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New Tacrine–Huperzine A Hybrids (Huprines): Highly Potent Tight-Binding Acetylcholinesterase Inhibitors of Interest for the Treatment of Alzheimer’s Disease

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Several new 12-amino-6,7,10,11-tetrahydro-7,11-methanocycloocta[b]quinoline derivatives (tacrine–huperzine A hybrids, huprines) have been synthesized and tested as acetylcholinesterase (AChE) inhibitors. All of the new compounds contain either a methyl or ethyl group at position 9 and one or two (chloro, fluoro, or methyl) substituents at positions 1, 2, or 3. Among the monosubstituted derivatives, the more active are those substituted at position 3, their activity following the order 3-chloro > 3-fluoro > 3-methyl > 3-hydrogen. For the 1,3-difluoro and 1,3-dimethyl derivatives, the effect of the substituents is roughly additive. No significant differences were observed for the inhibitory activity of 9-methyl vs 9-ethyl derivatives monosubstituted at positions 1 and/or 3. The levorotatory enantiomers of these hybrid compounds are much more active (eutomers) than the dextrorotatory forms (distomers) as AChE inhibitors. Compounds rac-20, (−)-20, rac-26, (−)-26, rac-30, (−)-30, and rac-31 showed human AChE inhibitory activities up to 28.5-fold higher than for the corresponding bovine enzyme. Also, rac-19, (−)-20, (−)-30, and rac-31 were very selective for human AChE vs butyrylcholinesterase (BChE), the AChE inhibitory activities being 438–871-fold higher than for BChE. Several hybrid compounds, specially (−)-20 and (−)-30, exhibited tight-binding character, showing higher activity after incubation of the enzyme with the inhibitor than without incubation, though the reversible nature of the enzyme–inhibitor interaction was demonstrated by dialysis. The results of the ex vivo experiments also supported the tight-binding character of compounds (−)-20 and (−)-30 and showed their ability to cross the blood–brain barrier. Molecular modeling simulations of the AChE–inhibitor complex provided a basis to explain the differences in inhibitory activity of these compounds.

Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative illness that affects up to 5% of people over 65 years, rising to 20% of those over 80 years.¹ Until now, most treatment strategies have been based on the cholinergic hypothesis of cognitive dysfunction of AD, which postulates that the cognitive impairment associated with AD results from a deficit of the cholinergic function in the brain.² Accordingly, enhancement of the central cholinergic neurotransmission has been regarded as one of the most promising approaches for treating AD patients, mainly by means of reversible acetylcholinesterase (AChE) inhibitors. The prototype for the centrally acting AChE inhibitors was tacrine³ (2), the first drug to be approved in the United States (Cognex) for the treatment of AD. However, its severe side effects, such as hepatotoxicity and gastrointestinal upset, represent an important drawback. The results of the studies on tacrine spurred the development of other centrally acting reversible AChE inhibitors, such as the recently marketed galantamine⁴ (Nivalin), donepezil⁵ (Aricept), and rivastigmine⁶ (Exelon), or the natural product (−)-huperzine A⁷–⁹ (3), which is currently undergoing extensive clinical trials, showing considerable promise for the palliative treatment of AD.

Bivalency is an effective strategy for improving drug potency and selectivity, when multiple recognition sites for the same substrate exist. In this sense, important efforts have been made to develop new AChE inhibitors of increased potency and selectivity, able to bind simultaneously to the catalytic and peripheral sites of AChE and not only to the catalytic site as is the case for the above-cited drugs. Several homodimeric and heterodimeric tacrine-based compounds have been recently synthesized. Among these compounds, a homodimeric compound containing two units of tacrine connected by a heptamethylene chain turned out to be 149-fold more potent than tacrine inhibiting rat brain AChE.¹⁰–¹² The same strategy was used for the synthesis of some hybrid AChE inhibitors, composed of a unit of tacrine and a key fragment of huperzine A, connected by an oligomethylene chain (optimum: decamethylene), obtaining compounds up to 13-fold more potent than (−)-hup-
AChE from bovine erythrocytes. Likewise, the AChE pound 15 times more active than tacrine in inhibiting was also found to be advantageous, leading to a con-


duction of a fluorine substituent at position 3 (Figure 1). Moreover, the intro-

duction of a fluorine substituent at position 9. The structure of these compounds do not seem adequate to simultaneously bind to both the active sites and the peripheral sites of AChE. Several of these compounds exhibited higher AChE inhibitory activity than tacrine (2) and (−)-huperzine A (3) (Figure 1), particularly when a methyl (rac-15) or ethyl (rac-21) group was attached to position 9. Moreover, the intro-
duction of a fluorine substituent at position 3 (rac-18) was also found to be advantageous, leading to a compound 15 times more active than tacrine in inhibiting AChE from bovine erythrocytes. Likewise, the AChE inhibitory activity of the levorotatory enantiomers was roughly twice that of the racemic mixtures, while the dextrorotatory enantiomers were much less active. Molecular modeling of the interaction of these compounds with Torpedo californica AChE (TcAChE) suggested that they behave as true tacrine–huperzine A hybrids, since the 4-aminooxquinoline and bicyclo[3.3.1]nonane subunits roughly occupy the same positions of the corresponding moieties in tacrine and (−)-huperzine A, respectively, as determined from their crystallographic complexes with AChE. Later, replacement of fluorine by chlorine at position 3 (rac-30) was found to improve the inhibitory activity, leading to an inhibition constant (Ki) for human AChE around 30 pM, which means an affinity around 1200-fold higher than that of tacrine.

