Novel Tacrine−Melatonin Hybrids as Dual-Acting Drugs for Alzheimer Disease, with Improved Acetylcholinesterase Inhibitory and Antioxidant Properties

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Abstract: Tacrine and melatonin are well-known drugs with activities as an acetylcholinesterase (AChE) inhibitor and free radical scavenger, respectively. In this work, we report new hybrids of both drugs that display higher in vitro properties than the sum of their parts. As selective inhibitors of human AChE, their IC_{50} values range from sub-nanomolar to picomolar. They exhibit a higher oxygen radical absorbance capacity than does melatonin and are predicted to be able to cross the blood−brain barrier to reach their targets in the central nervous system.

Alzheimer's disease (AD), the most common dementia in elderly people, is a complex neurodegenerative disorder of the central nervous system. AD patients present a progressive loss of cholinergic synapses in brain regions associated with higher mental functions, mainly located in hippocampus and neocortex. It causes brain atrophy and a progressive failure of intellectual abilities. The histopathological hallmarks of the disease are senile plaques and neurofibrillary tangles. Plaques are massive extracellular deposits of aggregated amyloid-β peptide, while tangles are intracellular residues of abnormally phosphorylated protein-tau.[1]

In the last two decades, several rational pharmacological strategies have emerged, including cholinergic and noncholinergic interventions.[2] Among the cholinergic hypothesis, the first approved drugs for the management of the disease were cholinesterase inhibitors tacrine, donepezil, rivastigmine, and galanthamine that increase neurotransmission at cholinergic synapses in the brain and thereby improve cognition.[3]

Despite the many published clinical trials of approved acetylcholinesterase inhibitors (AChE-I) for AD treatment, the practical effectiveness of these drugs remains controversial. Recent AD trials concluded that AChE-I therapies are not cost-effective.[4] However, other recent clinical studies showed that patients treated with cholinesterase inhibitors did not show the widespread cortical atrophic changes associated with AD, providing empirical evidence of neuroprotection by cholinesterase inhibitors.[5] These effects might be related to their primary mode of action or their interaction with other neuronal targets, such as N-methyl-D-asparate (NMDA), nicotinic, or muscarinic receptors.[5c,6] For these reasons, the interest in cholinesterase inhibitors has increased in the last few years.

Furthermore, several studies have shown that the antioxidant defense system in elderly people loses its capacity to neutralize oxidative species, and then oxidative stress can act as a risk factor for the initiation and progression of AD.[7] Recent research demonstrated that oxidative damage is an event that precedes the appearance of other pathological hallmarks of the disease, namely, senile plaques and neurofibrillary tangles.[8] Thus, drugs that specifically scavenge oxygen radicals may have a particular therapeutic efficacy,[9] and several antioxidants have been tested in clinical trials.[10]

Tacrine (**1**), the first approved drug for AD, is a potent nonselective inhibitor of both AChE and butyrylcholinesterase (BuChE). Although this lack of selectivity and its hepatotoxicity have reduced its therapeutic use,[11] the search for tacrine analogues is still of interest in AD.[12] Melatonin (**2**) is a pineal neurohormone whose levels decrease during aging, especially in AD patients. It has been reported to possess strong antioxidant actions and is able to directly scavenge a variety of reactive oxygen species.[13] Moreover, recent studies have shown that melatonin has protective effects against Aβ-induced apoptosis in microglial cells and improves learning and memory in rats.[14]

Our research in the AD field[15] is currently focused on dual-acting drugs, capable of combining AChE inhibition and antioxidant properties in a single small molecule (**3**−**11**). In this work, we planned to use a high-quality moiety for each biological activity, tacrine and melatonin. According to the catalytic site of AChE, which is located at the bottom of a deep gorge,[16] we considered binding these fragments using carbon chains that could be accommodated into the enzyme cavity (Chart 1).

Scheme 1 depicts the general procedure for the synthesis of tacrine−melatonin derivatives. Substituted 9-chloro-1,2,3,4-tetrahydroacridines **12**−**15** were synthesized as previously described.[17] Their treatment with the appropriate α,ω-amino acid in *n*-pentanol and subsequent hydrolysis of the pentyl ester derivatives with sodium hydroxide gave the intermediate acids **16**−**19a**,**b**. Reaction of these acids with different amines (tryptamine, serotonin, or 5-methoxytryptamine), using (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate as a coupling reagent, afforded the desired tacrine-indol derivatives **3**−**11a,b**, which were characterized by ^{1}H NMR, ^{13}C NMR, mass spectra, and elemental analyses.

