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# Preparation and in vitro screening of symmetrical bispyridinium cholinesterase inhibitors bearing different connecting linkage—initial study for Myasthenia gravis implications

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ABSTRACT

Reversible inhibitors (e.g., pyridostigmine bromide, neostigmine bromide) of carbamate origin are used in the early treatment of Myasthenia gravis (MG) to block acetylcholinesterase (AChE) native function and conserve efficient amount of acetylcholine for decreasing number of nicotinic receptors. Carbamate inhibitors are known for many undesirable side effects related to the reversible inhibition of AChE. In contrast, this paper describes 20 newly prepared bispyridinium inhibitors of potential concern for MG. Although some compounds from this series have been known before, they were not assayed for cholinesterase inhibition yet.

The newly prepared compounds were evaluated in vitro on human erythrocyte AChE and human plasmatic butyrylcholinesterase (BChE). Their inhibitory ability was expressed as $IC_{50}$ and compared to standard carbamate drugs. Three compounds presented promising inhibition (in μM range) of both enzymes in vitro similar to the used standards. The novel inhibitors did not present selectivity between AChE and BChE. Two newly prepared compounds were chosen for docking studies and confirmed apparent π–π or π–cationic interactions aside enzyme's catalytic sites. The kinetics assay confirmed non-competitive inhibition of AChE by two best newly prepared compounds.



Inhibitors of acetylcholinesterase (EC 3.1.1.7; AChE) are widely scoped for various purposes such as Alzheimer disease (AD).[1] Differently, quaternary AChE inhibitors (e.g., pyridostigmine bromide **1**, neostigmine bromide **2**; Fig. 1) are used for the treatment of Myasthenia gravis (MG).[2] Namely, quaternary AChE inhibitors **(1–2)** take part as a symptomatic treatment in the mild stage of MG. In fact, MG is autoimmunity disease with progressive decrease of peripheral AChE nicotinic receptors dealing with muscle weakness and painless.[3] Quaternary AChE inhibitors are targeted to competitively block AChE vital function and consequently enable the excess of acetylcholine on decreasing amount of nicotinic receptors.[4]

Although **1–2** are widely used in the treatment of MG, they are known for many side effects including gastrointestinal effects (nausea, intestinal obstruction), increased bronchial secretion, cardiac arrhythmia or cholinergic crisis.[5] Mentioned side effects of **1–2** are related to the reversible inhibition of AChE. This reversible inhibition is common for carbamate inhibitors (e.g., **1–2**) that are binding to serine oxygen in the AChE active site.[6] The resulting carbamylated enzyme intermediate inhibits AChE activity until a water molecule attacks the carbonyl to reactivate enzyme and produces a carbamic acid derivative. The spontaneous regeneration of carbamylated enzyme proceeds in the range of minutes.

Furthermore, the blood–brain barrier penetration is not required for MG treatment to decrease central side effects and ensure necessary peripheral activity.[7] For this purpose, **1–2** contain quaternary nitrogen. This structural feature is important for increased peripheral effect of mentioned drugs, where the charged compounds are penetrating in minor ratio.[8] In fact, the BBB crossing of **1–2** is limited, but may proceed through disruptive mechanisms.[9] Thus, **1–2** may still exhibit strong central side effects related to carbamylation of brain AChE that results in cholinergic crisis.[6]

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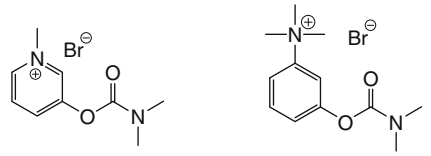


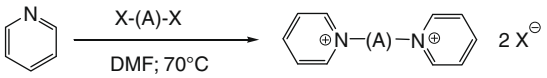
pyridostigmine bromide (**1**) neostigmine bromide (**2**)

**Figure 1.** Carbamate compounds are already used for early Myasthenia gravis treatment.

On the other hand, there is huge variety of compounds reversibly inhibiting AChE that might be used in the MG treatment.[10] In contrast to carbamates, their effect should originate for competitive reversible inhibition of AChE aside the active serine.[11] Moreover, their increased peripheral effect and decreased BBB crossing should be ensured. More valuably, the selective AChE inhibition instead of dual AChE/BChE (butyrylcholinesterase; EC 3.1.1.8) inhibition might keep the non-specific esterase (BChE) active for other toxic substrates.[12] Our approach originates from design, synthesis, in vitro screening and docking studies of selected bisquaternary pyridinium compounds connected by various linkers.

