Rational Approach To Discover Multipotent Anti-Alzheimer Drugs

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Abstract: The coupling of two different pharmacophores, each endowed with different biological properties, afforded the hybrid compound lipocrine (**7**), whose biological profile was markedly improved relative to those of prototypes tacrine and lipoic acid. Lipocrine is the first compound that inhibits the catalytic activity of AChE and AChE-induced amyloid-β aggregation and protects against reactive oxygen species. Thus, it emerged as a valuable pharmacological tool to investigate Alzheimer's disease and as a promising lead compound for new anti-Alzheimer drugs.

Alzheimer's disease (AD), the most common cause of dementia, is a complex neurological affection that is clinically characterized by loss of memory and progressive deficits in different cognitive domains. The consistent neuropathologic hallmark of the disorder, generally noted on postmortem brain examination, is a massive deposit of aggregated protein breakdown products, amyloid-β (Aβ) plaques and neurofibrillary tangles. Even if the primary cause of AD is still speculative, Aβ aggregates are thought to be mainly responsible for the devastating clinical effects of the disease.[1] In recent years, significant research has been devoted to the role of free radical formation, oxidative cell damage, and inflammation in the pathogenesis of AD, providing new promising targets and validated animal models.[2] To date, however, the enhancement of the central cholinergic function is the only clinically effective approach.[3,4] The intensive research of drugs that can improve the cholinergic transmission in AD has produced so far four approved acetylcholinesterase (AChE) inhibitors, that is, tacrine (TC),[5] donepezil,[6] rivastigmine,[7] and galantamine.[8] However, these drugs have been approved for the symptomatic treatment of AD because they do not address the etiology of the disease for which they are used.

It is therefore necessary to discover pharmacological instruments that are able to act as far upstream as possible in the neurodegenerative cascade and, because of the multifaceted etiology of AD, able to hit different selected targets. The aim of this communication is to provide new multipotent compounds, i.e., single molecules that can exhibit more pharmacological properties simultaneously, such as the enhancement of the cholinergic transmission and inhibition of Aβ accumulation and oxidative stress, leading to a synergic and effective treatment of AD.

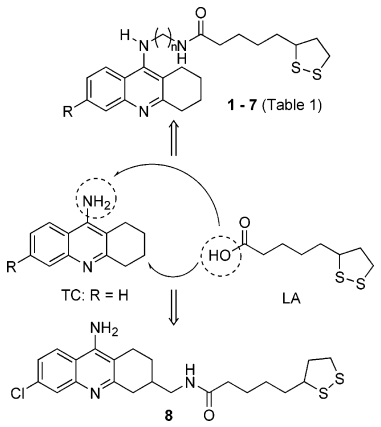

Figure 1. Design strategy for **1**−**8**.

To this end, we applied a design strategy in which distinct pharmacophores of two different drugs were combined in the same structure leading to hybrid molecules. In principle, each pharmacophore of these new drugs should retain the ability to interact with its specific site(s) on the target and consequently to produce specific pharmacological responses that taken together should block or hopefully cure the neurodegenerative process leading to AD. To obtain proof of concept for this proposal, we chose TC and lipoic acid (LA) as prototype drugs to be combined in the same structure because of their well-established biological properties (Figure 1). In fact, LA is a universal antioxidant,[9−12] which was shown to protect neurons against cytotoxicity induced by Aβ[13] and to stabilize cognitive functions in patients with AD,[14] whereas TC was the first AChE inhibitor approved for AD treatment.[5] AChE is the enzyme involved in the hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses in the central and peripheral nervous system. Inhibitors of AChE activity promote an increase in the concentration and the duration of action of synaptic ACh, thus causing an enhancement of the cholinergic transmission through activation of the synaptic nicotinic and muscarinic receptors. However, achievement of potent inhibitors of the AChE catalytic site would not represent a significant improvement unless there is a concomitant inhibition of the peripheral anionic site (PAS) of the enzyme, which is associated with the neurotoxic cascade of AD through AChE-induced Aβ aggregation.[15,16] For this reason, the introduction onto the TC structure of a side chain, namely, an LA fragment following the two routes shown in Figure 1, should hopefully combine antioxidant properties with the ability to interact with PAS.

Compounds **1**−**8** were synthesized by coupling tetrahydroacridine intermediates **9**−**16** with LA in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), according to Scheme 1 (see Supporting Information). No attempt was made to separate the enantiomers of **1**−**7** because it was reported that stereochemistry is not relevant for the protective effect of LA against oxidative cell damage.[10] In addition, concerning **8**, the diastereomers were not

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Scheme 1. Synthesis of **1**−**8**

