Novel Multipotent Tacrine−Dihydropyridine Hybrids with Improved Acetylcholinesterase Inhibitory and Neuroprotective Activities as Potential Drugs for the Treatment of Alzheimer's Disease

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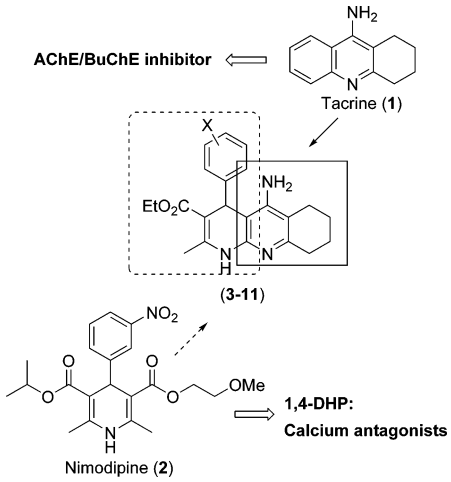
Abstract: In this work we describe the synthesis and biological evaluation of the tacrine−1,4-dihydropyridine (DHP) hybrids (**3**−**11**). These multipotent molecules are the result of the juxtaposition of an acetylcholinesterase inhibitor (AChEI) such as tacrine (**1**) and a 1,4-DHP such as nimodipine (**2**). Compounds **3**−**11** are very selective and potent AChEIs and show an excellent neuprotective profile and a moderate Ca^{2+} channel blockade effect. Consequently, these molecules are new potential drugs for the treatment of Alzheimer's disease.

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by progressive memory loss, decline in language skills, and other cognitive impairments. Although the etiology of AD is not well-known, there are diverse factors such as amyloid-β (Aβ) deposits, τ-protein aggregation, oxidative stress, and low levels of acetylcholine (ACh) that are thought to play significant roles in the disease.[1] The cholinergic theory of AD suggests that the selective loss of cholinergic neurons in AD results in a deficit of ACh in specific regions of the brain that mediate learning and memory functions.[2] The primary approach for treating AD has therefore focused on increasing the levels of acetylcholine in the brain by using acetylcholinesterase inhibitors (AChEI) such as tacrine, donepezil, galantamine, and rivastigmine.[3] On the other hand, it is well-known that Ca^{2+} overload is the main factor initiating the processes leading to cell death. Several lines of evidence show that calcium dysfunction, involved in the pathogeny of AD,[4] augments Aβ formation[5a] and τ hyperphosphorylation.[5b] Ca^{2+} entry through L channels causes calcium overload and mitochondrial disruption, which lead to the activation of the apoptotic cascade and cell death.[6] Hence, blocking the entrance of Ca^{2+} through this specific subtype of Ca^{2+} channel could be a good strategy to prevent cell death.

The multitarget approach in drug design[7] for the treatment of AD includes novel tacrine−melatonin hybrids,[8a] dual inhibitors of AChE and monoamine oxidase (MAO),[8b] dual AChEI and serotonin transporters,[8c] and potent cholinesterase inhibitors with antioxidant and neuroprotective properties.[8d]

Chart 1. Selection of Multipotent Hybrid Molecules (**3**−**11**), Based on the Juxtaposition of an AChEI and a 1,4-DHP, Such as Tacrine (**1**) and Nimodipine (**2**)



Since 1,4-dihydropyridines (DHPs) selectively block L-type voltage-dependent Ca^{2+} channels (VDCC), we considered the synthesis and pharmacological study of new multipotent hybrid molecules, based on an AChEI and a DHP such as tacrine (**1**) and nimodipine (**2**) (Chart 1), to be novel and of great interest.[9] Besides inhibition of AChE and blockade of VDCC, which could prevent Ca^{2+} overload and subsequent cell death, we were also interested in compounds targeted to prevent oxidative stress. Recent research has demonstrated that oxidative damage is an event that precedes the appearance of other pathological hallmarks of AD.[10] Thus, drugs that scavenge oxygen radicals may have a particular therapeutic efficacy.[11,12]

In this Letter we report our preliminary results on the synthesis and biological evaluation, including AChE/BuChE inhibition, propidium iodide displacement, Ca^{2+} blockade, and neuroprotective activity, of novel tacrine−DHP hybrids ("tacripyrines") (**3**−**11**) (Chart 1). From this study, we conclude that tacripyrines (**3**−**11**) are very selective and potent AChEIs, show excellent neuroprotective profiles and moderate Ca^{2+} channel blockade effects, and consequently, can be considered as new potential drugs for further development, targeted to the treatment of Alzheimer's disease.

