Pyra[3,2-c]quinoline–6-Chlorotacrine Hybrids as a Novel Family of Acetylcholinesterase- and β-Amyloid-Directed Anti-Alzheimer Compounds

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Two isomeric series of dual binding site acetylcholinesterase (AChE) inhibitors have been designed, synthesized, and tested for their ability to inhibit AChE, butyrylcholinesterase, AChE-induced and self-induced β-amyloid (Aβ) aggregation, and β-secretase (BACE-1) and to cross blood–brain barrier. The new hybrids consist of a unit of 6-chlorotacrine and a multicomponent reaction-derived pyrano[3,2-c]quinoline scaffold as the active-site and peripheral-site interacting moieties, respectively, connected through an oligomethylene linker containing an amido group at variable position. Indeed, molecular modeling and kinetic studies have confirmed the dual site binding of these compounds. The new hybrids, and particularly 27, retain the potent and selective human AChE inhibitory activity of the parent 6-chlorotacrine while exhibiting a significant in vitro inhibitory activity toward the AChE-induced and self-induced Aβ aggregation and toward BACE-1, as well as ability to enter the central nervous system, which makes them promising anti-Alzheimer lead compounds.

Introduction

In the past decade, the design of novel classes of inhibitors of the enzyme acetylcholinesterase (AChE) as therapeutic interventions for Alzheimer’s disease (AD) has been mostly driven by the pivotal finding that AChE can bind the β-amyloid peptide (Aβ), thereby promoting Aβ aggregation as an early event in the neurodegenerative cascade of AD.1,2 The Aβ proaggregating effect of AChE results in cognitive impairment in doubly transgenic mice expressing human amyloid precursor protein (APP) and human AChE.3,4 Blockade of the peripheral site of AChE, the Aβ recognition zone within the enzyme,5 was therefore expected to affect the AChE-induced Aβ aggregation and could be a potential strategy to modulate the progression of AD.

On the basis of these premises, novel classes of AChE inhibitors (AChEIs) targeting the peripheral site have emerged as promising disease-modifying anti-Alzheimer drug candidates.6 Of particular interest are those AChEIs able to simultaneously bind to both peripheral and catalytic sites, which are separated by about 14 Å, as they are located at the mouth and at the bottom of the gorge leading to the active site.7 Apart from the Aβ antiaggregating effects arising from blockade of the peripheral site, dual binding site AChEIs are usually endowed with a potent AChE inhibitory activity because of the increased number of drug–target interactions, thus overcoming the low activity of selective peripheral site AChEIs.8–18 Indeed, in vitro inhibitory activities of AChE and AChE-induced Aβ aggregation have been reported for different families of dual binding site AChEIs,19–24 which in cases such as memquin25,26 and NP-6125 have been shown to reduce brain amyloid burden and increase cognition in animal models of AD. Some dual binding site AChEIs such as memquin26,27 or bis(7)-tacrine3,6,13,36,37 are undergoing preclinical evaluation, while NP-61 entered phase I clinical trials for AD in the U.K. in April 2007.38 Prompted by these results and the tremendous potential of dual binding site AChEIs to impact both the course of AD and its symptomatology, the design and synthesis of novel families of dual binding site AChEIs have been actively pursued in the past years.28,29 The design of dual binding site AChEIs is carried out by linking through a tether of suitable length an active-site interacting unit, usually derived from a known active site AChE, with a peripheral-site interacting unit suited to interact with Trp286 (human AChE (hAChE) numbering), the
characteristic residue of the peripheral site. With a few exceptions in which a peripheral-site interacting unit containing an aliphatic amine, protonated at physiological pH, or a quaternary ammonium group establishes cation−π interactions with Trp286, in most cases the peripheral-site interacting unit contains aromatic moieties able to establish π−π stacking interactions with Trp286, in some cases reinforced by concomitant cation−π interactions due to the presence of protonatable or quaternary nitrogen atoms in the aromatic system. The prototype of peripheral site AChEi is propidium (1, Chart 1), which binds the AChE peripheral site in two orientations related by a flip of 180° around the phenanthridinium pseudosymmetry axis.53,54 The driving force for the binding of 1 to the peripheral site is the π−π stacking, reinforced by cation−π interactions, between the phenanthridinium moiety and Trp286, which is supplemented by a hydrogen bond between one of the aromatic amino groups and His287.