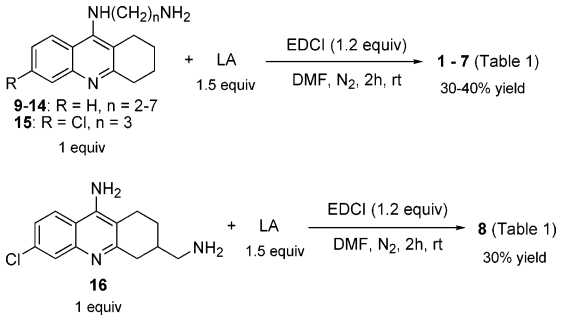

Table 1. Inhibition of AChE and BChE Activities by **15**, Tacrine (TC), Lipoic Acid (LA), and Hybrid Derivatives **1**−**8** (See Figure 1 for Structures)

			$IC_{50} \pm SEM$ (nM)[a]	
compd	n	R	AChE	BChE
1	2	H	97.0 ± 3.6	47.5 ± 1.8
2	3	H	6.96 ± 0.45	12.0 ± 0.6
3	4	H	35.2 ± 2.2	5.04 ± 0.32
4	5	H	38.4 ± 2.3	1.48 ± 0.35
5	6	H	30.1 ± 1.5	3.24 ± 0.29
6	7	H	32.7 ± 1.3	8.58 ± 0.57
7	3	Cl	0.253 ± 0.016	10.8 ± 2.5
8			1090 ± 180	329 ± 28
15			21.5 ± 0.8	2580 ± 60
TC			424 ± 21	45.8 ± 3.0
LA			>1000000	>1000000

[a] Human recombinant AChE and BChE from human serum were used. IC_{50} values represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements, each performed in triplicate. See Supporting Information for details.

separated because it was the weakest AChE inhibitor among the investigated compounds.

To determine the potential interest of **1**−**8** for the treatment of AD, their AChE inhibitory activity was determined by the method of Ellman et al.[17] on human recombinant AChE. Moreover, to study further the biological profiles of **1**−**8**, their butyrylcholinesterase (BChE) inhibitory activity was also assessed by the same method on BChE from human serum. To allow comparison of the results, **15**, TC, and LA were used as the reference compounds (Table 1). It is evident that all compounds were effective inhibitors of AChE and BChE and significantly more potent than prototype TC with the exception of **8**. Modifying the chain length between the two nitrogen atoms of the lateral chain, affording **1**−**7**, affected the affinity for AChE and BChE. Optimum inhibition of AChE was observed for **2**, having three methylene units. As expected,[18] the insertion of a chlorine atom into the acrydine system, affording **7** (lipocrine), produced an 85-, 1676-, and 28-fold increase in AChE inhibition relative to **15**, TC, and **2**, respectively. As one would expect, LA did not inhibit either enzyme. The finding that **8** was 4308-fold less potent than **2** in inhibiting AChE activity suggests that the insertion of the lipoyl fragment on the nitrogen atom at position 3 of **16** resulted in a highly negative effect on the interaction mechanism with the enzyme.

Inhibition of AChE activity by **7** was very fast and not time-dependent because 50% of enzyme inactivation produced by 0.253 nM following a 1 min incubation was not significantly different ($p > 0.01$) from the inhibition observed up to a 40 min incubation. The estimates of

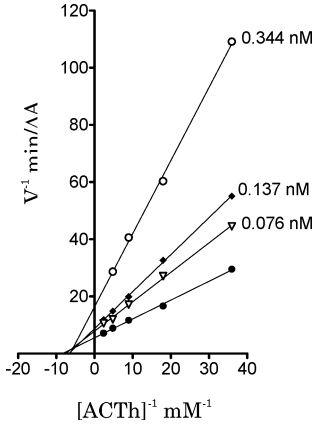

Figure 2. Steady-state inhibition of AChE hydrolysis of acetylthiocholine (ACTh) by **7**. Lineweaver−Burk reciprocal plots of initial velocity and substrate concentrations are presented. Reciprocal plots of initial velocity in the absence of inhibitor gave an estimate of k_{app} for acetylthiocholine of 170 ± 15 μM (four experiments). Lines were derived from a weighted least-squares analysis of the data points.

competitive inhibition constants K_i calculated for **7** and TC were 0.155 ± 0.046 and 0.151 ± 0.016 μM, respectively, whereas the graphical analysis of steady-state inhibition data for **7** is shown in Figure 2. An analysis of the Lineweaver−Burk reciprocal plots of **7** reveals that there are an increasing slope and an increasing intercept with higher inhibitor concentration. The inhibitory behavior of **7**, as deduced from Figure 2, is strictly similar to that displayed by some reported bis-tetrahydroaminoacridine inhibitors of AChE. These compounds bind simultaneously at the catalytic and the peripheral sites of AChE and are characterized by a linear mixed type of enzyme inhibition.[19] Therefore, we concluded that **7** causes a mixed type of inhibition, i.e., inhibition of both the active site and a second distal site of the enzyme. Once verified that **7** may interact also with PAS of AChE, we verified whether there is a concomitant inhibition of Aβ aggregation induced by AChE through a thioflavin T-based fluorometric assay.[16,20] It turned out that TC and LA, i.e., the pharmacophoric moieties combined in **7**, were not able to inhibit at 100 μM the Aβ aggregation induced by AChE, whereas 100 μM **15** caused only a 25 ± 5% inhibition. In contrast, **7** was only 3-fold less potent than propidium, which is the most potent inhibitor of AChE-induced Aβ aggregation so far available,[16] as revealed by an analysis of their IC_{50} values of 45.0 ± 14.6 and 12.6 ± 0.5 μM, respectively. Furthermore, **7** was significantly more potent than all the other AChE inhibitors ever tested,[16] including AP2238, an inhibitor purposely designed to bind the catalytic and the peripheral sites of AChE (35% of inhibition at 100 μM).[21]