In this Letter, the preparation of 20 symmetrical bisquaternary AChE inhibitors (**3–22**) is reported (Fig. 2). Although the synthesis of such compounds is trivial, their structural differences are important for comprehension of key factors influencing inhibition of AChE and/or BChE (Table 1).[13] Moreover, these compounds were previously not evaluated as AChE or BChE inhibitors.[14–20]

Namely, presented compounds differ in the structure of the connecting linker. The key length and/or spatial orientation of connecting linker are highly important factors for molecular interactions among the enzyme active sites. Subsequently, the optimal structure of the linker depends not only on the length, but is usually related to presented π-electrons (double bond or aromatic residues) or heteroatom (hydrogen-bonding interactions), too.[21]




X = Cl, Br

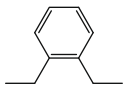
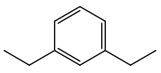
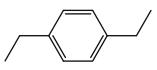
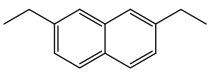
| (A) | **Compound** (anion) |
|---|---|
| $(CH_2)_{1\text{-}12}$ | **3-14** (Br) |
| $CH_2OCH_2$ | **15** (Cl) |
| $(CH_2)_2O(CH_2)_2$ | **16** (Br) |
| (*E*)-$CH_2CH{=}CHCH_2$ | **17** (Br) |
| (*Z*)-$CH_2CH{=}CHCH_2$ | **18** (Cl) |
|  | **19** (Br) |
|  | **20** (Br) |
|  | **21** (Br) |
|  | **22** (Br) |

**Figure 2.** Prepared bisquaternary pyridinium salts bearing different linkers.

**Table 1**
Inhibitory results of tested compounds (a—no significant inhibition in the selected concentration scale)

| Compound | AChE $IC_{50}$ ± SD (μM) | BuChE $IC_{50}$ ± SD (μM) | AChE $K_{i1}/K_{i2}$ (μM/l) |
|---|---|---|---|
| Pyridostigmine (**1**) | 40 ± 7.8 | 16000 ± 260.8 | — |
| Neostigmine (**2**) | 0.1 ± 0.02 | 0.8 ± 0.13 | — |
| **3** | a | a | — |
| **4** | a | a | — |
| **5** | a | a | — |
| **6** | 505 ± 98.5 | 9800 ± 159.7 | — |
| **7** | 1270 ± 247.6 | 120 ± 19.7 | — |
| **8** | 63 ± 12.3 | 130 ± 21.2 | — |
| **9** | 241 ± 47.0 | 78 ± 12.7 | — |
| **10** | 31 ± 6.0 | 29 ± 4.7 | — |
| **11** | 2 ± 0.4 | 6 ± 0.9 | — |
| **12** | 0.4 ± 0.08 | 5 ± 0.8 | 0.02/0.03 |
| **13** | 0.7 ± 0.14 | 7 ± 1.1 | — |
| **14** | a | a | — |
| **15** | 1770 ± 345.2 | 583 ± 95.0 | — |
| **16** | 2010 ± 631.2 | 2360 ± 384.7 | — |
| **17** | 2290 ± 392.0 | 541 ± 88.2 | — |
| **18** | 1990 ± 388.1 | 1270 ± 207.0 | — |
| **19** | 636 ± 124.0 | 345 ± 56.2 | — |
| **20** | 2360 ± 460.2 | 1380 ± 224.9 | — |
| **21** | 1540 ± 300.3 | 529 ± 86.2 | — |
| **22** | 0.2 ± 0.04 | 0.8 ± 0.13 | 0.14/0.14 |

The pyridinium part of the molecule was chosen among the other heteroaromatic rings due to its small size and universality. Moreover, the pyridinium ring is plain structure with π-electrons that may interact with many amino-acid residues (Phe, His, Trp, Tyr). These amino-acid residues are well known for their principal function in the enzyme active sites via non-covalent interactions (AChE or BChE).[22]

Additionally, the peripheral effect of AChE inhibitors valuable for MG treatment is preferred, because the reduced amount of such inhibitor in the central nervous system decreases side effects via minor interactions with the brain AChE. For this reason, the charged molecules were chosen. Moreover, the monoquaternary compounds are penetrating BBB at least in 10%, whereas the bisquaternary compounds were previously found to penetrate the blood–brain barrier (BBB) in less than 5%.[8,23] Hence, the bisquaternary molecules were designed to maintain the peripheral inhibitory activity against AChE.

The new inhibitors (**3–22**; Fig. 2) were prepared via standard synthetic strategy.[24] The solution of pyridine (1 ml, 12.4 mmol) and corresponding alkylating agent (5.6 mmol) in DMF (10 ml) was stirred at 70 °C. The reaction mixture was cooled to the room temperature, portioned with acetone (50 ml) and cooled in refrigerator (5 °C) overnight. The crystalline or amorphous crude product was collected by filtration, washed with acetone (3 × 20 ml) and recrystallized from MeCN. NMR, ESI-MS and elemental analysis determined the purity of all compounds.

The bisquaternary pyridinium compounds were assayed for their inhibitory ability in standard inhibition test using human erythrocyte AChE (hAChE) and human plasmatic BChE (hBChE).[25] The $IC_{50}$ values of all compounds are listed in Table 1.

The commercial compound **1** presented satisfactory inhibition of hAChE in μM range. On the other hand, compound **1** showed no inhibition of hBChE. Differently, compound **2** resulted as strong inhibitor both of hAChE and hBChE and had approximately threefold lower $IC_{50}$ compared to **1** for hAChE. From these in vitro results, compound **2** seems to be more valuable for MG treatment among two tested commercial reversible inhibitors.

Concerning the inhibition of the prepared compounds towards AChE, there may be seen at least three trends of inhibitory ability within the whole series. Firstly, compounds **3–5** and **14** did not