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Novel potent pyridoxine-based inhibitors of AChE and BChE, structural analogs of pyridostigmine, with improved in vivo safety profile

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ABSTRACT

We report a novel class of carbamate-type ChE inhibitors, structural analogs of pyridostigmine. A small library of congeneric pyridoxine-based compounds was designed, synthesized and evaluated for AChE and BChE enzymes inhibition in vitro. The most active compounds have potent enzyme inhibiting activity with IC_{50} values in the range of 0.46–2.1 μM (for AChE) and 0.59–8.1 μM (for BChE), with moderate selectivity for AChE comparable with that of pyridostigmine and neostigmine. Acute toxicity studies using mice models demonstrated excellent safety profile of the obtained compounds with LD_{50} in the range of 22–326 mg/kg, while pyridostigmine and neostigmine are much more toxic (LD_{50} 3.3 and 0.51 mg/kg, respectively). The obtained results pave the way to design of novel potent and safe cholinesterase inhibitors for symptomatic treatment of neuromuscular disorders.

Disorders of neuromuscular synaptic transmission (Myasthenia Gravis, Lambert Eaton Syndrome and Congenital Myasthenic Syndromes) are a group of diseases in which an abnormality of neurotransmitter-receptor interaction or an abnormality of neurotransmitter release at the neuromuscular junction (NMJ) provokes the muscles weakness. In many instances the diseases are mediated by an autoantibody directed to a specific epitope at the NMJ.[1] In others, there is a genetically acquired abnormality of the structure of the NMJ.[2] While primary therapy for the majority of the immune mediated disorders involves immunosuppressive drugs with or without thymectomy, patients are also treated with inhibitors of acetyl- and butyrylcholinesterase (BChE) for symptomatic improvement of muscles weakness.[3] For Congenital Myasthenic Syndromes, medications by inhibitors of cholinesterases (ChEs) are the standard of care.[4] Effectiveness of ChEs inhibition in symptomatic treatment of muscles weakness is based on their ability to potentiate the effects of neurotransmitter acetylcholine (ACh) due to a decrease in the rate of its enzymatic hydrolysis. When a selective acetylcholinesterase (AChE) inhibitors are used to increase the ACh concentration in the synaptic cleft, the functional improvement is better than with a non-selective inhibitor of ChEs, during which inhibition of BChE counteracts the positive action of AChE inhibition.[5]

Currently, the most frequently used ChEs inhibitor in treatment of muscle weakness symptoms is pyridostigmine[3] (Fig. 1). This pseudo-reversible carbamylating agent does not cross the blood–brain barrier, and its selectivity for human AChE is poor [K_i ratio (BChE/AChE) ≈ 6].[6] Neostigmine (Fig. 1) is another nonspecific ChEs inhibitor. Due to strong muscarinic side effects, it is less frequently used than pyridostigmine for the treatment of muscle weaknesses.[7] However, anaesthetists traditionally use neostigmine in daily routine practice to reverse the action of nondepolarizing muscle relaxants.[8]

There are serious safety concerns regarding the therapeutic use of ChEs inhibitors.[3] In particular, carbamates stimulate delayed neuropathy or make it more severe, when they are dosed after applying organophosphate (OP) neuropathic doses, inducing promotion of delayed neuropathy.[9] Thus, pyridostigmine bromide has been FDA approved for military use during combat situations as an agent to be given prior to exposure to the nerve agent soman in order to increase survival. However, used in particular during the first Gulf War, pyridostigmine itself has been implicated as a causal factor in Gulf War syndrome.[10]

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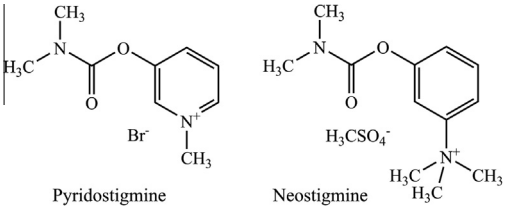


Figure 1. Carbamate ChEs inhibitors for treatment of neuromuscular disorders.

Because the number of specific AChE inhibitors for the treatment of muscle weakness is limited, and due to safety concerns, design and discovery of new compounds is of great interest.

In our group, we have systematically studied chemistry and biology of the biologically active pyridoxine derivatives.[11] In this work, we have synthesized a series of novel AChE/BChE inhibitors based on pyridoxine scaffold (Scheme 1). 1,3-Dioxepino[5,6-*c*] pyridines **2a–j** were obtained from pyridoxine hydrochloride **1** according to literature methods.[12]

The carbamylated intermediates were obtained by treatment of **2a–j** with NaOH in ethanol followed by addition of dimethylcarbamoyl chloride in dimethylformamide. The intermediates were then alkylated by methyl bromide in dimethylformamide. The crude products **3a–j** were recrystallized from ethanol to obtain the desired compounds in 45–80% yield.

