin polymerization. Combination of cognitive-stimulating and

### Table 4: Effect of γ-carboline conjugates with carbazoles on the characteristics of mitochondria and tubulin polymerization to microtubules.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ΔΨm, %</th>
<th>Vsw</th>
<th>Va355</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>R1</td>
<td>R2</td>
<td>Cl (g/m)</td>
</tr>
<tr>
<td>3a</td>
<td>H</td>
<td>CH3</td>
<td>H</td>
</tr>
<tr>
<td>3b</td>
<td>H</td>
<td>CH3</td>
<td>H</td>
</tr>
<tr>
<td>3c</td>
<td>CH3</td>
<td>CH3</td>
<td>H</td>
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<tr>
<td>3d</td>
<td>CH3</td>
<td>CH3</td>
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<td>H</td>
</tr>
<tr>
<td>3f</td>
<td>F</td>
<td>CH3</td>
<td>H</td>
</tr>
<tr>
<td>3g</td>
<td>H</td>
<td>CH3</td>
<td>Cl</td>
</tr>
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<td>3i</td>
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<td>CH3</td>
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<td>F</td>
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<td>Cl</td>
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<tr>
<td>3k</td>
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<td>3t</td>
<td>F</td>
<td>CH3</td>
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</tr>
<tr>
<td>3u</td>
<td>F</td>
<td>CH3</td>
<td>Br</td>
</tr>
<tr>
<td>Dimebon</td>
<td>rsd</td>
<td>rsd</td>
<td>0.4</td>
</tr>
<tr>
<td>2,8-dimethyltetrahydro-γ-carboline</td>
<td>1.08</td>
<td>18.2 ± 1.1</td>
<td>1</td>
</tr>
<tr>
<td>Carbazole</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

ΔΨm is depolarization after 10-min incubation with 30 μM of compounds for:

Cl (g/m), energization with RC complex I substrates (glutamate, malate); CII (s/r), energization with RC complex II substrate (succinate) in the presence of rotenone; Vsw, rate of Ca2+-induced swelling of mitochondria in the presence of 30 μM of compounds, rel.u. to the control; Va355, rate of absorbance change in a tubulin–microtubule suspension, i.e. tubulin polymerization rate, percent of the control (0.1 mM); rsd, 30 μM of compound reduces spontaneous depolarization of mitochondria.

The bold values correspond to the most active compounds.
neuroprotective potentials makes compound 5e the most promising candidate for the subsequent optimization in the drug design for the treatment of neurodegenerative diseases.

**Methods**

**Synthesis**

Compounds 3a–o and 5a–f were synthesized according to Scheme 1 by procedures described previously [52, 53]. Physical properties, elemental analysis, and spectral data of synthesized compounds have been presented there.

**Biological assay**

*In vitro AChE, BChE and CaE inhibition*

Acetylcholinesterase (AChE, EC 3.1.1.7, from human erythrocyte), butyrylcholinesterase (BChE, EC 3.1.1.8, from equine serum), carboxylesterase (CaE, EC 3.1.1.1, from porcine liver), acetylthiocholine iodide (ATCh), butyrylthiocholine iodide (BTCh), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and 4-nitrophenol acetate (4-NPA), were purchased from Sigma-Aldrich (Germany).

AChE and BChE activities were measured by the method of Ellman and coworkers as described earlier [82]. The assay solution consisted of 0.1 M K/Na phosphate buffer pH 7.5, 25 °C with the addition of 0.33 mM DTNB, 0.02 unit/mL of AChE or BChE and 1 mM of substrate (ATCh or BTCh, respectively). Assays were carried out with a blank containing all components except ATCh and BTCh.

The activity of CaE was determined spectrophotometrically by the release of 4-nitrophenol at 405 nm [83]. The assay solution consisted of 0.1 M K/Na phosphate buffer pH 8.0, 25 °C, with the addition of 1 mM 4-nitrophenyl acetate and 0.02 unit/mL of CaE. Assays were carried out with a blank containing all components except CaE.

