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Synthesis and Pharmacological Evaluation of Huprine−Tacrine Heterodimers: Subnanomolar Dual Binding Site Acetylcholinesterase Inhibitors

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Abstract: A series of huprine−tacrine heterodimers has been developed by connection of huprine Y, a compound with one of the highest affinities for the active site of acetylcholinesterase yet reported, with tacrine, a compound with known affinity for the peripheral site of the enzyme, through a linker of appropriate length to allow simultaneous interaction with both binding sites. These compounds exhibit human acetylcholinesterase and butyrylcholinesterase inhibitory activities with IC_{50} values in the subnanomolar and low nanomolar range, respectively.

Combination into a single molecule of two identical or different structural entities or fragments of well-known biologically active natural products or synthetic drugs with the aim of either increasing the potency of the parent compounds or combining complementary actions has remained a strategy largely unexplored for long time. However, in the last years pharmacomodulation of biologically active compounds through conjunctive approaches has become an area of very active research in different fields of medicinal chemistry.[1]

In the particular field of acetylcholinesterase inhibitors (AChEIs), so far the most important class of drugs for the treatment of Alzheimer's disease (AD), two different concepts of conjunctive approaches have been used with the same goal of increasing affinitiy for the enzyme.

On one hand, the increase of affinity for AChE has been pursued through a simultaneous interaction of the drug with both the active site of the enzyme and the so-called peripheral site, a less well-defined area located at the entrance of the catalytic gorge of AChE, approximately 14 Å distant from the active site.[2] For this purpose, several homo- and heterodimers containing two identical or different structural units of known AChEIs connected by a linker of suitable length to locate both components at the most appropriate distance for simultaneous interaction with both binding sites have been developed.[3] So far, the most interesting of these bis-ligands is the so-called bis(7)-tacrine, **2** (Figure 1), a dimer of tacrine, **1**, developed by the group of Pang and Carlier, in which the two component units are connected through an heptamethylene chain. The great increase in AChE inhibitory potency of **2** (IC_{50} = 1.5 nM, using rat brain AChE) relative to the parent compound (149-fold more potent than tacrine) dramatically illustrates the power of the dual binding site strategy.[4] An increase in affinity can still be achieved by placing in the linker a suitable functionality able to interact with the aromatic residues that are lining the wall of the AChE gorge and which would constitute a third binding site within the enzyme. Thus, Campiani et al. have reported that replacement of the central methylene group of bis(7)-tacrine by a protonatable methylamino group capable of providing additional specific interactions with this mid-gorge recognition site leads to a dramatic increase in potency (667-fold more potent than tacrine).[5] Analogously, the group of Valenti and Recanatini has developed a very interesting AChE inhibitor, AP2238, consisting of benzylamino and coumarin moieties as the active and peripheral site binding units, respectively, connected by a *p*-phenylene linker able to interact with the aromatic residues of the enzyme gorge. In addition to its high AChE inhibitory potency (IC_{50} = 44.5 nM, using human AChE), AP2238 exhibits a significant β-amyloid anti-aggregating action.[6]

A second concept of pharmacomodulation of known AChEIs through conjunctive approaches involves the simultaneous occupation of the binding zones of two different AChEIs within the active site of the enzyme, when their recognition sites are separated but adjacent. For this purpose, we designed some time ago a series of tacrine−huperzine A hybrids, the so-called huprines, by combination of the 4-aminoquinoline moiety of tacrine with the carbobicyclic substructure of (−)-huperzine A, (−)-**3** (Figure 1).[7] Huprines tightly bind at the base of the active site gorge of AChE, where its 4-aminoquinoline moiety occupies the same position of the corresponding substructure of tacrine, thus sharing all of the features that modulate the binding of tacrine to AChE, and additionally they partially share the same binding pocket of (−)-huperzine A.[8] As a result, as compared with the parent compounds tacrine and (−)-huperzine A, whose binding zones within the active site of AChE are close and partially overlap, the additional contacts of huprines can account for their greatly increased inhibitory activities relative to the parent compounds. The most active huprines prepared to date, the so-called huprine Y, **4** (Figure 1), and its 9-ethyl-analogue, huprine X, are, in racemic form, up to 260- and 330-fold more potent as human AChE inhibitors than the parent compounds tacrine and (−)-huperzine A, respectively.