Since these derivatives bind to AChE with very high affinity, it is of interest to explore in more detail the effect of these structural changes on the AChE inhibi-
tory activity. This study reports the synthesis and pharmacological data of a new series of rac-huprines of general structure 1, having R = Me or Et and one or two identical substituents (Me, F, or Cl) at different positions (1, 2, 3, or 1,3) of the benzene ring. The pharmacological analysis includes: (a) inhibitory activity of bovine and human AChE and human butyrylcholinesterase (BuChE), (b) neuromuscular studies focused on the ability to revert the neuromuscular blockade induced by d-tubocurarine, (c) time dependence and reversibility of the AChE inhibitory activity, and (d) ex vivo AChE inhibitory activity studies. Finally, molecular modeling studies have been performed to explain the differences in inhibitory activity of the more active compounds.

Results and Discussion

Chemistry. The synthesis of the new compounds (rac-19, rac-24–rac-29, and rac-31) was carried out by Friedländer reaction of the known enones rac-4a and rac-4b21 and the corresponding aminobenzonitrile 7–12, under aluminum trichloride catalysis, usually in 1,2-dichloroethane as solvent under reflux. Enones rac-4a and rac-4b were easily prepared from the commercially available bicyclo[3.3.1]nonane-3,7-dione by reaction with the appropriate organomagnesium, organolithium, or organocopper reagent to give a 3-alkyl-2-oxa-1-adamantanol, which was then mesylated and submitted to a silica gel promoted fragmentation reaction. Aminobenzonitriles 7,22 9,23 10,24 11,25 12,26 and 1427 were prepared by the described procedures, while 12 is a commercial compound. Not unexpectedly on steric grounds, the yield of these reactions was low in the cases where the product contained a chlorine (rac-28 and rac-31) or methyl (rac-24) substituent at position 1, despite working under more forcing conditions (100 °C, under pressure for rac-28 and rac-31, 1,2-dibromoethane under reflux for rac-24). Products having a fluorine atom at position 1 (rac-19, rac-25, and rac-27) could be obtained under the standard conditions although in medium to low yields, probably due to the lower steric hindrance of the fluorine atom as compared with the chlorine atom or the methyl group.
As previously observed, only the anti-regioisomers were detected in these reactions. This could be the result of a kinetic control during the cyclization step as we pointed out, but it could also be the result of the acid-catalyzed isomerization of the syn-regioisomer under the reaction conditions, a matter which is actually being studied. Compound rac-26 was separated into its enantiomers by chiral MPLC under similar conditions to those reported for other members of this series.

All of the new compounds were fully characterized through their spectroscopic data (IR, 1H and 13C NMR spectra, and elemental analysis). Assignment of the 1H and 13C NMR spectra was carried out through the COSY 1H/1H and COSY 1H/13C spectra and by comparison with assignments previously carried out for other members of the same or related series of compounds. The 13C and 1H NMR data and elemental analyses of the new compounds are collected in Tables 1–3, respectively, of the Supporting Information.

**Pharmacology.** To determine the potential interest of the new huprines for the treatment of AD, their AChE inhibitory activity was assayed by the method of Ellman et al. on AChE from bovine and human erythrocytes. To establish their selectivity, their BChE inhibitory activity on human serum BChE was also assayed by the same method. For the more active compounds the time dependence of the inhibition was determined after a period of incubation of 30 min. The reversibility of the inhibition process was also studied. Moreover, the ex vivo AChE inhibitory activity in mouse brain was evaluated after treating animals with these compounds. Finally, most of them were further analyzed in a peripheral cholinergic synapse, such as the skeletal neuromuscular junction. In this analysis, the ability to reverse the d-tubocurarine-induced neuromuscular blockade, a well-known effect of AChE inhibitors, was tested.

Table 1 summarizes the data comparing bovine and human AChE and human BChE inhibition as well as the ratio between AChE and BChE inhibitory activities for the new huprines, and tacrine and (−)-huperzine A, as reference compounds. The reversion of the neuromuscular blockade for most of the hybrid compounds and tacrine is also shown. Most of the new compounds (rac-19, rac-24, rac-26, and rac-27) and rac-20 and rac-30, whose syntheses were previously reported, are clearly more active than tacrine and (−)-huperzine A as bovine AChE inhibitors. As previously observed in this series of compounds, the levorotatory enantiomers [(−)-20, (−)-26, and (−)-30] are more active than the corresponding racemic mixtures, while the dextrorotatory enantiomers are clearly less potent. With the exception of rac-29, which is the sole compound substituted at position 2, the rest of compounds (rac-25, rac-28, and rac-31) are slightly more potent than (−)-huperzine A. In contrast to tacrine and (−)-huperzine A, which are less active toward human than bovine AChE, rac-20, (−)-20, rac-30, (−)-30, and rac-31 are more potent toward human AChE. Regarding the BChE inhibitory activity, tacrine is 5-fold more active toward human BChE than toward human AChE, while (−)-huperzine A is highly selective for human AChE. Among the huprines, rac-19, rac-20, (−)-20, (−)-30, and rac-31 showed a high selectivity for human AChE, while the rest of the new hybrid compounds showed an intermediate selectivity for this enzyme.