The new tacrine−melatonin derivatives were evaluated as inhibitors of AChE and BuChE, following the method of Ellman.[18] Initially, compounds were tested with enzymes of animal source, namely, AChE from bovine erythrocytes and BuChE from horse serum, which show a high degree of homology with the corresponding human enzymes.[19] Tacrine and melatonin were also evaluated for comparative purposes (Table 1).

All the new compounds are potent inhibitors of nonhuman cholinesterases at the low nanomolar level, better than tacrine. As expected, melatonin did not inhibit either enzyme. In general, compounds with a 6-methylene linker between the amine and the amide groups showed better inhibition of AChE than molecules with a 5-methylene chain. Compounds derived from 6-chlorotacrine and 6,8-dichlorotacrine combined high potency and selectivity toward AChE.

A selection of tacrine−melatonin hybrids was further evaluated using human enzymes. Tacrine was also tested, and data are presented in Table 2.

All tested hybrids inhibited the human AChE (h-AChE) more efficiently than the bovine enzyme, showing IC_{50} values ranging from the sub-nanomolar to the picomolar order (8×10^{-10} −

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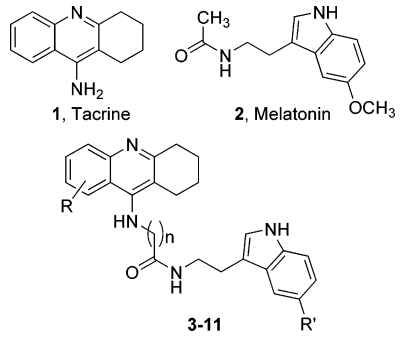
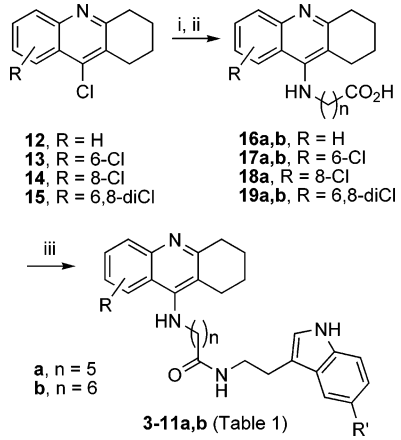
Chart 1. Structures of New Tacrine−Melatonin Hybrids **3**−**11** and Their Parent Drugs



Scheme 1. Synthesis of Tacrine−Melatonin Derivatives[a]



[a] Reagents and conditions: (i) $H_2N(CH_2)_nCO_2H$ (1 equiv), *n*-pentanol, reflux, 18−24 h; (ii) NaOH 2 N, reflux, 6 h; (iii) $H_2N(CH_2)_2$-indole (1.3 equiv), BOP (1.3 equiv), $(CH_3CH_2)_3N$ (2.6 equiv), CH_2Cl_2, r.t., 8−16 h.

Table 1. Inhibition of AChE and BuChE by Tacrine−Melatonin Hybrids **3**−**11**, Tacrine (**1**), and Melatonin (**2**)[a]

				$IC_{50} \pm SD$ (nM)[b]	
	R	n	R′	AChE[c]	BuChE[d]
3a	H	5	H	4.0 ± 0.2	12 ± 1
3b	H	6	H	1.0 ± 0.1	0.95 ± 0.05
4a	6-Cl	5	H	2.0 ± 0.1	5.2 ± 0.3
4b	6-Cl	6	H	0.2 ± 0.01	8.1 ± 0.3
5a	8-Cl	5	H	65 ± 3	15 ± 1
6a	6,8-diCl	5	H	3.5 ± 0.2	8.2 ± 0.5
6b	6,8-diCl	6	H	2.0 ± 0.1	85 ± 4
7a	H	5	OCH_3	9.0 ± 0.3	2.0 ± 0.1
7b	H	6	OCH_3	2.3 ± 0.1	2.5 ± 0.1
8a	6-Cl	5	OCH_3	12 ± 1	55 ± 2
9a	8-Cl	5	OCH_3	25 ± 1	22 ± 1
10b	6,8-diCl	6	OCH_3	5.0 ± 0.3	100
11a	H	5	OH	35 ± 2	3.5 ± 0.2
1				40 ± 2	10 ± 0.4
2				>100	>100

[a] See Figure 1 for structures. [b] Results are presented as the mean (n = 3) ± SD. [c] AChE from bovine erythrocytes. [d] BuChE from horse serum.

8×10^{-12} M). They inhibited human BuChE (h-BuChE) with $IC_{50} = 1 - 250$ nM, exhibiting an interesting selectivity toward h-AChE.

