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Short communication

Pyridonepezils, new dual AChE inhibitors as potential drugs for the treatment of Alzheimer's disease: Synthesis, biological assessment, and molecular modeling

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ABSTRACT

The synthesis, biological assessment and molecular modeling of new *pyridonepezils* **1**–**8**, able to inhibit human acetylcholinesterase (hAChE) and human butyrylcholinesterase (hBuChE), are described. The new compounds have been designed as hybrids resulting from a conjunctive approach that combines the *N*-benzylpiperidine moiety, present in donepezil, and the 2-amino-6-chloropyridine heterocyclic ring system, connected by an appropriate polymethylene linker. Compounds **1**–**8** were prepared by reaction of 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile (**13**) [or 2-amino-6-chloropyridine-3,5-dicarbonitrile (**14**)] with 2-(1-benzylpiperidin-4-yl)alkylamines (**9**–**12**). The biological evaluation of molecules **1**–**8** showed that compounds **1**–**6** are potent AChE inhibitors, in the submicromolar, while compounds **7** and **8** are on the nanomolar range, the most potent, 2-amino-6-((3-(1-benzylpiperidin-4-yl)propyl)amino)pyridine-3,5-dicarbonitrile (**7**), showing a IC_{50} (hAChE) = 9.4 ± 0.4 nM. Inhibitors **2**–**8** are permeable as determined in the PAMPA assay. Compared to donepezil, compound **7** is in the same range of inhibitory activity for hAChE, and 703-fold more selective for hAChE than for hBuChE. Molecular modeling investigation on *pyridonepezil* **7** supports its dual AChE inhibitory profile, binding simultaneously at the catalytic active and at peripheral anionic sites of the enzyme. The theoretical ADME analysis of *pyridonepezils* **1**–**8** has been carried out. Overall, compound **7**, a potent and selective dual AChEI, can be considered as a candidate with potential impact for further pharmacological development in Alzheimer's therapy.

1. Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder [1]. AD is characterized by the gradual development of forgetfulness, progressing to disturbances in language, disorientation, and mutism. The symptomatic course of the disease is generally five or more years of stepwise decline in memory and attention span [2]. Typical pathological hallmarks are extracellular senile plaques, consisting principally of amyloid-β (Aβ), and intracellular neurofibrillary tangles, which are composed of phosphorylated tau protein. Moreover, the basal nucleus of Meynert undergoes profound neuron loss, the neocortex exhibits a loss of cholinergic fibers and receptors, and a decrease of both choline acetyltransferase and acetylcholinesterase (AChE) enzyme activity [2,3].

Since the symptoms of AD were associated with an altered cholinergic function, research has been focused on the basal forebrain cholinergic system [3]. As a result, the cholinergic hypothesis was developed, which postulated that a loss of cholinergic function in the central nervous system contributed significantly to cognitive decline associated with advanced age and AD [4]. Thus, drugs capable of inhibiting AChE might potentiate central cholinergic function, therefore improving cognition and perhaps even some of the behavioral problems experienced by AD patients [5].

AChE inhibitors (AChEI) may inhibit AChE *via* a competitive mechanism, by interacting with the catalytic active site (CAS) of the enzyme, *via* a non-competitive mechanism, by binding with the peripheral anionic site (PAS), or *via* both mechanisms, by exerting a dual binding AChE inhibition [5]. For a while the treatment with AChE inhibitors (AChEI) was reported to produce only symptomatic improvement, having no effect in the course of the disease [6].

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However, other studies indicated that AChE interacts with Aβ by an hydrophobic environment close to the PAS, thus promoting Aβ fibril formation [7,8]. Moreover, AChE–Aβ complexes increase Aβ-dependent neurotoxicity [9]. These reports arose a new interest in AChEI, demonstrating their ability to enhance the release of non-amyloidogenic soluble derivatives of amyloid precursor protein (APP) *in vitro* and *in vivo*, and possibly to slow down the formation of amyloidogenic compounds in the brain [10]. The increase of soluble APP (APPs) was also consistent with AChE inhibition [11]. Numerous clinical trials have shown the safety and efficacy of AChEIs in the treatment of AD. Besides, there is growing evidence from preclinical studies indicating that these agents can attenuate neuronal damage and death from cytotoxic insults, and therefore might affect AD pathogenesis [12].