In this paper we report that combination of the above-mentioned two strategies results in a highly increased affinity relative to huprines and tacrine. Herein, we describe the synthesis and pharmacological evaluation

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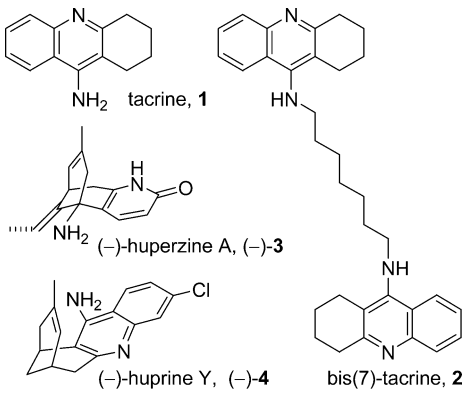


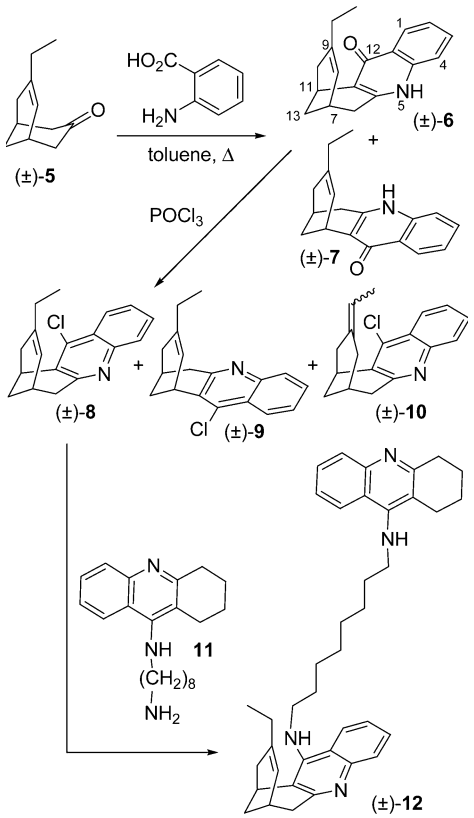
Figure 1. Structures of bis(7)-tacrine and (−)-huprine Y and their parent compounds.

of a series of huprine-based heterodimers consisting of a huprine moiety as the active site binding unit, a tacrine-related unit as the peripheral site binding unit, and a linker of suitable length and nature to allow dual site binding, and, in some cases, also binding to the midgorge recognition site.

The design of the huprine-based heterodimers was carried out taking into account the extremely high affinity of huprines X and Y for the active site of AChE and the pharmacological data reported for the tacrine-based homo- and heterodimers, which involve both the use of tacrine[9] or 6-chlorotacrine[10] as the peripheral site ligand and a tether length of six to eight methylene groups[4b] for an optimal AChE inhibitory activity. The use of the 4-methyl-4-azaheptamethylene chain[5] as the linker was also considered for a possible interaction with the AChE gorge.