The results in Table 1 for the bovine AChE inhibitory activity allow us to conclude that: (a) with the exception of rac-25 (1-F,9-Et), for a given substituent (Me or Et) at position 9 the monosubstituted (Cl, F, or Me) derivatives at position 1 or 3 are more active than their parent compounds (e.g., rac-20 (3-Cl,9-Me), rac-18 (3-F,9-Me), and rac-16 (3-Me,9-Me) are 15.4-, 7.6-, and 5.2-fold more active than rac-15 (3-H,9-Me), respectively, and rac-30...
(3-Cl,9-Et), rac-26 (3-F,9-Et), and rac-23 (3-Me,9-Et) are 13.9-, 5.2-, and 3.2-fold more active than rac-21 (3-H,9-Et), respectively; (b) the AChE inhibitory activity varies in the order Cl > F > Me [e.g., rac-20 (3-Cl,9-Me) is 2.0- and 2.9-fold more active than rac-18 (3-F,9-Me) and rac-16 (3-Me,9-Me), respectively, while rac-30 (3-Cl,9-Et) is 2.7- and 4.3-fold more active than rac-26 (3-F,9-Et) and rac-23 (3-Me,9-Et), respectively]; (c) for a given substituent at the benzene ring, the derivative substituted at position 3 is more active than that substituted at position 1 [e.g., rac-30 (3-Cl,9-Et) is 5.8-fold more active than rac-28 (1-Cl,9-Et), rac-26 (3-F,9-Et) is 6.3-fold more active than rac-25 (1-F,9-Et), rac-23 (3-Me,9-Et) is 2.5-fold more active than rac-22 (1-Me,9-Et), and rac-18 (3-F,9-Me) is 3.7-fold more active than rac-17 (1-F,9-Me)]; (d) substitution at position 2 leads to less active compounds [e.g., rac-29 (2-Cl,9-Et) is 6.7-fold less active than the parent compound rac-21 (2-H,9-Et)]; (e) for the 1,3-difluoro and 1,3-dimethyl derivatives the effect of the substitutions on the AChE inhibitory activity is roughly additive; (f) in all cases, the levorotatory enantiomers are the eutomers (e.g., (−)- rac-20 (3-Cl,9-Me), (−)- rac-26 (3-F,9-Et), and (−)- rac-30 (3-Cl,9-Et) are 3.8-, 1.1-, and 2.1-fold more active than their racemic mixtures, while (+)- rac-20, (+)- rac-26, and (+)- rac-30 are 8.5-, 18.8-, and 145-fold less active than their racemic mixtures. These values are only indicative since no corrections were made to take into account the ee’s of the enantiomeric compounds. The above results for the racemic compounds parallel those reported by Kawakami et al. for other tacrine derivatives.

The results in Table 1 also indicate that, in general, the more active compounds toward bovine AChE show an increased activity toward human AChE, showing subnanomolar IC50 values in several cases [e.g., rac-20, (−)- rac-20, rac-26, (−)- rac-26, rac-30, (−)- rac-30, and rac-31 exhibit human AChE inhibitory activities 2–28.5-fold higher than for the bovine enzyme], whereas (−)-huperzine A and tacrine exhibit higher inhibitory activity toward bovine AChE.

Likewise, although it is not fully established that the selectivity in inhibiting AChE vs BChE results in low peripheral cholinergic effects in AD patients, it is worth noting that these compounds, particularly rac-19, (−)- rac-30, and rac-31, are 438–871-fold more active inhibiting human AChE than human BChE, being much more selective than tacrine (Table 1).

Several of these compounds have been submitted to neuromuscular studies. Most of the tested compounds are more potent than tacrine in reversing the neuromuscular blockade induced by d-tubocurarine (Table 1). The most and the least active compounds among the new tested huprines are (−)- rac-20 and (−)- rac-30, which are 2269- and 249-fold more active than tacrine, respectively. Among all of the hybrids so far prepared, only rac-18 (AI50 = 8.0 nM) is more active than (−)- rac-20 in reversing the neuromuscular blockade. The discrepancy observed between the AChE inhibitory potency of racemates and enantiomers and the reversion of the neuromuscular blockade was discussed in a preceding paper.

Inspection of the data in Table 2 shows that the inhibitory activity is affected by incubation of the enzyme with the inhibitor before addition of substrate. Worthy of note, the 3-chloro derivatives (−)- rac-20 and (−)- rac-30 showed IC50 values much lower after incubation of the AChE with the inhibitor for 30 min than when AChE activity was measured without previous incubation.

To determine the reversibility of the inhibition process, AChE was incubated for 30 min at 25°C with enough quantity of either (−)- rac-20 or (−)- rac-30 to give approximately 60% inhibition in each case and the samples were then dialyzed. Activity was not fully recovered after dialysis overnight at 4°C. This fact and the high potency exhibited by these compounds indicated that they could behave as tight-binding inhibitors. To check this possibility, enzyme–inhibitor mixtures were dialyzed at 37°C for 18 h. This resulted in complete recovery of the activity, confirming that a reversible tight-binding inhibitory process was involved (Figure 2). The preceding results indicate that the AChE inhibitory activity is affected by the incubation time, specially for the eutomers (−)- rac-20 and (−)- rac-30, this effect being likely due to a low dissociation constant of the corresponding AChE–inhibitor complex.