Some of us recently reported the synthesis of the multicomponent reaction-derived pyrano[3,2-c]quinoline scaffold 2 (Chart 1).55 We thought that a 5-phenyl-substituted derivative thereof would resemble the 6-phenylphenanthridinium moiety of propidium and could serve as the peripheral-site interacting unit of a novel family of dual binding site AChEIs. Because the nitrogen atom of this tricyclic moiety is not expected to be protonated at physiological pH (Table S1, Supporting Information), this aromatic system should establish π−π stacking with Trp286. Noteworthy, the neutral character of this moiety could result in a better penetration into the central nervous system (CNS).

Herein, we describe the synthesis, pharmacological evaluation, and molecular modeling of a novel family of potent dual binding site AChEIs that combine a 5-phenylpyrano[3,2-c]quinoline moiety with 6-chlorotacrine, 3 (Chart 1), a potent AChEI already used in other dual binding site AChEIs,22,23,31,32,39,44,52,56,57 through an amido-containing oligomethylene linker. The pharmacological evaluation of these novel compounds includes AChE and butyrylcholinesterase (BChE) inhibition, as well as inhibition of the AChE- and self-induced Aβ aggregation and inhibition of β-secretase (BACE-1), which altogether comprises an interesting set of effects shared by some dual binding site AChEIs. To prove the starting hypothesis on a good blood–brain barrier (BBB) permeability, the brain penetration of the novel hybrids has been assessed using an artificial membrane assay.

Chemistry

The structures of the novel 5-phenylpyrano[3,2-c]quinoline–6-chlorotacrine hybrids 18–27 are shown in Scheme 2. Alignment of the 5-phenylpyrano[3,2-c]quinoline system with the phenanthridinium moiety of propidium in its complex with mouse AChE55 (Figure S1, Supporting Information) revealed that if the novel hybrids were to interact with AChE by placing the tricyclic system in a way similar to that of the tricyclic system of propidium, position 9 of the pyrano[3,2-c]quinoline system would be suitable for attachment of the linker, which from this point could snake down the active site gorge. An ester group at position 9 was chosen as a suitable functionalization to allow attachment of the linker by reaction with an appropriate aminoalcohol tetracrine. Thus, tricyclic ester 11 (Scheme 1) was designed as the precursor of the peripheral-site interacting unit of the novel hybrids 18–22 (Scheme 2).

Recently, Martínez and co-workers developed a novel series of indole–tacrine hybrids as dual binding site AChEIs, which contained an amido group within the linker either directly bound to the indole system (i.e., the peripheral-site interacting unit) or separated by one to three atoms (methylene groups in most cases), and reported important differences in the AChE inhibitory activity upon shift of the amido group within the tether chain while keeping the same total length of the linker.23 Specifically, separation of the amido from the indole ring by two methylene groups increased up to 2300-fold the AChE inhibitory potency relative to the indole directly bound counterpart. In view of these results, we designed the tricyclic ester 12 (Scheme 1) as the precursor of the parallel series of hybrids 23–27 (Scheme 2), containing linkers of the same total length as 18–22 but with the amido group shifted two positions within the tether. Regarding the length of the linker, oligomethylene chains of 6–10 and 4–8 members for hybrids 18–22 and 23–27, respectively, were considered suitable to provide the dual site binding.
The synthesis of hybrids 18–27 was envisaged through the coupling of tricyclic esters 11 and 12 with readily available aminoalkyltacrines 17. A straightforward access to 11 and 12 was carried out through a Povarov multicomponent reaction. Thus, reaction of 3,4-dihydro-2H-pyran-4-carboxylic acid with ethyl p-amino benzoate, 5, and p-chlorobenzaldehyde, 7, yielded the desired tricyclic ester 11 in 75% yield. The Povarov reaction of methyl 3-(4-aminophenyl)propanoate, 4, and benzaldehyde under Y(OTf)3 catalysis in CH2CN afforded in 76% yield a 1.3:1 diastereomeric mixture of pyranotetrahydroquinolines 9, whose 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation yielded the desired tricyclic ester 11 in 75% yield. The Povarov reaction of methyl 3-(4-aminophenyl)propanoate 6 with 4 and benzaldehyde 8 under Sc(OTf)3 catalysis, followed by DDQ oxidation of the diastereomeric mixture of pyranotetrahydroquinolines 10 and catalytic hydrogenation of the unseparable mixture of the desired ester 12 and its cinname derivative, formed by the competitive oxidation of the ethylene bridge, afforded in 82% overall yield the tricyclic ester 12 (Scheme 1). This latter compound lacked the chlorine atom at the phenyl substituent, present in 11. However, this chlorine atom, easily removable in the catalytic hydrogenation conditions, was not expected to play a major role in the interaction of this moiety with the AChE peripheral site.