Considering the non-cholinergic aspects of AChE related to the PAS [7–9] in associating with Aβ, an attractive target for the design of new antidementia drugs emerged. Peripheral or dual site inhibitors of AChE may simultaneously alleviate the cognitive deficit in AD patients and prevent the assembly of Aβ, which will delay the neurodegenerative process [13]. This strategy was pursued for several medicinal chemists who have been developing new compounds with dual AChE inhibitory activity [14–17], based on well known AChEIs, such as tacrine [18], rivastigmine [19], donepezil [20] and galanthamine [21]. A recent clinical study of patients with moderate-to-severe Alzheimer's disease found that continued treatment with donepezil could improve cognition and function for even severe patients [22].

Several classes of donepezil hybrids, such as donepezil–tacrine [23,24], donepezil–aminoacids [25], indanone hybrids [26], donepezil–aminothienoquinoline [27], or indanone and aurone derivatives [28], have been developed as dual binding site AChE inhibitors. The success of the dual binding site strategy is evidenced by the increased AChE inhibitory potency of these hybrids compared to the parent compounds from which they have been designed.

Based on this rational design, we have recently described the highly multipotent donepezil–indole **ASS234** [29] and the donepezil–pyridine hybrid **ASS280** [30] (Chart 1), showing that these molecules act as dual AChEI, binding simultaneously the CAS and PAS of the enzyme. The simple aminopyridine **II** has been also reported [31] (Chart 1).

Herein, we describe the synthesis, pharmacological evaluation [AChE and butyrylcholinesterase (BuChE) inhibition, Blood Brain Barrier (BBB) permeability using the PAMPA protocol, theoretical ADME analysis], and molecular modeling of *pyridonepezils* **1**–**8** (Table 1), a novel class of highly potent donepezil–aminopyridine hybrids of type **I** (Chart 1). These new hybrids were designed by combining the *N*-benzylpiperidine moiety of donepezil with 2-aminopyridine moiety of compound **II** [31] (Chart 1).

2. Results and discussion

2.1. Chemistry

The synthesis of *pyridonepezils* 2-amino-6-((2-(1-benzylpiperidin-4-yl)alkyl)amino)-4-phenylpyridine-3,5-dicarbonitriles **1**–**4**, and 2-amino-6-((2-(1-benzylpiperidin-4-yl)alkyl)amino)pyridine-3,5-dicarbonitriles **5**–**8** was easily achieved by reaction of readily available precursors, such as commercial **9**, known **10** [32] and **11** [30b], or new **12**, amines, with 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile **13** [33] and 2-amino-6-chloropyridine-3,5-dicarbonitrile **14** [34], respectively (Scheme 1) (see Supplementary material).

Compound **12** was prepared starting from alcohol **15** [29]. Reaction of **15** with DPPA, DBU and sodium azide in DMF gave azide **16**. Hydrolysis of azide **16** in the presence of triphenylphosphine/water/THF gave amine **12** (Scheme 2).

2.2. In vitro evaluation of human cholinesterase inhibition

The *in vitro* activity of the *pyridonepezils* **1**–**8** against hAChE and hBuChE was determined using Ellman's method [35]. For comparative purposes, donepezil was used as reference compound. The obtained IC_{50} values are summarized in Table 1. The IC_{50} values show that most of these molecules are potent, in the nanomolar range. Some of them are selective hAChE inhibitors. The most potent inhibitors were *pyridonepezils* **7** and **8** [IC_{50} (hAChE) = 9.4–70 nM]. Compared to donepezil, compound **7** was in the same range of inhibitory activity for hAChE, and 2.6-fold less active than donepezil for hBuChE. However, compounds **2**, **3**, **4**, **6** and **8** were 3.7-, 1.6-, 4.3-, 23-, and 1.5-fold more active against hBuChE, respectively. Inhibitors **1** and **5** (both with $n = 0$) were extremely