The obtained compounds were tested in vitro for their ability to inhibit human AChE and BChE enzymes according to literature method.[13] The activity parameters are shown in Table 1. All the obtained compounds have expressed AChE and BChE inhibiting activity with IC_{50} values in the range of 0.46–500 μM (for AChE) and 0.59–1200 μM (for BChE). The most active compounds **3h–j** with long lipophilic C7–C10 substituents have IC_{50} values in the range of 0.46–2.1 μM (for AChE), and they are almost equipotent to pyridostigmine (IC_{50} 0.35 μM for AChE). The selectivity of compounds **3a–j** for AChE (IC_{50} ratio (BChE/AChE)) ranges from 1.3 to 6.9.

Then we performed a study of acute toxicity in mice. The results are presented in Table 2. In general, the studied compounds are significantly less toxic (LD_{50} in the range of 22–326 mg/kg) than pyridostigmine and neostigmine (LD_{50} 3.3 and 0.51 mg/kg, respectively).

The underlying idea of this work was to design effective ChEs inhibitors, possessing key structural motif of pyridostigmine and capable to occupy the same binding site of AChE/BChE enzymes. The variation of R-substituents allows for a modulation of activity and selectivity range, both in vitro and in vivo.

Table 1
In vitro AChE and BChE inhibition activity of **3a–j** compared to pyridostigmine[a]

Compound	IC_{50}, AChE human, μM	IC_{50}, BChE human, μM	Selectivity for AChE[b]	Log*P*[c]
3a	130 ± 15	400 ± 32	3.1	−0.57
3b	150 ± 13	1030 ± 86	6.9	−0.56
3c	500 ± 42	1190 ± 20	2.4	0.06
3d	280 ± 11	800 ± 67	2.9	0.01
3e	140 ± 12	400 ± 30	2.9	0.41
3f	260 ± 30	900 ± 80	3.5	0.51
3g	110 ± 10	310 ± 30	2.8	1.12
3h	2.1 ± 0.3	8.1 ± 0.7	3.9	1.99
3i	1.3 ± 0.1	2.0 ± 0.3	1.5	2.39
3j	0.46 ± 0.05	0.59 ± 0.06	1.3	3.68
Pyridostigmine	0.35 ± 0.03	1.0 ± 0.1	2.9	

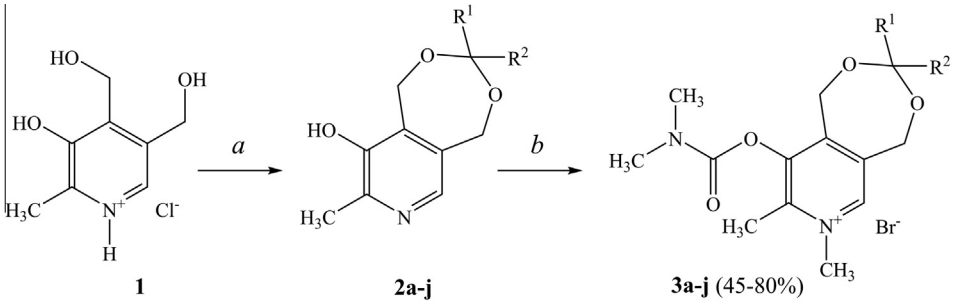
[a] Values are expressed as mean ± standard error of three independent measurements, each performed in duplicate; acetylthiocholine and butyrylthiocholine concentration was 1 mM.
[b] (IC_{50} BChE)/(IC_{50} AChE).
[c] Log*P* values are calculated using HyperChem 8 software.

Table 2
In vivo acute toxicity data in mice

Compound	LD_{50} (mg/kg)
3a	50
3b	224
3c	326
3d	150
3e	100
3f	98
3g	50
3h	38
3i	47
3j	22
Pyridostigmine	3.3
Neostigmine	0.51

We have found that the observed activity is well correlated with calculated log*P* values for AChE (Fig. 2) as well as for BChE enzyme (data not shown). The observed structure–activity relationships can be related to different binding efficacy to the enzymes' active sites. The long lipophilic substituents may play an important role for stabilization of the molecules in the active center.