The synthesis of tacripyrines (**3**−**11**) was easily achieved, in excellent yields, by the Friedländer reaction[13] between the unknown ethyl esters of 6-amino-4-aryl-5-cyano-2-methyl-1,4-dihydropyridine-3-carboxylic acids (**12**−**20**)[14] and cyclohexanone under standard conditions[15] (Scheme 1). Compounds **3**−**11** are racemic hexahydrobenzo[*b*][1,8]naphthyridines substituted at C-4 by an aromatic ring incorporating different types of substituents. These molecules have been conveniently characterized by their analytical and spectroscopic data (see Supporting Information).

The new tacripyrines were evaluated as inhibitors of AChE from electric eel (*Electrophorus electricus*) and of AChE from human serum, following the method of Rappaport,[16] and as inhibitors of BuChE from human serum, following the method of Ellman.[17] To allow comparisons of the results, tacrine (**1**) was used as the reference compound (Table 1). As shown, all tacripyrines are more potent inhibitors of AChE at the nanomolar

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Scheme 1. Synthesis of Tacrine−DHP Hybrids ("Tacripyrines") **3**−**11**

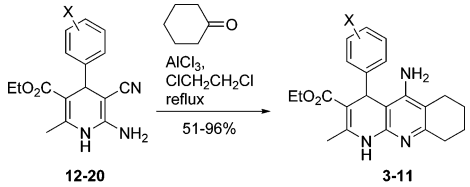

Table 1. Inhibition of AChE and BuChE by Tacripyrines **3**−**11** and Tacrine (**1**)

		IC_{50} (nM)[a]			
	X	AChE[b] electric eel	BuChE[c] human serum	AChE[d] human erythrocytes	selectivity:[e] IC_{50}(BuChE)/ IC_{50}(AChE)
tacrine		180 ± 20	36 ± 4	147 ± 11	0.24
3	H	80 ± 2.0	>100000	122 ± 21	>820
4	4-F	52 ± 9.1	>100000	193 ± 31	>518
5	2-NO_2	304 ± 2.0	4800 ± 40	338 ± 13	14.2
6	3-NO_2	110 ± 2.0	>100000	191 ± 29	>524
7	4-NO_2	600 ± 7.0	34400 ± 110	309 ± 21	111
8	4-Me	91 ± 4.0	>100000	169 ± 15	>592
9	2-MeO	160 ± 6.5	>100000	234 ± 32	>427
10	3-MeO	61 ± 9.3	>100000	58 ± 7	>1724
11	4-MeO	45 ± 5.0	>100000	105 ± 15	>952

[a] IC_{50} values are the mean ± SEM of at least three independent measurements. [b] From electric eel (*Electrophorus electricus*). [c] From human serum. [d] From human erythrocytes. [e] Human AChE and BuChE.

level than tacrine, and a simple structure−activity relationship could be obtained. Only **5** and **7**, with a nitro group at C-2′ and C-4′ in the aromatic ring, are less potent than tacrine; however, they are the only molecules able to inhibit BuChE, showing IC_{50}s of 4.8 and 34.4 μM, respectively. In fact, hybrids **3**, **4**, **6**, **8**−**11** are highly selective AChE inhibitors, exhibiting no inhibition of BuChE, with BuChE/AChE selectivities higher than 952, 518, and 1724 for the most potent AChE inhibitors (**11**, **4**, and **10**, respectively). The effect of the position and type of the susbtituent in the aromatic ring is also worthy of comment. It was concluded that the most potent (**11**) or the poorest (**7**) AChEIs have an electron-donating group (OMe) or an electron-withdrawing group (NO_2), respectively, at C-4′. Very interestingly, from C-3′ to C-2′, regardless of the type of substituent (from **10** to **9** or from **6** to **5**), AChE inhibition drops significantly; however, note that **6** with a nitro group at C-3′ is a better AChEI than molecule **9** with a methoxy substituent at C-3′. It is also clear that lowering the electron-donating power of the substituent at the critical C-4′ position has a definitive importance for potent AChE inhibitors, a fact that is more evident when comparing AChE inhibition of **11** (IC_{50} = 45 nM) with **4** (IC_{50} = 52 nM) and **8** (IC_{50} = 91 nM), compounds bearing a C4′-F and a C4′-Me group, respectively.