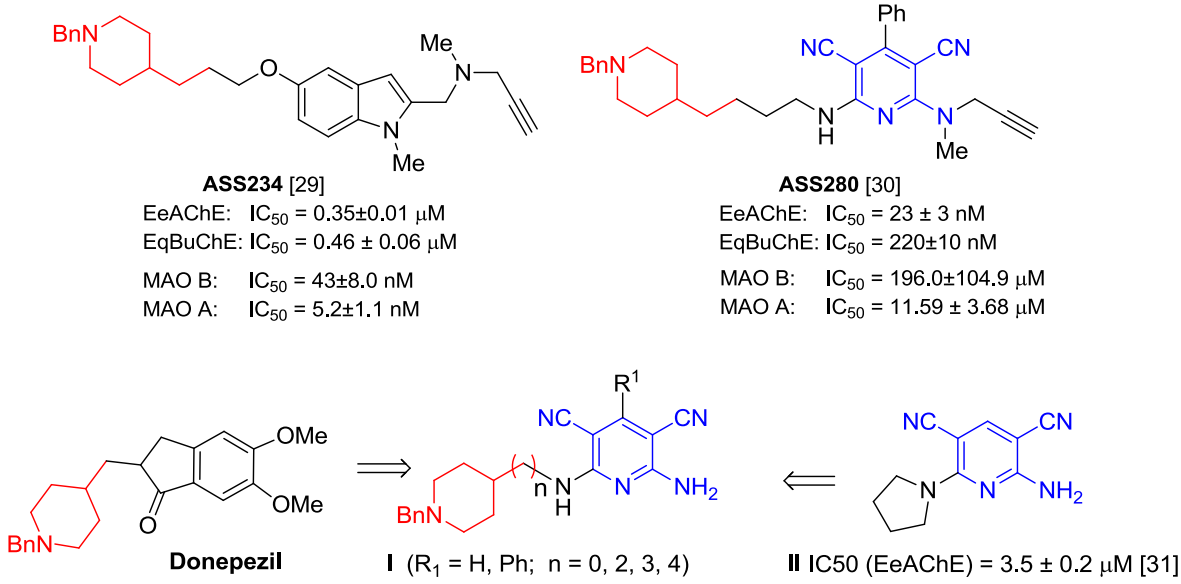


Chart 1. General structure of the multipotent MAO/ChE inhibitors **ASS234**, **ASS280**, donepezil and the novel dual ChE inhibitors **I** and 2-aminopyridine derivative **II**.

Table 1
IC_{50} (μM)[a] for the inhibition of human acetylcholinesterase (hAChE) and human butyrylcholinesterase (hBuChE) and experimental permeability (P_e 10^{-6} cm s^{-1})[b] in the PAMPA-BBB assay for *pyridonepezils* **1**–**8** with their predictive penetration in the CNS.

Compd.	R	*n*	hAChE	hBuChE	S.I[c]	P_e	Prediction
1	Ph	0	8.8 ± 0.4	>10	–	Insoluble	Insoluble
2	Ph	2	0.39 ± 0.04	0.67 ± 0.04	1.7	34.0 ± 0.3	CNS+
3	Ph	3	0.24 ± 0.05	1.5 ± 0.08	6.3	31.6 ± 1.6	CNS+
4	Ph	4	0.12 ± 0.02	0.58 ± 0.04	4.8	25.7 ± 0.3	CNS+
5	H	0	3.1 ± 0.8	>10	–	17.0 ± 0.3	CNS+
6	H	2	0.22 ± 0.08	0.26 ± 0.07	1.2	14.4 ± 0.8	CNS+
7	H	3	0.0094 ± 0.0004	6.6 ± 0.7	703	20.8 ± 1.1	CNS+
8	H	4	0.070 ± 0.005	1.7 ± 0.5	24	21.2 ± 0.7	CNS+
Donepezil			0.010 ± 0.002	2.5 ± 0.07	250	–	–
II[d]			3.5 ± 0.2	>10	–	–	–