Aminoalkyltacrines 17a–g were synthesized in 35–78% yield following a procedure that involves amination of dichloroacridine 15 with commercially available αω-diamines 16a–g in refluxing 1-pentanol (Scheme 2). In this reaction, significant amounts of dimers of 6-chlorotacrine, 28a–g, were formed despite the fact that an excess of diamines (4 equiv) was used.

Hydrolysis of esters 11 and 12 (Scheme 1), followed by treatment of the corresponding carboxylic acids 13 and 14 with 1 equiv of ethyl chloroformate and 2.2 equiv of Et3N in CH2Cl2, and reaction of the resulting mixed anhydrides with 1 equiv of aminoalkyltacrines 17c–g and 17a–e, respectively, afforded hybrids 18–22 and 23–27 in low to moderate yields (19–37% and 25–48% yield, respectively). The novel hybrids 18–27 were fully characterized as dihydrochlorides through their spectroscopic data, HRMS, and elemental analyses. Not unexpectedly, in a study to assess the potential chemical instability at physiological pH of the new hybrids due to the presence of a hydrolyzable amido group, hybrid 23 did not undergo any noticeable decomposition in 1:1 acetonitrile/Sorensen phosphate buffer at pH 7.4 up to 4 days at 37 °C (see Supporting Information).

Pharmacology and Molecular Modeling

Cholinesterase Inhibition. AChE Inhibition. The AChE inhibitory activity of hybrids 18–27 was assayed by the method of Ellman et al. on AChE from bovine (bAChE) and human (hAChE) erythrocytes (Table 1). The hybrids of the first series (18–22) are potent inhibitors of both bAChE and hAChE, with IC50 values in the low nanomolar range in most cases. The most potent hAChE inhibitor was 20, and shortening or lengthening of the linker led to a 3- to 7-fold decrease of inhibitory activity. The hybrids of the second series (23–27) are also potent inhibitors of both enzymes. In contrast with the first series, hybrids 23–27 turned out to be 2- to 3-fold more potent toward the human enzyme. Also, unlike the first series, no significant dependency on the length of the linker was found for the hAChE inhibitory activity of 23–27. Hybrids 24 and 25 were the most potent hAChE inhibitors of the second series, being roughly equipotent to compound 20, in which the total length of the linker is equivalent to that of 25. Although dual site binding to AChE is expected to increase the inhibitory potency relative to the monomeric parent compounds from which they were designed, this assumption is not always fulfilled. Indeed, the hAChE inhibitory activity of hybrids 20, 24, and 25 is comparable with that of the parent 6-chlorotacrine and is clearly higher than the activity measured for propidium and the tricyclic ester precursors 11 and 12.

Molecular Modeling Studies. To gain insight into the molecular determinants that modulate the hAChE inhibitory activity of the novel hybrids, the binding mode of compounds 20, 25, and 27 was investigated by means of docking computations.

The conformation of the active site gorge appears to be highly conserved in different X-ray crystallographic structures. In particular, the observed structural changes at the catalytic binding site are small except for those of Tyr337 (in TcAChE, TcAChE complexes with tacrine and its structurally related analogue huprine X, where the 9-aminotetrahydroacridine unit is stacked against Trp86 and its protonated pyridine nitrogen atom is hydrogen-bonded to His447 (His440 in TcAChE), and the chlorine atom fits a hydrophobic pocket formed by Trp84, Met443, and Pro446 (Trp432, Met436, and Ile439 in TcAChE). In fact, a common pose is found for the tacrine moiety in the X-ray structures of a variety of dual binding site inhibitors, including bis(5)-tacrine and bis(7)-tacrine, tacrine(8)-4-aminoquinoline, NF595, tacrine(10)-hupyrindine, and T22PAc. The structural features of the binding of the pyrylium moiety of the hybrids at the peripheral site are
in the apo form of the enzyme,53,75 and in complexes with bovine or human erythrocytes) or BChE (from human serum) activity.