Comparing **3**−**6**, which do not bear any substituent in the indole ring, it is possible to establish the best modifications in the tacrine fragment for potency and for selectivity toward h-AChE. The presence of a chlorine atom in position 6 improved both factors, compound **4b** being a low sub-nanomolar h-AChE inhibitor with a good selectivity to this enzyme. When the chlorine atom was attached to position 8 of the tacrine ring (**5a**) inhibition of AChE dropped, revealing that this change in the structure is not well tolerated by the active center. Finally, disubstituted 6,8-dichlorotacrine derivative **6b** was the most potent and selective h-AChE inhibitor of the series, pointing out that the two chlorine atoms could work synergistically in the active center. In relation to structural modifications in the indole fragment, a methoxy or a hydroxy group in the 5-position gave rise to compounds with inferior values for both potency and selectivity toward AChE.

Table 2. Inhibition of Human AChE and BuChE (nM) and Oxygen Radical Absorbance Capacity (ORAC, Trolox Equivalents) by Selected Tacrine−Melatonin Hybrids **3**−**11**, Tacrine (**1**), and Melatonin (**2**)[a]

	$IC_{50} \pm SD$ (nM)[b]		h-AChE selectivity[c]	Trolox equiv[d]
	h-AChE	h-BuChE		
3b	0.5 ± 0.02	6.8 ± 0.3	14	3.3 ± 0.1
4b	0.1 ± 0.005	35 ± 2	350	2.1 ± 0.03
5a	0.87 ± 0.04	23 ± 2	26	4.0 ± 0.1
6b	0.008 ± 0.0004	7.8 ± 0.4	975	2.5 ± 0.1
7b	0.65 ± 0.03	3.0 ± 0.2	5	2.7 ± 0.1
10b	0.04 ± 0.002	25 ± 1	625	1.7 ± 0.01
11a	0.45 ± 0.02	1.0 ± 0.1	2	3.2 ± 0.2
1	350 ± 10	40 ± 2	0.1	<0.01
2	n.d.	n.d.		2.3 ± 0.1

[a] See Figure 1 and Table 1 for structure definitions. [b] Results are the mean (n = 3) ± SD. [c] Selectivity for h-AChE = IC_{50} (h-BuChE)/IC_{50} (h-AChE). [d] Data are expressed as μmol of trolox equivalent/μmol of tested compound and are the mean (n = 3) ± SD.

To the best of our knowledge, compound **6b** is one of the most potent inhibitors of human AChE described, although recently femtomolar inhibitors of eel AChE were obtained by click chemistry.[20] This compound, derived from 6,8-dichlorotacrine and unsubstituted indole with a 6-methylene linker, exhibited an IC_{50} (h-AChE) = 0.008 nM and was about 1000-fold more active toward h-AChE than toward h-BuChE. Moreover, this inhibitor is 40 000 times more effective than tacrine and showed the required selectivity.

Since AChE is mainly located in the central nervous system and BuChE is more abundant in the peripheral system, the potency and selectivity toward AChE found in tacrine−melatonin hybrids are of paramount importance. These new molecules should be able to activate mostly the central cholinergic transmission improving mental abilities and lack the side effects related to nonselective cholinesterase inhibitors.[21]

The antioxidant activity of new tacrine−melatonin hybrids **3**−**11a**,**b** was determined by the oxygen radical absorbance capacity assay using fluorescein (ORAC-FL). Following the method described by Ou et al.,[22] recently optimized by Dávalos et al.,[23] peroxyl radicals were thermally generated from 2,2′-azobis-(amidinopropane) dihydrochloride and reacted with fluorescein to form nonfluorescent products at 520 nm. The antioxidant capacity of new compounds was determined by their competition with fluorescein in the radical capture, using a fluorescence microplate reader. Vitamin E analogue trolox was used as a standard, and the results were expressed as trolox equivalents (μmol of trolox equivalents/μmol of tested compound) (Table 2). Tacrine and melatonin were also checked for comparison. Tacrine showed negligible radical capture, whereas melatonin had an ORAC-FL value 2.3-fold higher than that of trolox. This activity fully agrees with the value previously described for melatonin (2.0 trolox equiv),[24] pointing out the reliability of our experiments.