[a] The *in vitro* test compound concentration required to produce 50% inhibition of hAChE and hBuChE. The result (IC_{50}) is the mean ± SEM of three independent experiments.
[b] PBS:EtOH (70:30). Data are the mean ± SD of three independent experiments.
[c] Selectivity Index = hBuChE IC_{50}/hAChE IC_{50}.
[d] Inhibition was done using EeAChE and eqBuChE enzymes.

selective for hAChE, but derivatives **2**–**4** and **6** showed equipotent, high activity for both hAChE and hBuChE. Compounds **7** and **8** were found to be 703- and 24-fold more selective for hAChE than for hBuChE, respectively.

Compared to compound **II** as reference, bearing also the 2-aminopyridine ring, most of compounds **1**–**8** are more active in both enzymes hAChE and hBuChE. These data suggest that the incorporation of *N*-benzylpiperidine fragment to the aminopyridine system increase the inhibitory potency for both ChE enzymes.

Compared to the already published donepezil derivatives, compound **7** proved to be the most active within all the published series of indanone hybrids [26], and in the same order of magnitude of donepezil–tacrine hybrids [23,24], while compounds **1**–**6** and donepezil–aminoacid derivatives [25] showed a similar inhibitory power.

Concerning the structure-activity relationships (SAR), the presence of a phenyl group at C4 decreases the AChE inhibitory activity. Thus, inhibitor **7** ($n = 3$) was found to be 25-fold more active than compound **3** ($n = 3$) bearing a phenyl group at C4. Regarding the effect of the linker, for compounds **1**–**4** bearing a phenyl group at C4, the inhibition of hAChE increases 74-fold on going from $n = 0$ to $n = 4$. In the absence of phenyl group at C4, as in compounds **5**–**8**, the inhibition of hAChE increases 334-fold on going from $n = 0$ to $n = 3$, and 45-fold on going from $n = 0$ to $n = 4$.

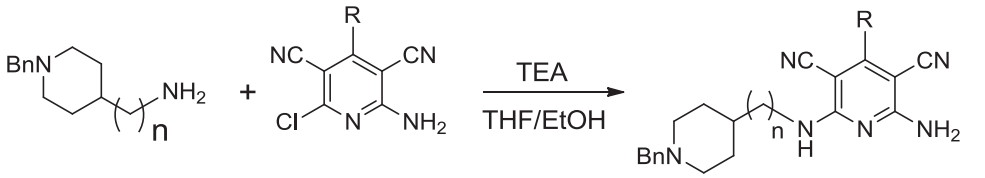
2.3. In vitro blood–brain barrier permeation assay

The ability of a drug to penetrate the Blood Brain Barrier (BBB) is of fundamental importance in drug design. In order to evaluate the brain penetration of *pyridonepezils* **1**–**8**, we used the PAMPA-BBB method as reported [36–38]. The *in vitro* permeabilities values (P_e) of these pyridonepezils and 15 commercial drugs through a lipid extract of porcine brain were determined using a mixture of PBS:EtOH (70:30). Assay validation was made by comparing the experimental permeability with the reported values for these commercial drugs, which gave a good lineal correlation, P_e (exptl) = 1.24 P_e (bibl) + 1.98 ($R^2 = 0.93$). Except compound **1**, which presents low solubility in the experimental conditions, all tested *pyridonepezils* showed good permeability values (P_e ranges from 17.0 × 10^{-6} to 34.0 × 10^{-6} cm s^{-1}), as the known CNS drugs used in the assay validation, pointing out that these molecules would cross the BBB by passive diffusion (Table 1).