Regarding the synthesis of the new heterodimers, we first considered the optimal synthetic approach described for the preparation of tacrine-based homo- and heterodimers, based on the aromatic nucleophilic substitution of 4-chloroquinolines with α,ω-diaminoalkanes.[4b] For this purpose, 4-chloroquinolines with the framework of huprines were needed. Although the substitution pattern of huprines leading to an optimal activity implies the presence of a chlorine atom at the benzene ring of the aminoquinoline system, the initial studies were directed toward the synthesis of derivatives unsubstituted at the benzene ring of the quinoline system. Thus, the new chloroquinoline (±)-**8** was prepared in 42% isolated overall yield by reaction of the known enone (±)-**5** with anthranilic acid in refluxing toluene with azeotropic removal of water, followed by treatment of the resulting 9:1 mixture of regioisomeric quinolones (±)-**6** and (±)-**7** with $POCl_3$ under reflux,[4b] and column chromatography separation of the desired (±)-**8** from the also formed isomers (±)-**9** and (±)-**10** (Scheme 1). Finally, reaction of (±)-**8** with the known amine **11**[9] in refluxing 1-pentanol[4b] afforded heterodimer (±)-**12** in 21% yield, after column chromatography. The low yield of this last step, which is not rare in the context of the synthesis of dual binding site heterodimers using this methodology,[9] and particularly, the low efficiency in the synthesis of chloroquinoline (±)-**8** due to the formation of significant amounts of regioisomers constituted important drawbacks of this synthetic approach, which made us consider an alternative strategy for the synthesis of huprine−tacrine heterodimers.

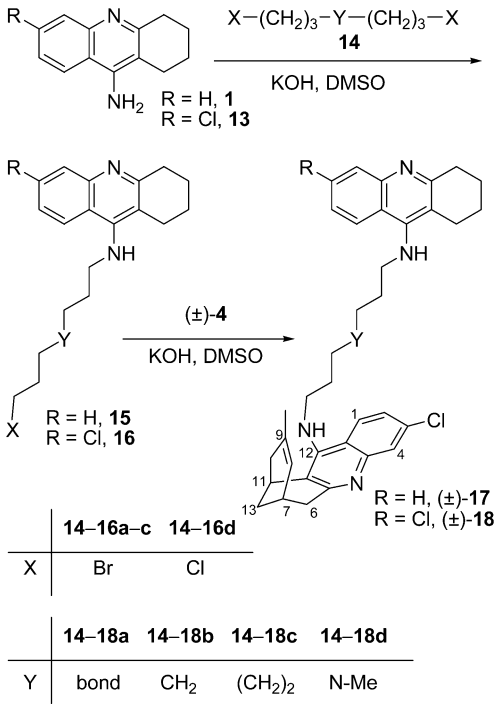
Scheme 1. Synthesis of the Huprine−Tacrine Heterodimer (±)-**12**



The initially reported methodology for the synthesis of tacrine-based homodimers, based on the nucleophilic substitution of α,ω-dihaloalkanes with 4-aminoquinolines,[4a] was later discarded in favor of the above-mentioned synthetic approach due to the formation of important amounts of byproducts arising from β-elimination or intramolecular cyclization reactions from the initially formed *N*-(ω-haloalkyl)tacrines under the basic reaction conditions.[4b] However, this problem was especially important when the tether length was lower than that corresponding to six methylenes, which was not our case. Moreover, as this synthetic approach required 4-aminoquinolines instead of 4-chloroquinolines, huprines could be used directly, with the advantage that huprines can be readily synthesized from enone (±)-**5** or its 7-methyl analogue on a multigram scale, in one step, high yields, and as single regioisomers.[7a]

In our case, the synthesis of the desired huprine−tacrine heterodimers was straightforward using this methodology. Thus, amination of the commercial dibromoalkanes **14a**−**c** and the known dichloroderivative **14d**[11] with tacrine, **1**, or 6-chlorotacrine, **13**,[12] in DMSO in the presence of KOH and 4 Å molecular sieves at room temperature afforded the alkylated tacrines **15a**−**d** or **16a**−**d** in 20−44% yield (Scheme 2), after column chromatography, accompanied in most cases by small amounts of the byproducts arising from them by β-elimination of HX. Reaction of compounds **15a**−**d** and **16a**−**d** under similar conditions with huprine Y, (±)-**4**, afforded the heterodimers (±)-**17a**−**d** and (±)-**18a**−**d**, respectively, in pure form (23−57% yield) after column chromatography, which were fully characterized as di- or trihydrochlorides through their spectroscopic data and elemental analyses (C, H, N, Cl).