Compounds were tested in 0.1−1 μM concentrations, showing potent peroxyl radical absorbance capacities ranging from 1.7- to 4-fold the value of trolox. In general, best results were obtained with derivatives bearing an unsubstituted indole or a 5-hydroxyindole, whereas compounds derived from 5-meth-

Table 3. Permeability ($P_e \times 10^{-6}$ cm s^{-1}) in the PAMPA-BBB Assay of 20 Commercial Drugs, Used in the Experiment Validation

compd	bibl[a]	exp[b]	compd	bibl[a]	exp[b]
testosterone	17.0	13.0	piroxicam	2.5	2.3
verapamil	16.0	13.0	hydrocortisone	1.9	3.2
imipramine	13.0	9.1	aldosterone	1.2	2.7
desipramine	12.0	11.0	lomefloxacin	1.1	1.3
astemizole	11.0	11.0	enoxacin	0.9	1.7
progesterone	9.3	8.6	atenolol	0.8	2.0
promazine	8.8	7.3	ofloxacin	0.8	1.8
chlorpromazine	6.5	6.1	isoxicam	0.3	1.6
clonidine	5.3	6.8	theophylline	0.1	1.5
corticosterone	5.1	7.2	cimetidine	0.0	1.2

[a] Taken from ref 25. [b] Data are the mean of three independent experiments and standard errors are within 5% of the mean.

Table 4. Permeability ($P_e \times 10^{-6}$ cm s^{-1}) in the PAMPA-BBB Assay for Tacrine−Melatonin Hybrids and Their Predictive Penetration in the CNS

comp	P_e ($\times 10^{-6}$ cm s^{-1})[a]	prediction
3a	11.0 ± 0.5	CNS+
3b	9.2 ± 0.4	CNS+
4a	7.7 ± 0.3	CNS+
5a	5.2 ± 0.1	CNS+
6a	8.4 ± 0.2	CNS+
7a	7.7 ± 0.1	CNS+
7b	8.6 ± 0.3	CNS+
8a	8.0 ± 0.2	CNS+
9a	8.9 ± 0.1	CNS+
11a	2.0 ± 0.1	CNS−

[a] Data are the mean ($n = 3$) ± SD.

oxyindole showed lower values. Comparing compounds **3**−**6**, which do not bear any substituent in the indole ring, it is possible to evaluate contributions of the tacrine fragment to antioxidant properties. Unsubstituted tacrine compound **3b** showed an ORAC-FL value of 3.3 trolox equiv, whereas the activity of 6-chloro- and 6,8-dichlorotacrine derivatives (**4b** and **6b**, respectively) clearly dropped (ORAC-FL ≈ 2.3 trolox equiv). It is worth mentioning that when the chlorine atom was attached to position 8 in the tacrine fragment we found the best radical scavenger of this work, **5a** that is 4 times more active than trolox.

To evaluate the brain penetration of new compounds we used a parallel artificial membrane permeation assay for blood−brain barrier (PAMPA-BBB) using a lipid extract of porcine brain, as recently described by Di et al.[25] Assay validation was made comparing experimental permeabilities of 20 commercial drugs with reported values (Table 3).

A plot of experimental data versus bibliographic values gave a good linear correlation, P_e (exp.) = 0.7331P_e (bibl.) + 1.4816 ($R^2 = 0.9536$). From this equation and taking into account the limit established by Di et al. for blood−brain barrier permeation,[25] we found that compounds with a permeability above 4.4×10^{-6} cm s^{-1} could be able to cross the blood−brain barrier.

Then, new tacrine−melatonin hybrids were tested in the PAMPA-BBB assay, and the results are presented in Table 4. With the only exception of **11a**, derived from 5-hydroxyindole, all new tacrine−melatonin hybrids could cross the blood−brain barrier and reach their biological targets located in the central nervous system.

In conclusion, we have developed new tacrine−melatonin hybrids, which were potent inhibitors of human AChE and showed high oxygen radical absorbance capacity. Compound **6b** was the most potent inhibitor of human AChE here described that was 40 000-fold more potent than tacrine, exhibiting an IC_{50} of 8 pM. In addition, it was about 1000-fold less active toward human BuChE, displaying an interesting selectivity. In the oxygen radical absorbance capacity assay, this compound is 2.5-fold higher than trolox, a vitamin E analogue. In addition, these tacrine−melatonin hybrids are also predicted to be able to enter the central nervous system. Although there were differences in potencies for AChE inhibition and antioxidant properties, these new compounds can be considered interesting structures in the search for new agents of potential application in AD. Work is now in progress to outline the scope of these new tacrine−melatonin hybrids.

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Supporting Information Available: Experimental details for new compounds (synthesis and in vitro biological tests). This material is available free of charge via the Internet at http://pubs.acs.org.

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