6-chlorotacrine

- The gorge leading from the peripheral site to the catalytic atom, and the gorge leading from the peripheral site to the catalytic pocket is shown in gray.

Table 1. AChE and BChE Inhibitory Activities of the Hydrochlorides of 6-Chlorotacrine and Tricyclic Esters 11 and 12, Propidium Iodide, and the Dihydrochlorides of the Pyran[3,2-c]quinoline−6-Chlorotacrine Hybrids

<table>
<thead>
<tr>
<th>compd</th>
<th>bAChE IC50 (nM)</th>
<th>hAChE IC50 (nM)</th>
<th>hBChE IC50 (nM)</th>
<th>AChE selectivity</th>
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<tr>
<td>18·2HCl</td>
<td>20.4 ± 0.9</td>
<td>19.2 ± 1.5</td>
<td>1074 ± 178</td>
<td>56</td>
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<tr>
<td>19·2HCl</td>
<td>10.3 ± 0.4</td>
<td>18.3 ± 2.6</td>
<td>1931 ± 47</td>
<td>106</td>
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<tr>
<td>20·2HCl</td>
<td>10.4 ± 0.7</td>
<td>7.03 ± 0.3</td>
<td>331 ± 42</td>
<td>47</td>
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<tr>
<td>21·2HCl</td>
<td>24.0 ± 1.6</td>
<td>24.9 ± 1.5</td>
<td>1391 ± 31</td>
<td>56</td>
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<tr>
<td>22·2HCl</td>
<td>93.7 ± 3.2</td>
<td>50.0 ± 3.0</td>
<td>1622 ± 117</td>
<td>32</td>
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<tr>
<td>23·2HCl</td>
<td>48.1 ± 2.0</td>
<td>16.6 ± 1.0</td>
<td>586 ± 16</td>
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<td>24·2HCl</td>
<td>23.9 ± 1.3</td>
<td>9.64 ± 1.4</td>
<td>290 ± 9.8</td>
<td>30</td>
</tr>
<tr>
<td>25·2HCl</td>
<td>30.8 ± 1.9</td>
<td>11.1 ± 0.1</td>
<td>218 ± 3.2</td>
<td>20</td>
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<tr>
<td>26·2HCl</td>
<td>29.9 ± 0.6</td>
<td>14.4 ± 1.4</td>
<td>234 ± 7.8</td>
<td>16</td>
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<tr>
<td>27·2HCl</td>
<td>34.1 ± 1.0</td>
<td>14.0 ± 1.2</td>
<td>1076 ± 78</td>
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<tr>
<td>11·HCl</td>
<td>&gt; 10000</td>
<td>&gt; 10000</td>
<td>nd</td>
<td>Not determined</td>
</tr>
<tr>
<td>12·HCl</td>
<td>&gt; 10000</td>
<td>nd</td>
<td>nd</td>
<td>Not determined</td>
</tr>
<tr>
<td>propidium iodide</td>
<td>6289 ± 377</td>
<td>32300 ± 2200</td>
<td>13200 ± 400</td>
<td>0.4</td>
</tr>
<tr>
<td>6-chlorotacrine·HCl</td>
<td>5.73 ± 0.4</td>
<td>8.32 ± 0.7</td>
<td>916 ± 19</td>
<td>110</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard error of the mean of at least four experiments. IC50 inhibitory concentration (nM) of AChE (from bovine or human erythrocytes) or BChE (from human serum) activity. AChE selectivity = IC50(bBChE)/IC50(bAChE). Not determined. Data from ref 21.

angles close to −120° (χ1) and +50° (χ2) are found in complexes with bis(7)-tacrine,70 tacrine(8)-4-aminoquinoline,70 and NF595.71 At this point, it is worth stressing how the different lengths of the tether in bis(5)-tacrine and bis(7)-tacrine lead to a distinct arrangement of the indole ring of Trp286.70 Finally, an alternative orientation defined by dihedral angles close to −160° (χ1) and −120° (χ2) is found in the complex with syn-TZ2PA6.73 Overall, this analysis stresses the conformational plasticity of Trp286 and its capability to adopt different orientations depending on the chemical features of the ligand.