The ex vivo AChE inhibitory activity in mouse brain was determined for the more active compounds. Only one dose (10 μmol kg−1, ip) of the selected compounds was administered 20 min before the animals were sacrificed, and the percentage of AChE inhibition vs control was measured. Results in Table 3 show that huprines cross the blood–brain barrier. Both rac-20 and rac-30 are significantly more active than rac-25 and rac-
Table 3. Ex Vivo AChE Inhibitory Activity of Several Hydrochlorides of Huprines*  

<table>
<thead>
<tr>
<th>compd</th>
<th>% inhibition over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>rac-20</td>
<td>59.2 ± 8.2</td>
</tr>
<tr>
<td>(-)-20 (99% ee)</td>
<td>97.0 ± 0.6</td>
</tr>
<tr>
<td>(+)-20 (87% ee)</td>
<td>23.4 ± 1.2</td>
</tr>
<tr>
<td>rac-25</td>
<td>3.6 ± 1.0</td>
</tr>
<tr>
<td>rac-26</td>
<td>10.4 ± 4.3</td>
</tr>
<tr>
<td>rac-30</td>
<td>60.3 ± 7.3</td>
</tr>
<tr>
<td>(-)-30 (99% ee)</td>
<td>76.6 ± 3.8</td>
</tr>
<tr>
<td>(+)-30 (97% ee)</td>
<td>4.8 ± 1.2</td>
</tr>
<tr>
<td>tacrine</td>
<td>0</td>
</tr>
</tbody>
</table>

*In all cases, 10 μmol kg⁻¹ was used and animals were sacrificed 20 min posttreatment. The results are the percentage of inhibition of brain AChE activity of drug-treated mice vs untreated controls. Each value is the mean ± standard error of at least six animals.

Figure 3. Thermodynamic cycles used in free energy calculations to determine relative binding affinities between the inhibitors. 26 in this test. The levorotatory enantiomers are more active than the racemic mixtures, while the dextrorotatory enantiomers are much less active. In fact, the marked inhibitory activity of (-)-20 and (-)-30 supports the tight-binding character of these compounds. In contrast, tacrine does not show activity at the doses used, but this might be due to dissociation of the reversible enzyme–inhibitor complex owing to the necessary dilution of the tissue sample. The preceding results point out that (-)-20 and (-)-30 have a set of valuable pharmacological properties (very high human AChE inhibitory activity, high human AChE vs BChE selectivity, tight-binding nature, reversibility, ability to cross the blood–brain barrier, and high activity in reversing the neuromuscular blockade induced by d-tubocurarine), which make them suitable candidates as potential therapeutic agents for the treatment of AD.

Molecular Modeling Studies. Free energy calculations were performed (Figure 3) to determine the effect of the introduction of a chlorine atom at position 3 and of the methyl → ethyl replacement at position 9 on the binding of the huprine inhibitors to AChE. These substitutions involve contacts of the drug in two distant regions of the binding pocket, and the calculations allow us to examine the additivity of those structural changes on the binding affinities. The free energy profiles for the mutations in water and in the protein (Figure 3) varied smoothly, and no discontinuities were found. The free energy differences determined in the first (equilibration) and second (collection) halves of each window were almost identical. Finally, the thermodynamic cycles for mutations 3-H,9-Me → 3-F,9-Me → 3-F,9-Et → 3-H,9-Et and 3-F,9-Me → 3-Cl,9-Me → 3-Cl,9-Et → 3-F,9-Et in water and in the protein were closed with an error lower than 0.3 kcal mol⁻¹. Overall, all these analyses give us confidence in the computed relative binding affinities between inhibitors.

The predicted differences in binding free energy for conversion of the 3-F,9-Me and 3-F,9-Et compounds into their 3-chloro derivatives are given in Figure 4, which also shows the values for the corresponding Me → Et mutations. The results indicate that the effect of the substitutions at positions 3 and 9 on the binding affinity is largely additive, indicating that the active site can accommodate such structural changes without disturbing the binding. Replacement of F by Cl improves the binding to AChE by 1.0–1.2 kcal mol⁻¹ (Figure 4). This effect mainly arises from a better fit of the chlorine atom in the pocket formed by residues Leu333, Met436, Ile439, and Trp432, leading to an increase in the nonpolar/electrostatic interaction relative to the fluoro derivatives. Likewise, the methyl → ethyl mutation increases the drug binding by 0.5–0.6 kcal mol⁻¹ (Figure 4). In this case, the effect is largely due to a better desolvation of the ethyl group. Overall, the 3-chloro derivatives are predicted to bind AChE around 3.9 kcal mol⁻¹ better than the parent compound, which agrees with the free energy difference of 2.2 kcal mol⁻¹ estimated from the experimental bovine AChE inhibitory data.

A series of 1-ns molecular dynamics (MD) simulations was performed to explore the structural and energetic fluctuations of the AChE complexes with the 3-chloro derivatives. Table 4 gives the values of selected structural and energetic properties averaged from 100 structures collected during the last 0.5 ns. Inspection of Table 4 shows the lack of relevant structural alterations both in the mobile part and in the subset of residues forming the binding site. The pattern of interactions that define the proposed binding mode22 is maintained along all the simulation for the chloro derivatives, as noted in the geometrical and energetic parameters taken from the interaction with selected residues. All of these results reinforce our confidence in the suitability of the binding mode proposed to explain the binding of huprines with AChE.