The tested compounds were dissolved in DMSO; the incubation mixture contained 2% of the solvent. Eight different concentrations of the test compounds in the range of $10^{-11}$–$10^{-4}$ M were selected in order to obtain inhibition of AChE and BChE activity between 20% and 80%. The test compounds were added to the assay solution and preincubated with the enzymes for 5 min at 25 °C followed by the addition of substrate. A parallel control was made for the assay solution with no inhibitor. Measurements were performed in a BioRad Benchmark Plus microplate spectrophotometer (France). Each experiment was performed in triplicate. The results were expressed as the mean ± SEM. The reaction rates in the presence or absence of inhibitor were compared, and the percentage of residual enzyme activity due to the presence of test compounds was calculated. The IC50 (the concentration of inhibitor required to decrease the enzyme activity by 50%) values were determined graphically from inhibition curves (log inhibitor concentration vs percent residual enzyme activity) using the Origin 6.1 software.

**Kinetic analysis of BChE inhibition. Determination of steady-state inhibition constants**

To elucidate the inhibition mechanisms for the most active compounds, the BChE residual activity was determined in the presence of three increasing concentrations of the test compounds and six decreasing concentrations of the substrates. The test compounds were preincubated with the enzymes at 25 °C for 5 min, followed by the addition of the substrates. Parallel controls were made for an assay of the rate of hydrolysis of the same concentrations of substrates in the solutions with no inhibitor. The kinetic parameters of substrate
hydrolysis were determined. Measurements were performed in a BioRad Benchmark Plus microplate spectrophotometer (France). Each experiment was performed in triplicate. Results were fitted into Lineweaver-Burk double reciprocal kinetic plots of 1/V versus 1/[S] and values of inhibition constants $K_i$ (competitive component) and $\alpha K_i$ (noncompetitive component) were calculated using the program Origin 6.1.

**ABTS radical cation scavenging activity assay**

Radical scavenging activity of the compounds was assessed using an ABTS$^+$ radical decolorization assay. The procedure followed the method [80] with some minor modifications.

ABTS (2,2$’$-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt) was purchased from TCI (Tokyo, Japan), potassium persulfate (di-potassium peroxodisulfate), Trolox® (6-hydroxy-2,5,7,8-tetramethylochroman-2-carboxylic acid (Sigma-Aldrich), and ascorbic acid were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ethanol was HPLC grade. Aqueous solutions were prepared using deionized water.

Trolox and ascorbic acid were used as the antioxidant standards. Five millimolarity solutions of standards were prepared in DMSO for use as stocks. Fresh working solutions of the standards of known concentrations (1–100 μM) were prepared on the day of experiments and used for calibration and as positive controls for ABTS$^+$ scavenging activity.

ABTS was dissolved in deionized water to a 7 mM concentration. The solution of ABTS radical cation (ABTS$^+$) was produced by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate aqueous solution in equal quantities and allowing them to react for 12–16 h at room temperature in the dark. At the time of activity, ABTS$^+$ solution was diluted with ethanol to adjust an absorbance value of about 0.80 ± 0.02 at 734 nm. Fresh working ABTS$^+$ solution was prepared for each assay.

The radical scavenging capacity of the compounds was analyzed by mixing 10 μL of compound with 240 μL of ABTS$^+$ working solution. The reduction in absorbance was measured spectrophotometrically at 734 nm after 1 h of mixing the solutions using the microplate UV/VIS spectrophotometer BioRad xMark (Japan). EtOH blanks were run in each assay. Values were obtained from three replicates of each sample and three independent experiments.

The antioxidant activity is reported as Trolox equivalent antioxidant capacity (TEAC values) by comparing $(A_0-A_{test})$ of the test antioxidant with $(A_0-A_{Trolox})$ of the Trolox standard at concentration of 20 μM after reaction time of 1 h, where $A_0$ is the absorbance of a control lacking any radical scavenger and $A_{test/Trolox}$ is the absorbance of the remaining ABTS$^+$ in the presence of the test compound or Trolox:

$$\text{TEAC} = (A_0 - A_{\text{Trolox}}) / (A_0 - A_{\text{test}}).$$

For the most active compounds, we also determined the IC$_{50}$ values (compound concentration required for 50% reduction of ABTS radical). The IC$_{50}$ values were calculated using Origin 6.1 for Windows (OriginLab, Northampton, MA, USA).

**Rat liver mitochondria isolation**

Rat liver mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar strain rats fasted overnight. The isolation was carried out in an ice-cold isolation buffer (IB), pH 7.6 [81]. The mitochondrial protein concentration was determined using a biuret procedure with bovine serum albumin as the standard [84].