2.4. Molecular modeling of compound 7

To shed light in the effective AChE binding mode of the novel AChE inhibitors here reported, compound **7** was submitted to molecular modeling studies. Docking simulations were carried out with the software Autodock Vina [39] using hAChE (PDB:1B41).

The docking procedure was applied to the whole protein target ("blind docking"). To account for side chain flexibility during docking, flexible torsions in the ligands were assigned, and the acyclic dihedral angles were allowed to rotate freely. It is well known that protein conformational flexibility is an important aspect of ligand binding; thus, the incorporation of protein structural flexibility into the ligand binding procedure has been commonly used in our previous studies [30b]. Accordingly, Trp286, Tyr124, Tyr337 and Tyr72 residues were prescribed flexible in these



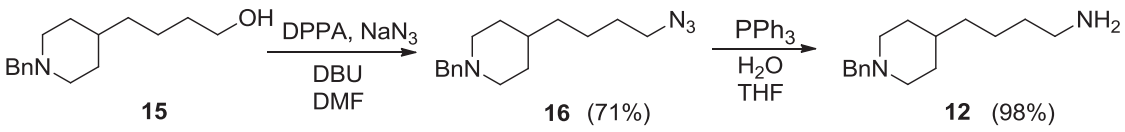
9 n = 0
10 n = 0
11 n = 3
12 n = 4

13 R = Ph
14 R = H

1 R = Ph, n = 0 (97%)
2 R = Ph, n = 2 (39%)
3 R = Ph, n = 3 (52%)
4 R = Ph, n = 4 (70%)

5 R = H, n = 0 (94%)
6 R = H, n = 2 (74%)
7 R = H, n = 3 (61%)
8 R = H, n = 4 (61%)

Scheme 1. Synthesis of *pyridonepezils* **1**–**8**.



Scheme 2.

works. However, when the ligand is large, this motion by itself is not sufficient to enlarge the gorge to enhance ligand access to the active center. Therefore, Asp74, Thr75, Trp86, and Tyr341 receptor residues were also selected to be flexible during docking simulation. These eight residues delineate the shape of the gorge entry and lining, as their motion may significantly enlarge the gorge mouth to facilitate ligand access to the catalytic site.

The binding mode of inhibitor **7** is shown in Fig. 1. It can be seen that the ligand can favorably interact with both the catalytic pocket and the PAS of the enzyme. In particular, the following major interactions, responsible for the inhibiting profile of the selected molecule, could be identified: (i) the benzyl group interacts by means of π–π stacking with the indole ring of Trp286; (ii) the pyridine ring interacts with Trp86 by means of a π–π stacking; (iii) the cyano group can establish H-bond interaction with the hydroxyl group of Tyr133; (iv) the secondary amino group donates a proton to the hydroxyl group of Tyr124 and forms a hydrogen bond; (v) the interaction of the primary amino group with Ser203 seems to be very crucial, because donates a proton to His447, an important step during the hydrolysis of ACh. Formation of this hydrogen bond probably masks the ability of Ser203 to participate in catalysis.

The interaction of this compound with hBuChE has also been carried out. hBuChE (PDB: 1P0I) was taken for the study. The preparation of the protein and the docking protocol applied was the same as that mentioned earlier. Major differences between AChE and BuChE are restricted to those residues that line the active site cleft. Docking results reveal that compound **7** has two major predicted binding modes at the active site of the enzyme (Fig. 2). Mode **I**, places the ligand in the binding pocket interacting with both the catalytic triad residue Ser198 and the PAS, while mode **II** accommodates the ligand interacting with the PAS. A close up of mode **I** in the vicinity of the catalytic triad, His438, Ser198, Glu325, uncovers a likely critical interaction where the cyano group (in *ortho* position with respect to the amino group) is hydrogen bonded to the Ser198 side chain, whereas the amino group can establish three hydrogen bonds with the side chain of Ser287 and the backbone of Leu286. Besides, the other cyano group is doubly hydrogen bonded to the Thr120 and to the protonated piperidine ring by an intramolecular hydrogen bond. Finally, the ligand interacts with Trp82 located in the PAS, forming a face-to-face π–π interaction with the benzyl moiety. Close examination of the first shell of residues surrounding **7** in mode **II** reveals a hydrogen bond interaction between the primary amino group and the hydroxyl group of Tyr332, located in the PAS, besides, the pyridine moiety interacts with Trp82 by means of π–π stacking. In this orientation, no interactions with the catalytic triad residues were found.