Free energy calculations were also performed to explore how the mutation Phe330 → Tyr, the corresponding residue in human AChE, may influence the affinity of the chloro derivatives. Figure 5 shows the computed free energy differences for the Phe330 → Tyr mutation in the isolated enzyme and in the AChE–drug complexes. Such a mutation is expected to stabilize around 0.3–0.4 kcal mol⁻¹ the binding of the inhibitors to the human enzyme relative to binding to TcAChE.
This can be seen in Table 4, which gives selected average structural and energetic parameters for the Phe330 → Tyr AChE complexes with the parent compound and its 3-chloro derivatives. The analysis of the structures showed that the hydroxyl group has no direct interaction with the drug, since it is solvated on average by one water molecule lying at around 3 Å along all the simulations. Indeed, results in Table 4 show that the interaction of the inhibitor with Tyr330 is slightly more favorable (around 0.7 kcal mol⁻¹) than with Phe330, as noted in the results of the free energy calculations (Figure 5).

In summary, the whole of results reflects the changes in the available experimental data originated by attachment of chlorine atoms at position 3 of the huprine derivatives. In all cases the enzyme–drug complex shows no relevant structural fluctuations and the pattern of interactions is fully preserved along the simulation. Indeed, the results determined for the Phe330 → Tyr mutation indicate that this change is expected to enhance slightly the binding to the enzyme. Overall, the results support the validity of the putative binding model for huprines to AChE, which gives a valuable basis to continue our current efforts in developing AChE inhibitors with improved inhibitory activity.

**Conclusion**

The AChE inhibitory activity of a series of huprines of general structure 1, having an Et or Me group at position 9, increases on substitution at positions 1 and/or 3 (benzene ring) with Cl, F, and/or Me substituents. The more active derivatives are those substituted at position 3 with a Cl atom (20 and 30). In the whole series, the levorotatory enantiomers are much more active than the dextrorotatory enantiomers. Several huprines and especially (−)−20 and (−)−30 proved to be more active toward human than bovine AChE. They are quite selective inhibiting human AChE vs human BChE, which may be indicative of reduced peripheral effects. Also, the tight-binding nature of the above two inhibitors has been evidenced by the dependence of their activity on the time of incubation of the enzyme with the inhibitor, although both of them have shown to be reversible inhibitors able to cross the blood–brain barrier.

**Table 4.** Selected Structural and Energetic Details for the Complexes of T. californica and the Mutated Phe330 → Tyr AChE with the Levorotatory Enantiomers of the Parent Compound (−)−15 and the Chloro Derivatives (−)−20 and (−)−30

<table>
<thead>
<tr>
<th>Property</th>
<th>(−)−15</th>
<th>(−)−20</th>
<th>(−)−30</th>
</tr>
</thead>
<tbody>
<tr>
<td>rmsd (all)</td>
<td>0.9 (±0.1)</td>
<td>0.7 (±0.1)</td>
<td>0.7 (±0.1)</td>
</tr>
<tr>
<td>rmsd (subset)</td>
<td>0.9 (±0.1)</td>
<td>0.7 (±0.1)</td>
<td>0.7 (±0.1)</td>
</tr>
<tr>
<td>d drug–Trp84</td>
<td>4.0 (±0.5)</td>
<td>4.1 (±0.5)</td>
<td>4.0 (±0.5)</td>
</tr>
<tr>
<td>α drug–Trp84</td>
<td>11.8 (±4.7)</td>
<td>11.8 (±4.7)</td>
<td>11.4 (±4.5)</td>
</tr>
<tr>
<td>d drug–Phe330(Tyr)</td>
<td>3.9 (±0.5)</td>
<td>3.8 (±0.4)</td>
<td>4.0 (±0.5)</td>
</tr>
<tr>
<td>α drug–Phe330(Tyr)</td>
<td>13.8 (±7.0)</td>
<td>10.4 (±5.3)</td>
<td>11.7 (±5.8)</td>
</tr>
<tr>
<td>d NH–OC(His440)</td>
<td>2.9 (±0.1)</td>
<td>2.9 (±0.1)</td>
<td>2.9 (±0.1)</td>
</tr>
<tr>
<td>d NH₂–O₁(Asp72)</td>
<td>5.0 (±0.6)</td>
<td>4.7 (±0.4)</td>
<td>4.6 (±0.4)</td>
</tr>
<tr>
<td>d NH₂–O₂(Asp72)</td>
<td>6.2 (±0.7)</td>
<td>5.6 (±0.5)</td>
<td>5.5 (±0.5)</td>
</tr>
<tr>
<td>d NH₂–OH(Tyr330)</td>
<td>3.4 (±0.3)</td>
<td>3.5 (±0.3)</td>
<td>3.5 (±0.3)</td>
</tr>
</tbody>
</table>

E₁ | −10.8 (±1.3) | −10.6 (±1.4) | −10.0 (±1.4) |
| E₂ | −5.4 (±0.9) | −6.7 (±1.0) | −6.9 (±0.9) |
| E₃ | −47.0 (±3.9) | −50.8 (±2.6) | −51.3 (±2.8) |
| E₄ | −17.3 (±1.1) | −18.0 (±1.1) | −18.0 (±1.2) |

Figure 5. Changes in the binding free energy (kcal mol⁻¹) of the (−)−enantiomer of the huprines (−)−15 (3-H,9-Me), (−)−20 (3-Cl,9-Me), and (−)−30 (3-Cl,9-Et) arising from mutation of Phe330 in TaChE to the corresponding Tyr residue in the human enzyme.