Scheme 2. Synthesis of the Huprine−Tacrine Heterodimers (±)-**17a**−**d** and (±)-**18a**−**d**



To determine the potential interest of the new huprine−tacrine heterodimers (±)-**12**, (±)-**17a**−**d**, and (±)-**18a**−**d** for the treatment of AD, their AChE inhibitory activity was assayed by the method of Ellman et al.[13] on AChE from bovine and human erythrocytes. The time dependence of the inhibition of bovine AChE was also determined after a period of incubation of 45 min, as an initial step to determine a possible irreversible or tight-binding activity of the compounds. Selectivity in inhibition of AChE vs butyrylcholinesterase (BChE), another cholinesterase which together with AChE coexists ubiquitously in humans, was regarded as a positive value in AChEIs, as it could result in low peripheral cholinergic effects in AD patients.[14] However, recent evidences have shown that in advanced AD patients, AChE activity is greatly reduced in specific brain regions, while BChE activity increases.[15] The increasing importance of BChE in the hydrolysis of the neurotransmitter acetylcholine, as the ratio AChE/BChE gradually decreases in these patients, makes inhibition of BChE an important target in the search for anti-Alzheimer agents. Consequently, inhibitory activity on human serum BChE of the huprine−tacrine heterodimers was also assayed by the same method.

All of the new heterodimers are clearly more active as bovine AChE inhibitors than the parent compounds tacrine (59−394-fold more potent), (−)-huperzine A (34−224-fold more potent), and (±)-huprine Y (2−13-fold more potent) (Table 1). Heterodimers **17**, bearing an unsubstituted tacrine unit, are clearly more potent bovine AChEIs than compounds **18**, bearing a 6-chloro-substituted tacrine unit, while the tether length for an optimal bovine AChE inhibitory activity is that equivalent to six methylenes. The heterodimer (±)-**17d** is the most active compound probably due to the combination of the best substitution pattern in the tacrine unit, an adequate tether length, and especially to the possibility of interaction of the protonated amino group of the linker with the aromatic residues of the AChE gorge. Compound (±)-**12** is the least active, what may be mainly due to the inadequate length of the tether, and the absence of the 3-chloro-substituent in the huprine unit.[7a] The bovine AChE inhibitory activity of the new heterodimers is poorly affected by incubation of the enzyme with the inhibitor before addition of substrate, in contrast with the results shown by some huprines which exhibit tight-binding properties.[7c]

Table 1. Pharmacological Data of Tacrine, (−)-Huperzine A, Huprine Y, and the Di- or Trihydrochlorides of the Huprine−Tacrine Heterodimers (±)-**12**, (±)-**17a**−**d**, and (±)-**18a**−**d**[a]

	IC_{50} (nM)			
	bovine AChE			
compound	0-min incub	45-min incub	human AChE	human BChE
(±)-**12**·2HCl	2.19 ± 0.22	1.67 ± 0.16	0.82 ± 0.09	5.55 ± 0.20
(±)-**17a**·2HCl	0.40 ± 0.04	0.27 ± 0.02	0.50 ± 0.01	7.69 ± 0.38
(±)-**17b**·2HCl	0.48 ± 0.05	0.36 ± 0.07	0.34 ± 0.02	6.70 ± 0.20
(±)-**17c**·2HCl	1.26 ± 0.13	0.65 ± 0.06[b]	0.33 ± 0.01	4.74 ± 0.09
(±)-**17d**·3HCl	0.33 ± 0.01	0.26 ± 0.02[b]	0.32 ± 0.02	16.4 ± 0.90
(±)-**18a**·2HCl	1.06 ± 0.09	0.80 ± 0.01	0.45 ± 0.06	14.0 ± 0.40
(±)-**18b**·2HCl	1.97 ± 0.28	1.60 ± 0.09	0.33 ± 0.04	7.38 ± 0.29
(±)-**18c**·2HCl	2.09 ± 0.11	1.31 ± 0.12[c]	0.40 ± 0.03	7.80 ± 0.07
(±)-**18d**·3HCl	0.54 ± 0.03	0.44 ± 0.03	0.29 ± 0.01	31.1 ± 0.10
tacrine·HCl	130 ± 10	*d*	205 ± 18	43.9 ± 17
(−)-huperzine A	74.0 ± 5.50	*d*	260 ± 18	> 10000
(±)-huprine Y·HCl	4.23 ± 0.86	*d*	0.78 ± 0.02	236 ± 44