By comparing the bioactive conformations for inhibitor **7**, the energy gap in hBuChE is approximately 29 kcal/mol higher than the calculated for the hAChE. This energetic penalty can be, at least in part, the reason for why compound **7** shows low hBuChE inhibitory activity.

2.5. Theoretical ADME analysis of pyridonepezils ***1*–*8***

Finally, a series of theoretical calculations allowed us to describe the ADME (Absorption, Distribution, Metabolism, and Excretion) properties of compounds **1**–**8** within the organism. All these four criteria influence the drug levels, its kinetics and exposure to the tissues, and hence the performance and pharmacological activity of

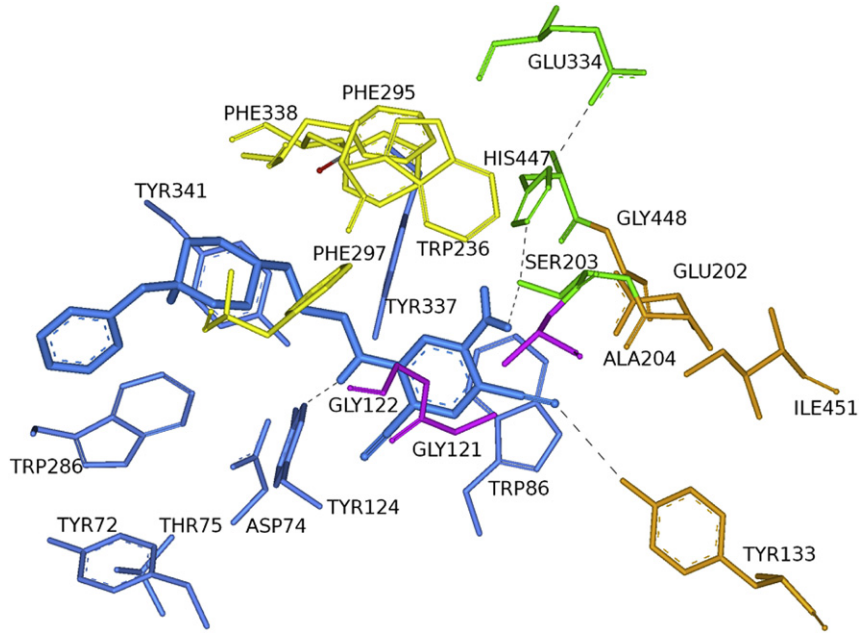


Fig. 1. Binding mode of inhibitor **7** at the active site of hAChE. Compound **7** is rendered as sticks and illustrated in blue. The side chains conformations of the mobile residues are illustrated in the same color as the ligand. Different subsites of the active site were colored: catalytic triad (CT) in green, oxyanion hole (OH) in pink, anionic subsite (AS) in orange, except Trp86, acyl binding pocket (ABP) in yellow and peripheral anionic subsite (PAS) in blue. Black dashed lines are drawn among atoms involved in hydrogen bond interactions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