(Figure 5). Accordingly, the relative binding affinities of the inhibitors for the mutated enzyme are expected to be slightly higher than the values computed for the T. californica enzyme. This suggests that the Phe330 → Tyr mutation does not alter remarkably the interaction pattern with the drug, and particularly that the hydroxyl group does not participate directly in drug binding.

To further corroborate these conclusions, a series of 1-ns MD simulations were conducted for the Phe330 → Tyr mutated enzyme complexed with the 3-chloro derivatives. Again, the analysis of 100 structures collected during the last 0.5 ns showed no significant structural or energetic alteration, and the inhibitor remained firmly bound to the binding site of the enzyme maintaining the pattern of interactions with neighboring residues. This can be seen in Table 4, which gives
New Tacrine–Huperzine A Hybrids (Huprines)

barrier. Moreover, all of these huprines proved to be much more active than tacrine in reversing the neuromuscular blockade induced by d-tubocurarine. The molecular modeling studies of the complexes of (−)-huprines having a chlorine atom at position 3 and an Et group at position 9 with TcAChE and the Phe330→Tyr mutated enzyme using the previously proposed model are in reasonable agreement with the experimental activity values. Despite the agreement with the experimental data, the definite validation of the binding model has to await a 3D X-ray structure of the complex.

Altogether, huprines (−)-20 and (−)-30 are very potent human AChE inhibitors. In conjunction with their AChE vs BChE selectivity, tight-binding but reversible character, and ability to reverse the neuromuscular blockade induced by d-tubocurarine, these findings make them very interesting candidates for further studies in connection with their possible use in the treatment of AD.

Experimental Section

Chemistry. General Methods. Melting points were determined in open capillary tubes with a MFB 595010M Gallenkamp melting point apparatus. H NMR spectra were recorded at 500 MHz on a Varian VXR 500 spectrometer, and 13C NMR spectra were recorded at 75.4 MHz on a Varian Gemini 300 spectrometer. The chemical shifts are reported in ppm (δ scale) relative to internal TMS, and coupling constants are reported in hertz (Hz). COSY H/H experiments were performed using standard procedures, while COSY H/13C were performed using the HMQC sequence with an indirect detection probe. For the 13C and 1H NMR data of the new huprines, see Tables 1 and 2, respectively, of Supporting Information.

IR spectra were run on a FT/IR Perkin-Elmer model 1600 spectrophotometer. Absorption values are expressed as wave numbers (cm⁻¹). Optical rotations were measured on a Perkin-Elmer model 241 polarimeter. The specific rotation has not been corrected for the presence of solvent of crystallization. Chiral HPLC analyses were performed on a Waters model 600 liquid chromatograph provided with a Waters model 486 variable λ detector, using a CHIRALCEL OD-H column (25 × 0.46 cm) containing the chiral stationary phase cellulose tris-(3,5-dimethylphenylcarbamate). Conditions A: mixture of hexane/EtOH/Et2O/NH4Cl in the ratio of 90:10:0.1 as eluent, flow 0.20 mL min⁻¹, λ = 235 nm. Chiral medium-pressure liquid chromatography (chiral MPLC) separations were carried out on an equipment which consisted of a pump (Buchi 688), a variable λ UV detector (Buchi), and a column (25 × 2.5 cm) containing microcrystalline cellulose triacetate (15–25 μm) as the chiral stationary phase. Column chromatography was performed on silica gel 60 ACC. (70–200 mesh, SDS, ref 21000027). For the TLC, aluminum-backed sheets with silica gel 60 F 254 (Merck, ref 05554) were used. AlCl3 was purchased from Aldrich. Analytical grade solvents were used for recrystallizations, while pure for synthesis solvents were used in all manipulations. Column chromatography. Pure-for-synthesis 1,2-dichloroethane and 1,2-dibromoethane were also used. Aminobenzonitrile 8 was purchased from ABCR while 7, 9, 10, 11, and 12 were prepared according to literature procedures. Elemental analyses were carried out at the Mycrolaboratory Service of the Centro de Investigación y Desarrollo, C.I.D., Barcelona, Spain, and are within ±0.4% of the theoretical values.