Experiments with human AChE (hAChE) were performed to compare their results with those of BuChE inhibition, since this last enzyme was also from human serum. As shown, the inhibition of hAChE by **3**−**11** was in general somewhat less potent than that with the electric eel, but the compound selectivity for AChE was preserved (Table 1).

Next, a series of experiments with **3**−**11** at 0.3 and 3 μM, using propidium iodide as ligand and designed to evaluate the binding of these new AChE inhibitors at the peripheral anionic site of the enzyme,[18] showed that for the most potent and selective AChE inhibitors (**11**, **4**, and **10**) the displacement of propidium iodide was not significant, suggesting that these compounds possibly bind preferentially at the catalytic site at the bottom of a deep gorge of AChE.[19]

Table 2. Effect of **3**−**11** on $[Ca^{2+}]_c$ Increase Elicited by 70 mM K^+ in SH-SY5Y Cells (% Inhibition with Respect to a Control without Any Drug)[a]

	X	% blockade in SH-SY5Y cells
nimodipine		45.5 ± 6.1***
3	H	36.64 ± 2.66*
4	4-F	22.22 ± 3.0 ns
5	2-NO_2	32.65 ± 4.2**
6	3-NO_2	33.61 ± 5.1***
7	4-NO_2	33.65 ± 3.0***
8	4-Me	29.83 ± 3.98 ns
9	2-MeO	46.50 ± 4.44***
10	3-MeO	31.04 ± 4.1**
11	4-MeO	32.75 ± 2.5**

[a] Data are expressed as the mean ± SEM of at least three different cultures in quadruplicate: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; ns, not significant. All compounds were assayed at 0.3 μM.

To sum up, from tacrine to our new tacripirines (**3**−**11**), AChE inhibitory activity is potentiated while BuChE inhibition dramatically drops, giving a new family of potent and selective AChE inhibitors. This fact is of great importance, as these new molecules should be able, primarily, to activate central cholinergic transmission. At the same time, they lack the side effects related to nonselective cholinesterase inhibitors.[20]

Concerning the Ca^{2+} channel blockade activity of tacripirines **3**−**11**, first we studied the Ca^{2+} influx induced by K^+ depolarization in SH-SY5Y neuroblastoma cells previously loaded with the fluorescent dye Fluo-4 AM. Fluo-4-loaded cells were incubated in the presence of **3**−**11** (0.3 μM) for 10 min and then stimulated with a concentrated solution of KCl so that the final concentration in the medium was 70 mM K^+. In all experiments, we tested additionally a positive control, such as nimodipine (0.3 μM), which caused 45.5% inhibition of K^+-evoked Ca^{2+} uptake.

The calcium channel antagonist activities exhibited by **3**−**11** are shown in Table 2. Most of the tested compounds gave significant Ca^{2+} blockade, the most potent being hybrid **9** with 46% inhibition, a blockade similar to that obtained for nimodipine at the same concentration.

These results prompted the investigation of the potential neuroprotective activity of the novel compounds **3**−**11**. Thus, we studied the effects of these new compounds on SH-SY5Y cells exposed for 24 h to a medium with a depolarizing concentration of KCl (70 mM), which induced Ca^{2+} overload and consequent cell death. Drugs at 0.3 μM were administered 24 h before incubation of the cells with high K^+ (70 mM, hypertonic) and maintained during the entire experiment. Thereafter, release of lactic dehydrogenase (LDH) was measured as a parameter of cell death.[21] As shown in Table 3, all the compounds afforded a degree of neuroprotection higher than tacrine and within the same range as nimodipine; tacripyrines **4**, **10**, and **11** were higher than 1,4-DHP **2**, showing protection values of around 38−48%.

Next, we evaluated the antioxidant activity of **3**−**11** at 0.3 μM on SH-SY5Y neuroblastoma cells exposed to 60 μM H_2O_2 for 24 h. From the results shown in Table 4, we conclude that again the new tacrine−DHP hybrids protect much more efficiently against free radicals than the parent compounds **1** and **2**. In fact, it can be observed that three of the most potent neuroprotectors in this assay (**11**, **4**, and **10**), with values between 45% and 55%, also proved to be among the most efficient in the previous test (see Table 3) and, very significantly, there is an excellent correlation between the best AChE inhibitors and the most efficient neuroprotective agents, a fact that, to the best of our knowledge, has been described in this work for the first time.