**Mitochondrial potential and permeability transition**

Safranine O (10 μM) was used as a membrane potential probe [85]. Fluorescence intensity at 580 nm (excitation at 520 nm) was measured with Victor3 multi-well fluorescence plate reader (Perkin Elmer, Germany).
Mitochondrial protein concentration was 0.2 mg/mL. The medium for measurements contained 75 mM sucrose, 225 mM mannitol, 10 mM K-HEPES (pH 7.4), 0.02 mM EGTA, 1 mM KH₂PO₄. After a 5-min incubation, 5 mM glutamate/malate or the 5 mM succinate and 0.5 μM rotenone were added to produce the mitochondrial potential. Then the compounds (30 μM) or the same volume of vehicle (DMSO) were injected to mitochondrial suspension. After 15–20 min, 12.5 μM CaCl₂ was added to each probe to induce the depolarization of mitochondria.

Ca²⁺-induced mitochondrial swelling was used to study the mitochondrial permeability transition induction. The mitochondrial swelling was determined by monitoring the absorbance at 620 nm using a Victor3 multi-well fluorescence plate reader (Perkin Elmer, Germany). The rat liver mitochondria (0.2 mg/mL) were incubated in a buffer containing 75 mM sucrose, 225 mM mannitol, 10 mM K-HEPES (pH 7.4), 0.02 mM EGTA, 1 mM KH₂PO₄, 5 mM succinate, and 0, 5 μM rotenone for energized conditions. The mitochondrial suspensions (0.2 mg/mL) were infused with the 25 μM CaCl₂ solution.

**Tubulin polymerization**

The assembly of tubulin into microtubules was carried out using pure tubulin from HTS-Tubulin polymerization assay kit (Cytoskeleton, Inc., Denver, USA). Tubulin polymerization assay was based on an adaptation of the original method of Shelanski et al. [86], who demonstrated that light is scattered by microtubules to an extent that is proportional to the concentration of microtubule polymer. The standard polymerization reaction contained 100 μL of a 4 mg/mL tubulin solution in 80 mM PIPES (pH 6.9), 0.5 mM EGTA, 2 mM MgCl₂, and 1 mM GTP. Polymerization was monitored by observing the change in the absorbance on a Victor microplate reader (Perkin Elmer, Finland) at λ = 355 nm. The parameter Vₘₐₓ = (dAₚᵢₚ/dt)ₘₐₓ normalized to control probes (%) was used to compare the action of the compounds. Electron microscopic monitoring was carried out on a Carl Zeiss Libra 120 Electron Microscope (Carl Zeiss Meditec AG, Jena, Germany) at 120 kV using negative contrasting [87].

**Statistical analyses**

Each experiment was performed in triplicate. Average results are presented as the mean ± S.D.

**Molecular modeling**

**Protein and ligand structure preparation**

To determine the protonation state of the piperidine nitrogen atom of γ-carboline moiety and other ionizable groups of the compounds, Marvin 14.9.1.0 (ChemAxon, http://www.chemaxon.com) was used to estimate pKᵃ values. Since they were found to be close to 7.4, protonated and neutral forms were used for molecular docking of all molecules.

Geometries of the ligands were quantum-mechanically (QM) optimized in Gamess-US package [88] using DFT method B3LYP and basis 6-31G*. Partial atomic charges were taken from QM results according to Mulliken scheme [89]. These optimized geometries and partial charges were used for molecular docking.

The X-ray structure of human BChE (PDB ID 1P0I [90]) was used. Previously the importance of saturation of BChE gorge with water molecules was demonstrated [91]. Protein structure was prepared, saturated with water molecules, and optimized using QM/MM method as reported previously [91, 92].

**Molecular docking**

Molecular docking with a Lamarckian Genetic Algorithm [93] was performed with Autodock 4.26 software [94]. Grid box for docking included the whole BChE active site and the gorge with dimensions...
15 Å × 20.25 Å × 18 Å with grid spacing 0.375 Å. The main of selected Lamarckian Genetic Algorithm parameters were 256 runs, 25 × 10⁶ evaluations, 27 × 10⁶ generations and population size 300. Docking positions with the lowest binding energies were used for analysis. Structural images were prepared with Accelrys Discovery Studio Visualiser 4.0 (http://www.accelrys.com), PyMOL (Schrödinger, LLC).

Acknowledgements: The study was supported by the grant of the Russian Science Foundation (project number 14-23-00160). Authors are grateful to the Lomonosov State University Research Super Computer Center [95] for providing computational facilities.

References