General Procedure for the Preparation of rac-Huprines from Enones rac-4 and 2-Aminobenzonitriles 7–12 and 14. To a suspension of anhydrous AlCl3 (1.8 mmol) and 2-aminobenzonitrile 7–12 or 14 (1.3 mmol) in 1,2-dichloroethane (2 mL) was added a solution of enone rac-4a or rac-4b (1 mmol) in 1,2-dichloroethane (10 mL) dropwise. The reaction mixture was stirred under reflux for 7 h, allowed to cool to room temperature, diluted with water (8 mL) and THF (12 mL), made basic by addition of 5 N NaOH, and stirred at room temperature for 30 min. The organic solvents were removed under reduced pressure, and the residue was filtered. The solid residue was submitted to column chromatography [silica gel (13 g), hexane/AcOEt/MeOH mixtures] to give the rac-huprine. A solution of this compound in MeOH was treated with a solution of HCl in Et2O or in MeOH (3 equiv), and the solvent was evaporated to give the corresponding hydrochloride, which was recrystallized from water.

rac-12-Amino-3,5,7,11-tetrahydro-9-methyl-7,11-methanocycloocta[b]quinoline Hydrochloride (rac-19 HCl). This compound was prepared according to the procedure described above, but using 1.1 equiv of AlCl3 and 0.8 equiv of aminobenzonitrile 10. The alkaline mixture was concentrated in vacuo, diluted with water and extracted with AcOEt. The combined organic extracts were dried over Na2SO4 and evaporated under reduced pressure to give a solid residue which was submitted to column chromatography. On elution with hexane/AcOEt 80:20, slightly impure rac 19 (110 mg) was obtained as a yellow solid which was again submitted to column chromatography [silica gel (11 g), hexane/Et2O, gradient elution]. On elution with hexane/ Et2O 75:25, pure rac-19 (60 mg, 30% yield) was isolated. Subsequent treatment with a solution of HCl (0.38 N solution in Et2O, 3 equiv), evaporation and recrystallization of the resulting solid from acetonitrile afforded rac-19 HCl·2H2O (13% overall yield): mp > 300 °C dec; IR 3500, 1600, 1590, 1580, 1538, 1550, 1480, 1450, 1315, 1270, 1230, 1190, 1170, 1150, 1040, 880, 790, 685, 500, 298, 2882, 2859, 2704, 2681) (CH, NH, NH+). 1H NMR spectra were recorded at 300 MHz on a Varian Gemini 500 spectrometer. Absorption values are expressed as wave numbers (cm⁻¹). Optical rotations were measured on a Perkin-Elmer model 241 polarimeter. The specific rotation has not been corrected for the presence of solvent of crystallization.

Chiral HPLC analyses were performed on a Waters model 600 liquid chromatograph provided with a Waters model 486 variable λ detector, using a CHIRALCEL OD-H column (25 × 0.46 cm) containing the chiral stationary phase cellulose tris-(3,5-dimethylphenylcarbamate). Conditions A: mixture of hexane/EtOH/Et2O/NH4Cl in the ratio of 90:10:0.1 as eluent, flow 0.20 mL min⁻¹, λ = 235 nm. Chiral medium-pressure liquid chromatography (chiral MPLC) separations were carried out on an equipment which consisted of a pump (Buchi 688), a variable λ UV detector (Buchi), and a column (25 × 2.5 cm) containing microcrystalline cellulose triacetate (15–25 μm) as the chiral stationary phase. Column chromatography was performed on silica gel 60 ACC. (70–200 mesh, SDS, ref 21000027). For the TLC, aluminum-backed sheets with silica gel 60 F 254 (Merck, ref 05554) were used. AlCl3 was purchased from Aldrich. Analytical grade solvents were used for recrystallizations, while pure for synthesis solvents were used in all manipulations. Column chromatography. Pure-for-synthesis 1,2-dichloroethane and 1,2-dibromoethane were also used. Aminobenzonitrile 8 was purchased from ABCR while 7, 9, 10, 11, and 12 were prepared according to literature procedures. Elemental analyses were carried out at the Mycrolaboratory Service of the Centro de Investigación y Desarrollo, C.I.D., Barcelona, Spain, and are within ±0.4% of the theoretical values.

Preparative Resolution of rac-26 by Chiral MPLC: (−)-(7R,11R)-26 and (−)-(7S,11S)-26. The chromatographic resolution of rac-26 was carried out by using the above-described MPLC eluting the racemical sample with a 0.1% solution of Et3N in EtOH. The sample of rac-26 (1.00 g) was introduced as free base in 2 portions (1 × 100 mg + 1 × 900 mg) using 96% Et3N (2 mL min⁻¹) as the sole eluent and solvent. The chromatographic fractions (5 mL) were analyzed by chiral HPLC under conditions A (tR = 28.66 min, k′ = 0.81; tR = 32.19 min, k′ = 1.03, α = 73).
A solution of (–)\textsuperscript{26} (235 mg, 95% ee) and (+)\textsuperscript{26} (200 mg, 94% ee) were obtained. The remaining product consisted of mixtures of both enantiomers with lower ee.

A solution of (–)\textsuperscript{26} (235 mg, 95% ee) in MeOH (10 mL) was treated with excess 0.77 N HCl in Et\textsubscript{2}O (10 mL) and the obtained residue (255 mg) was recrystallized from AcOEt/MeOH 4:1 afforded almost pure rac-\textsuperscript{27} (\(R\), \(S\))\textsuperscript{-}271 °C dec; IR 3600–2900 cm\textsuperscript{-1} (\textit{Eu} \(\text{Cl}_2\)). This compound was prepared as described for rac-\textsuperscript{26}.

Similarly, from (+)\textsuperscript{26} (200 mg, 94% ee), (+)\textsuperscript{26} HCl (0.38 N solution in Et\textsubscript{2}O, 3 equiv), evaporation and recrystallization of the resulting solid from AcOEt/MeOH 4:1 afforded almost pure rac-\textsuperscript{28} (CH, NH, NH\textsuperscript{+}), 95% ee by chiral HPLC on the liberated base.