On the basis of the preceding discussion, the binding modes of 20, 25, and 27 were investigated using three models of hAChE, in which Trp286 was imposed to adopt each one of the three above-mentioned conformational orientations (denoted as A, B, and C; Figure 1). Moreover, suitable restraints were introduced to fix the orientation of the 6-chlorotacrine moiety, thus enhancing the conformational sampling of the ligand at the peripheral binding site and along the gorge. Noteworthy, this restrained docking protocol was able to predict the X-ray binding mode of bis(5)-tacrine, bis(7)-tacrine, tacrine(8)-4-aminoquinoline, (R)- and (S)-tacrine(10)-hupyridone, and syn- and anti-TZ2PA6 within the first 10 poses and with root-mean square deviations less than 1.8 Å (Table S3, Supporting Information).

Following previous studies in the literature,79–81 the relative stabilities of the first 50 poses obtained in the docking of every ligand on each one of the targets were reranked using MM–PBSA calculations (Table S4, Supporting Information). In all cases the most favorable binding is found for target C, as this binding mode is favored by 1.4–7.6 and 2.4–9.9 kcal/mol when dielectric permittivities of 2 and 4 are considered for the interior of the ligand−hAChE complex, respectively, relative to the second most stable complex. Moreover, the results also show that there are small differences in the affinities estimated for the binding of hybrids 20, 25, and 27 to target C. Keeping in mind the range of uncertainty expected for MM–PBSA calculations,80,82 this finding is in agreement with the similar inhibitory potencies measured for these compounds (IC50 values ranging from 7 to 14 nM, Table 1).
displacement observed for the tetrahydroacridine systems can be ascribed to the positioning of the chlorine atom in 20 in the hydrophobic pocket formed by Trp439, Met443, and Pro446 (see above) and particularly to the active role played by the triazole ring in the tether of syn-TZ2PA6 in mediating interactions with specific residues along the gorge (see Figure 2), which also explain the different arrangement of the linker observed for syn-TZ2PA6 and for the two poses of 20. At the peripheral binding site the pyrano[3,2-c]quinoline moiety adopts two main orientations (Figure 2), which can be roughly interconverted by a 180° rotation through the C5-C9 axis. At first sight, this finding might be surprising, keeping in mind the well-defined arrangement observed for the phenanthridinium moiety of syn-TZ2PA6 in the X-ray structure, but this can be ascribed to additional interactions involving the buried phenanthridinium amino group that are absent in the case of the pyrano[3,2-c]quinoline moiety. Accordingly, the stacking of the pyrano[3,2-c]quinoline moiety might involve distinct arrangements provided that there exists a significant overlap with the aromatic rings of Trp86 and Tyr72, and that steric clashes with neighboring residues are avoided. Similar overall arrangements are found for hybrids 25 and 27 (see Figures S2 and S3, Supporting Information).

To further explore the proposed binding mode, a series of 10 ns molecular dynamics simulations were run for compounds 20 and 25, which were chosen as representative members of the two series of dual binding site inhibitors. The simulations were run for each of the two potential binding modes shown in Figure 2 for compound 20 (and Figures S2 and S3 for 25). Only the trajectories run for the ligand with the pyran ring oriented toward the bulk solvent yielded stable trajectories, as noted by inspection of the time dependence of both the potential energy and the root-mean-square deviation of selected atoms in the protein backbone, the binding site, and the ligand in the ligand-receptor complexes (Figure S4, Supporting Information). There is a large resemblance between the snapshots collected at the end of the trajectories and those used as starting structures (Figure 3). The main difference lies in the orientation of the tether, which adopts a more extended conformation at the end of the trajectories. Nevertheless, the tacrine unit remains stacked between the aromatic rings of Trp86 and Tyr72, and the pyrano[3,2-c]quinoline unit retains the π-π stacking with Trp286 and Tyr72. Finally, a well-defined interaction pattern between the amido functionality and residues lining the entrance of the gorge is not observed. This finding is in contrast with the binding mode reported from modeling studies for a series of structurally related indole-tacrine hybrids containing an amido group within the linker, where this group was proposed to participate in hydrogen-bond interactions with several residues in the gorge. These distinct features could presumably stem from the different arrangement of the pyrano[3,2-c]quinoline and indole units at the peripheral binding site.