Increased selectivity for AChE results in the functional improvement in symptomatic treatment of muscles weakness.[5] Our experimental results indicate that the selectivity of the obtained compounds for AChE (IC_{50} ratio (BChE/AChE)) ranges from 1.3 to 6.9, and for some compounds it outperforms selectivity of pyridostigmine (2.9). The structure-selectivity factors are not clearly



a: $R^1 = R^2 = H$; **b**: $R^1 = H$, $R^2 = Me$; **c**: $R^1 = R^2 = Me$; **d**: $R^1 = H$, $R^2 = Et$; **e**: $R^1 = H$, $R^2 = Pr$;
f: $R^1 = H$, $R^2 = i$-Pr; **g**: $R^1 = H$, $R^2 = t$-Bu; **h**: $R^1 = H$, $R^2 = n$-C_7H_{15}; **i**: $R^1 = H$, $R^2 = n$-C_8H_{17};
j: $R^1 = H$, $R^2 = CH(CH_3)C_9H_{19}$

Scheme 1. Synthesis of ChE inhibitors studied in this Letter. Reagents and conditions: *a* – H^+, $R^1C(O)R^2$, toluene; *b* – (1) NaOH in EtOH/H_2O; (2) $(CH_3)_2NC(O)Cl$ in DMF, rt; (3) CH_3Br in DMF, 60 °C, 3 d.

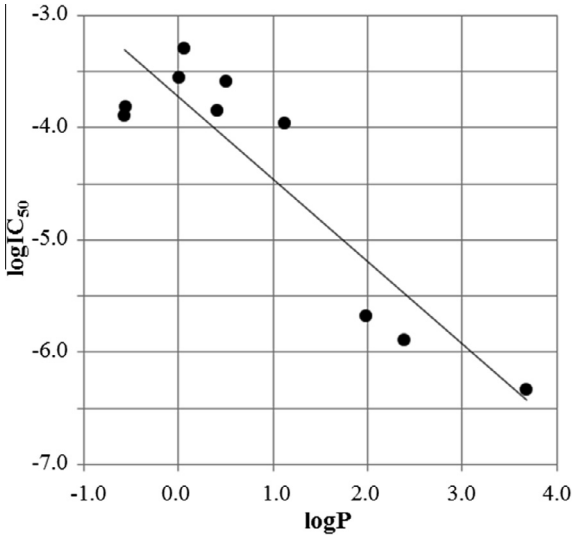


Figure 2. Correlation of calculated log*P* with in vitro AChE inhibition activity of **3a–j** (R^2 = 0.83, calculated using OriginPro 8 software).

understood, though the most potent inhibitors with the long lipophilic substituents demonstrate decreased selectivity (1.5 and 1.3 for the most active inhibitors **3i** and **3j**, respectively).

In vivo activity can be modulated due to different stability of the 1,3-dioxepin cycle, which is relatively easily hydrolyzed by hydrolase enzymes under physiological conditions. A modified pharmacokinetics due to different compound lipophilicity can also contribute to their efficacy in body.

The molecular mechanism of action of carbamates, such as pyridostigmine and neostigmine, involves formation of covalent bond between the carbamoyl fragment and the serine residue in the active center of ChEs. As a result, these therapeutic agents reduce ACh hydrolysis rate, and thereby increase its level in synaptic clefts improving nerve impulse transmission. Besides, these agents are capable to prevent the irreversible binding of inhibitors, such as organophosphorus compounds, to ChEs. Actually, these compounds are able to phosphylate (i.e. either phosphorylate or phosphonylate) serine residues of ChEs in non-reversible way, whereas the carbamylated serine residue is less stable and the carbamyl moiety can be split from the enzyme by spontaneous hydrolysis (decarbamylation time is 30–40 min).[14] Therefore, carbamates are considered pseudo-reversible ChEs inhibitors. Molecular docking study of one member of the studied group of cholinesterase inhibitors was recently performed.[15] According to this study, nucleophilic attack of the Ser203 hydroxyl on the carbamate carbon leads to formation of a covalent conjugate and inhibition of enzymatic activity of ChEs. Thus, the presumable mechanism of action of the studied cholinesterase inhibitors is the same that other drugs of carbamate type possess.

We have observed that the studied compounds are significantly less toxic than pyridostigmine and neostigmine. Compounds **3h–j** bearing long lipophilic C7–C10 substituents are the most potent ChEs inhibitors in the series, and, at the same time, the most toxic ones in in vivo experiments. This is a reasonable result of AChE inactivation, which leads to acetylcholine accumulation, hyperstimulation of nicotinic and muscarinic receptors, and disrupted neurotransmission. However, in contrast to good correlation between logIC_{50} values and compound's lipophilicity (R^2 = 0.83, Fig. 2), the correlation between logLD_{50} and the calculated log*P* values is not so expressed (R^2 = 0.51). Such a more complicated in vivo picture can be related to different toxicity of the decarbamylation products and their further metabolites, as well as different pharmacokinetics and biodistribution of the active agents.

In conclusion, the obtained results pave the way to design of novel carbamate-type cholinesterase inhibitors for symptomatic treatment of neuromuscular disorders with improved safety profile.

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Supplementary data

Supplementary data (synthetic procedure and analytical characteristics of newly synthesized compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.070.

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