**Time Dependence and Reversibility of Bovine AChE Inhibitory Activity.** The time dependence of the inhibitory process was determined for several of the more active compounds with at least 12 increasing inhibitor concentrations after an incubation period of 30 min. After this time the AChE inhibitory activity was assayed by the method of Ellman. In this set of experiments, the appearance of product was monitored at 412 nm in a Perkin-Elmer Lambda 2 spectrophotometer equipped with an automatic six-cell changer, in the absence and presence of different inhibitor concentrations.

**Ex Vivo AChE Inhibitory Activity.** Groups of 6 or 1 mice were treated with each compound at 10 nmol kg\textsuperscript{-1} ip. The animals were sacrificed 30 min later by cervical dislocation and frozen on dry ice. Residual AChE activities were determined as previously described by the method of Ellman using brain homogenate preparations as a source of the enzyme. Percent of inhibitions was calculated by comparing AChE activity in brain of drug-treated mice with activity from untreated controls.

**Neuromuscular Studies.** Right and left phrenic nerve-hemi-diaphragms were removed from male Sprague–Dawley rats (250–300 g) and placed in a Crouzet chamber and kept at 30 °C in a humidified atmosphere of 95% \textit{N}_2 and 5% \textit{O}_2. After a 30-min equilibration period, the hemidiaphragms were exposed to various test drugs for 30 min. At the end of each test period, the amount of choline liberated was measured and expressed as percentage of the control.

**Biochemical Studies.** AChE inhibitory activity was evaluated spectrophotometrically at 25 °C by the method of Ellman, using AChE from bovine erythrocytes and acetylthiocholine iodide (0.53 mM) as substrate.
achieved. The effect of each drug was evaluated after 15 min of exposure. To avoid the possible carry-over effects, only one concentration of inhibitor was tested on each preparation. Several drug concentrations were evaluated for each AChE inhibitor. To quantify the reversal effect of each drug, the antagonism index (AI or % of antagonism) was determined for each concentration and the AI% (drug concentration that gives a 50% value of AI) was calculated by nonlinear regression analysis. The drug purchased from Sigma.

Molecular Modeling: Methods. The simulation system was based on the structure of the complex between TeAChE and the hybrid compounds (−)−18 (3-fluoro-9-methyl-substituted) and (−)−26 (3-fluoro-9-ethyl-substituted). This structure has been determined from molecular modeling studies, and its definite validation must await a 3D X-ray structure of the complex. However, the results obtained up to now agree with the available experimental evidence. The enzyme was modeled in its physiological active form with neutral His440 and deprotonated Glu327, which together with Ser200 form the catalytic triad. The standard ionization state at neutral pH was considered for the rest of ionizable residues with the exception of Asp392 and Glu443, which were neutral, and His471, which was protonated, according to previous numerical titration studies. The geometry of the chloro derivatives (−)−20 and (−)−30 was fully optimized at the Hartree–Fock level with the 6-31G(d) basis set using the Gaussian-94 program. According to the pK_a = 8.9, measured for (−)−30, which must be very similar to those of the other huperzines of this work, the protonated species of the hybrid compounds was considered in calculations.

Free energy calculations were performed to predict the relative binding affinities of the hybrid 3-chloro derivatives. To this end, we investigated the effect of the changes fluoride → chlorine at position 3 and methyl → ethyl at position 9 on the binding free energy. Indeed, we examined the effect of mutating Phe330 in TeAChE to Tyr, which is the corresponding residue in human AChE, on the binding affinities of the 3-chloro derivatives. Such a mutation is the only relevant difference concerning the residues directly involved in the binding pocket between T. californica and human enzymes. This simulation allows us to determine which changes in the binding affinity can be due to the use of the protein structure of a different organism.

Free energy calculations were performed using thermodynamic integration (TI) coupled to molecular dynamics (MD) for sampling of the AChE−drug configurational space following the standard algorithm implemented in AMBER-5. The starting structures in TI-MD calculations were the equilibrated active forms of the 3-fluoro derivatives determined in our previous study, which consist of the protein, the inhibitor, and a cap of 670 TIP3P water molecules centered in the force-field. The van der Waals parameters for chlorine at position 3 and methyl at position 9 on the carboxylate group were taken from those defined for related atoms in the OPLS force field. SHAKE was used to maintain all the bonds at their equilibrium distances, which allowed to use an integration time step of 2 fs. A cutoff of 11 Å was used for nonbonded interactions. Calculations were performed using the AMBER5 computer program.

To analyze the structural and energetic features of the interaction between the drugs and the T. californica or mutated enzyme, a series of 1-ns MD simulations were performed using the same technical features already noted and the final structures obtained from the TI-MD calculations. The trajectory was stored every 1 ps for subsequent analysis of complexes.

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Supporting Information Available: Tables of 1^C and 1^H NMR data of the hydrochlorides of rac 19, rac 24–rac 29, and rac 31 (Tables 1 and 2), elemental analyses of all new compounds (Table 3), and bonded and nonbonded parameters for the chlorine atom in the huperzines (−)−20 and (−)−30 (Table 4). This material is available free of charge via the Internet at http://pubs.acs.org.

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