Clearly, this finding, together with the results observed for **15**, TC, and LA separately, is relevant because an association of 100 μM TC and 100 μM LA or 100 μM **15** and 100 μM LA produced only a weak inhibition (15 ± 6% or 30 ± 7%, respectively) of AChE-induced Aβ aggregation, suggesting that marked Aβ aggregation inhibition may be achieved only when the two prototypes are combined into the same structure, as in **7**.

The cytotoxicity effects of LA, **7**, and **15** were first determined by colorimetric MTT [3-(4,5-dimethyl-2-

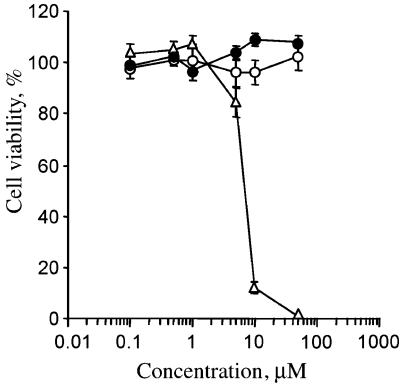

Figure 3. Effects of compounds on cell viability in neuronal cells. The cell viability was determined by the MTT assay (as described in Supporting Information) after 24 h of incubation with various concentrations of LA (●), **7** (○), and **15** (△). The results were expressed as a percentage of control cells. Values are reported as the mean ± SD of three independent experiments.

Table 2. Effects of the Hybrid Compound **7** and the Two Parent Compounds LA and **15** on Intracellular ROS Formation in Neuronal Cells[a]

	intracellular ROS, %		
concn, μM	LA	**7**	**15**
0	86.00 ± 9.46	86.00 ± 9.46	86.00 ± 9.46
0.1	91.25 ± 2.99	88.75 ± 8.41	99.50 ± 3.54
0.5	90.25 ± 6.65	95.00 ± 5.20	99.67 ± 7.02
1	83.00 ± 7.58	77.00 ± 8.76	96.67 ± 8.02
5	79.50 ± 8.06	61.67 ± 9.02*	82.50 ± 7.78
10	74.33 ± 3.51	51.25 ± 9.21**	tox[b]
50	58.50 ± 9.19*	30.50 ± 9.04**	tox[b]

[a] The results are expressed as percentage increase of intracellular ROS evoked by exposure to *tert*-butyl hydroperoxide. Values are the mean ± SD of three independent experiments (treated vs untreated; (*) $p < 0.01$, (**) $p < 0.001$). [b] tox = cytotoxicity. See Supporting Information for details.

thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay in human neuronal-like cells, SH-SY5Y, as described by Mosmann et al.[22] As reported in Figure 3, treatment of SH-SY5Y cells with LA and **7** (0.1−50 μM) did not show modified cell viability. By contrast, the treatment of SH-SY5Y cells with **15** (0.1−50 μM) produced a strong decrease of cell viability for 10 μM (88%) and 50 μM (99%). The intracellular antioxidant activity of LA, **7**, and **15** against formation of reactive oxygen species (ROS) in SH-SY5Y cells after treatment with *tert*-butyl hydroperoxide, a compound used to induce oxidative damage, was then assessed. A range of concentrations of tested compounds that did not affect cell viability (0.1−50 μM for LA and **7**; 0.1−5 μM for **15**) were used. As shown in Table 2, treatment of SH-SY5Y cells with LA showed a significant ($p < 0.01$) decrease of ROS formation only with the highest concentration used (50 μM), while the treatment with **7** produced a strong dose-dependent inhibitory effect on the ROS formation. Significant inhibitory effects by **7** with respect to basal values were reached for 5 μM ($p < 0.01$), 10 μM ($p < 0.001$), and 50 μM ($p < 0.001$). When treated with **15** (0.1−5 μM), the neuronal cells did not show any difference on ROS formation. Taken together, these results show that LA and **7** did not affect the neuronal viability while **15** exerted neurotoxic effects. In addition, LA and **7** (but not **15**) were able to protect neuronal cells against ROS formation evoked by oxidative stress, with **7** being the most active against ROS formation (64% inhibition at 50 μM).

In conclusion, the present investigation has shown that it is possible to obtain multipotent drugs for the treatment of AD: lipocrine (**7**) emerged in in vitro models as an effective candidate to be investigated in vivo for its multiple biological properties, namely, inhibition of AChE and BChE activities, inhibition of AChE-induced Aβ aggregation, and ability to protect cells against ROS.

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Supporting Information Available: Full experimental procedures and characterization data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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