[a] Values are expressed as mean ± standard error of the mean of at least four experiments. IC_{50} inhibitory concentration (nM) of AChE (from bovine or human erythrocytes) or BChE (from human serum) activity. [b] $P < 0.05$. [c] $P < 0.01$ vs 0-min incubation (Student's *t*-test). [d] Not determined.

Moreover, all of the new huprine−tacrine heterodimers are clearly more potent human BChE inhibitors than tacrine (1.4−9.3-fold more potent), (−)-huperzine A (> 322−>2110-fold more potent), and (±)-huprine Y (8−50-fold more potent), exhibiting IC_{50} values in the low nanomolar range. Although the higher AChE vs BChE inhibitory activity of the first tacrine-based homo- and hetero-dimers was initially ascribed to the lack of a peripheral binding site in BChE,[4a,9] recent molecular modeling studies have led to the hypothesis of the presence of a peripheral interaction site also in human BChE, in which the aromatic residue F278 would be responsible for $\pi-\pi$ interactions with aromatic moieties of tacrine-based heterodimers.[5] Indeed, the higher BChE inhibitory activity of bis(7)-tacrine and several tacrine-based heterodimers,[5] as well as that of the new huprine−tacrine heterodimers, relative to tacrine seems

All of the new huprine−tacrine heterodimers are also clearly more active as human AChEIs than tacrine (250−707-fold more potent), (−)-huperzine A (317−897-fold more potent), and (±)-huprine Y [1.6−2.7-fold more potent, with the exception of (±)-**12** which is nearly equipotent], all of them exhibiting IC_{50} values in the subnanomolar range. As previously observed in huprines, most of the new heterodimers exhibit an increased human vs bovine AChE inhibitory activity (up to 6-fold more potent). The substitution pattern at the tacrine unit seems to have little influence on the human AChE inhibitory activity of these compounds, while the optimal tether length is that equivalent to seven or eight methylenes. The presence of the protonatable amino group in the linker leads to a modest increase in human AChE inhibitory activity.

to validate this hypothesis. The presence in our compounds of an unsubstituted tacrine unit seems to lead to a higher BChE inhibitory activity, while the optimal tether length seems to be that equivalent to seven or eight methylenes. In this case, the presence of a protonatable amino group in the linker seems to have a detrimental effect on the BChE inhibitory activity, which could be due to the fact that many aromatic residues in the gorge of human AChE are replaced with aliphatic residues in human BChE.[5]

Thus, a series of huprine−tacrine heterodimers has been synthesized as a novel class of AChEIs, formally derived from tacrine and (−)-huperzine A by combining two different strategies to increase affinity (extended binding in the active site of AChE and simultaneous binding to the peripheral site), which results in a greatly increased affinity relative to the parent compounds. Introduction of a protonatable amino group in the linker results in a still increased bovine and human AChE inhibitory activity likely as a result of the additional interaction with the AChE gorge as a third recognition site. Interestingly, these compounds are also highly potent BChE inhibitors, exhibiting IC_{50} values in the low nanomolar range.

In a near future, some studies will be done to assess possible complementary actions in the huprine−tacrine heterodimers, such as ability to cross the blood-brain barrier[7c,16] and neuroprotective[17,18] and β-amyloid antiaggregating effects,[6] which together with their herein established combined pharmacological profile of highly potent AChE and BChE inhibitors would make these compounds very interesting drug candidates for the treatment of AD.

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Supporting Information Available: Experimental details (chemistry and pharmacology). This material is available free of charge via the Internet at http://pubs.acs.org.

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