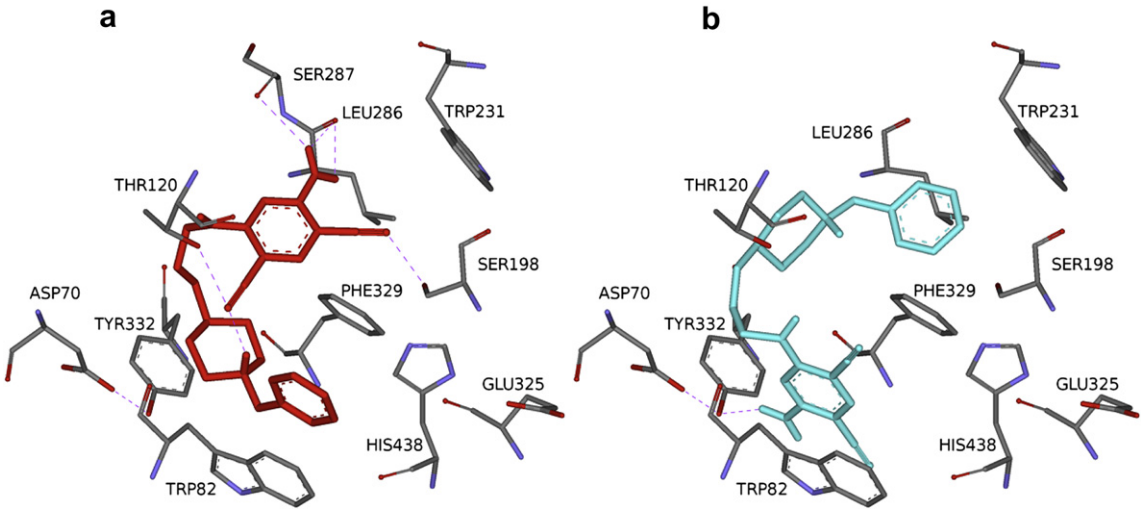


Fig. 2. Two major binding modes of inhibitor **7** at the active site of hBuChE. Mode **I** (a): The compound is rendered as sticks and illustrated in red; mode **II** (b): The compound is rendered as sticks and illustrated in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a drug. The ADME properties were calculated by QikProp (version 3.5, Schrödinger, LLC, New York, NY, 2012). About 45 physically significant descriptors and pharmacologically relevant properties of these compounds were predicted and some of the important properties were analyzed. Lipinski's rule of five (RO5) [40] is a rule of thumb to evaluate the drug likeness of a molecule based on some molecular descriptors representing ADME properties (see Table S1, Supplementary material). All the compounds (**1**–**8**) showed significant values for the properties analyzed and showed drug-like characteristics based on Lipinski's rule of five (see Table S1). Compounds used for neurological disorders treatment are generally CNS acting drugs. CNS drugs show values of molecular weight, HB donor, acceptors and rotatable bonds, etc., in general, in a smaller range than general therapeutics. Factors that are relevant to the success of CNS drugs were also analyzed. Except compound **4**, all the compounds fulfill molecular weight, show a log Po/w < 5 and the number of hydrogen bonds donors and acceptors are in the limits. The solubility (QPlogS) of organic molecules in water has a significant impact on many ADME-related properties like uptake, distribution, transport, and eventually bioavailability. Seven compounds (**1**–**2**, **4**–**8**) present solubility values within the limits (−6.5–0.5), while compounds **3** and **4** show values of −6.9 and −7.3, respectively, being in the limits of aqueous solubility.

3. Conclusions

To sum up, we have reported the synthesis and cholinesterase inhibition of new *pyridonepezils* **1**–**8**. The hAChE and hBuChE inhibition of these molecules were evaluated and compared to donepezil. Most of the assayed compounds showed higher activity. The most potent, inhibitor **7**, was found to be 1.4-fold more active than donepezil. Compounds **1** and **5** (both with $n = 0$) were extremely selective for AChE, but *pyridonepezils* **2**–**4** and **6** showed equipotent, high activity for both hAChE and hBuChE. Molecules **7** and **8** were found to be potent and selective for hAChE than for hBuChE. Concerning SAR, compound **7** ($n = 3$), bearing a hydrogen at C4, was found to be more active than the analog **3** ($n = 3$) with phenyl group at C4. Finally, the BBB study shows that all tested compounds would cross the BBB by passive diffusion. Work is now in progress in our laboratory to prepare and evaluate new *pyridonepezils* of type **I**, and the results will report in due course.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.09.030.

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