Table 3. Cell Viability for Tacripirines **3**−**11**, Expressed as Reduction in the Increase of LDH Released in the Presence of 70 mM K^+ [a]

	X	LDH release (% of control)	% protection
tacrine		90.4 ± 4,1 ns	13.4 ± 7.2
nimodipine		75.00 ± 2.24***	35.93 ± 2.84
3	H	75.02 ± 2.56***	35.92 ± 3.53
4	4-F	61.22 ± 2.10***	48.79 ± 3.99
5	2-NO_2	88.29 ± 2.99 ns	16.59 ± 4.18
6	3-NO_2	77.87 ± 2.74***	31.8 ± 3.81
7	4-NO_2	63.91 ± 2.34***	45.03 ± 4.02
8	4-Me	72.43 ± 1.89***	39.70 ± 2.47
9	2-MeO	77.61 ± 1.66***	32.23 ± 2.21
10	3-MeO	68.96 ± 1.33***	38.77 ± 3.43
11	4-MeO	67.63 ± 2.24***	41.30 ± 4.95

[a] Data are expressed as the mean ± SEM of at least three different cultures in quadruplicate. LDH released was calculated for each individual experiment, considering 100% the extracellular LDH released in the presence of vehicle. To calculate % protection, LDH release was normalized as follows. In each individual triplicate experiment, the LDH release obtained in nontreated cells (basal) was subtracted from the LDH released upon 70 mM K^+ treatment and normalized to 100%. Then that value was subtracted from 100. All the compounds were assayed at 0.3 μM: (***) $p < 0.001$; ns, not significant.

Table 4. Cell Viability for Tacripirines **3**−**11**, Expressed as Reduction in the Increase of LDH Released in the Presence of 60 μM H_2O_2 [a]

	X	LDH release (% of control)	% protection
catalase		18.93 ± 1.77***	88.34 ± 2.80
tacrine		100.34 ± 3,37 ns	0
nimodipine		75.42 ± 2.12***	36.03 ± 2.82
3	H	76.61 ± 1.83***	30.60 ± 1.96
4	4-F	64.17 ± 1.53***	46.11 ± 2.02
5	2-NO_2	71.91 ± 1.23***	37.18 ± 1.61
6	3-NO_2	77.19 ± 1.69***	30.09 ± 2.18
7	4-NO_2	72.43 ± 2.99***	35.48 ± 3.78
8	4-Me	59.60 ± 0.92***	53.68 ± 1.62
9	2-MeO	63.78 ± 1.35***	48.23 ± 2.24
10	3-MeO	65.14 ± 1.23***	44.80 ± 1.53
11	4-MeO	56.55 ± 1.94***	55.78 ± 2.32

[a] Data are expressed as the mean ± SEM of at least three different cultures in quadruplicate. LDH released was calculated for each individual experiment, considering 100% the extracellular LDH released in the presence of vehicle. To calculate % protection, LDH release was normalized as follows. In each individual triplicate experiment, the LDH release obtained in nontreated cells (basal) was subtracted from the LDH released upon 70 mM K^+ treatment and normalized to 100%. Then that value was subtracted from 100. All the compounds were assayed at 0.3 μM: (***) $p < 0.001$; ns, not significant.

In conclusion, we have developed a new family of tacrine−DHP hybrids that we have named tacripyrines, which are potent and selective inhibitors of AChE and show potent neuroprotection activity. The fact that our compounds provide protection against two stimuli with different mechanisms of action (calcium overload or free radical generation) indicates that they could be exerting their effects on the cascade of apoptosis/cell death beyond the particular mechanism of each toxic agent. The possibility exists that they are acting by inducing the expression of proteins implicated in cell survival, as we could demonstrate in a previous work from our laboratory.[22] This and other possibilities should be further explored. From the present study, we conclude that **11** was the most potent AChEI, being 4-fold more potent than tacrine and exhibiting an IC_{50} of 45 nM. In addition, it was about 1000 times less active toward BuChE, displaying a striking selectivity. In the neuroprotective assays, the protection afforded by this compound was 1.5-fold higher than that for nimodipine in the presence of H_2O_2 at the concentration assayed. In summary, the new compounds can be considered as interesting new chemical entities with potential therapeutic application for AD patients.

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Supporting Information Available: Synthetic procedures, analytical characterization for **12**−**20** and **3**−**11**, and pharmacological protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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