Kinetic Analysis of AChE Inhibition. The mechanism of AChE inhibition was investigated in vitro using compound 20, the most potent inhibitor of the two series. Graphical
Inhibition of Spontaneous Aβ Aggregation. A number of dual binding site AChE inhibitors exhibit a significant inhibitory activity on Aβ self-aggregation.23,26,27,29,32–34 The new hybrids significantly inhibit the self-induced Aβ aggregation when tested at equimolar ratio with Aβ, with percentages of inhibition ranging from 12% to 49% (Table 2). In the same assay conditions, 6-chlorotacrine and the tricyclic ester 12 turned out to be, respectively, almost and completely inactive, while propidium was more potent than the new hybrids. Hybrids of the second series were generally more potent than their counterparts of the first series, with the sole exception of 24 which was 2-fold less potent than 19. A clear dependency of the spontaneous Aβ antiaggregating activity on the length of the linker was not observed. Compounds 23, 26, and 27 were the most potent hybrids. It might be hypothesized that a two-methylene linker between the amido group and the phenylpiranof[3,2-c]quinoline moiety facilitates the formation of hydrogen bonds, with Aβ leading to an increased affinity. The expected IC50 values for the most potent hybrids must be around 50 μM, while the strongest inhibitors of spontaneous Aβ aggregation among known dual binding site AChEIs show potencies in the low micromolar range.26,27,33,34 Thus, 23, 26, and 27 can be considered moderate inhibitors of Aβ1-42 self-aggregation.

Inhibition of BACE-1. BACE-1 has been largely investigated as a therapeutic target for disease-modifying agents in AD, since BACE-1 is involved in the proteolytic cleavage of APP to Aβ and BACE-1 knockout mice did not show any adverse phenotype. Although highly active in vitro peptide inhibitors with poor pharmacokinetics are known, potent brain permeable inhibitors have not been developed yet. Therefore, there is an increasing need for small organic BACE-1 inhibitors able to cross the BBB.89 The ability of the novel hybrids to inhibit in vitro human recombinant BACE-1 was also investigated. Some dual binding site AChEIs such as donepezil,90 bis(7)-tacrine,91 lipocrine,92 memoquin,26 and AP224393 exhibit BACE-1 inhibitory activity, which increases their potential as disease-modifying anti-Alzheimer drug candidates. In particular, since the new hybrids might structurally resemble to some extent bis(7)-tacrine, which showed in vitro and in vivo activity,91 their BACE-1 inhibitory activity was first screened at a single concentration (2.5 μM) by a fluorometric assay.94

Hybrid 27 was the most potent BACE-1 inhibitor, exhibiting a 78% inhibition. Compounds 19, 20, 25, and 26 were less active than 27 (15–34% inhibition), and the rest of the hybrids, the tricyclic ester 12, and 6-chlorotacrine were inactive (Table 2). In general the BACE-1 inhibitory activity of the hybrids of the second series was higher than that of their counterparts of the first series. For the second series, the inhibitory activity increased along with the tether length. The IC50 value of the most active hybrid (27) was 1.81 ± 0.48 μM, it being more active than bis(7)-tacrine in vitro (IC50 = 7.5 μM).91 Thus, 27 emerged as a promising anti-Alzheimer drug candidate endowed with a potent hAChE inhibitory activity (nanomolar range) and a significant in vitro inhibitory effect on both Aβ formation and AChE-induced and self-induced Aβ aggregation.

In Vitro Blood–Brain Barrier Permeation Assay. Brain penetration is a major issue for successful CNS drugs. In the past years, several in silico/in vitro methods have been used to predict the BBB permeation potential of test compounds. Among them, the parallel artificial membrane permeation assay (PAMPA-BBB) described by Di et al.95 predicts passive BBB permeation with high success, high throughput, and reproducibility. To evaluate the brain penetration of the pyrano[3,2-c]quinoline–6-chlorotacrine hybrids herein described, we used the PAMPA-BBB assay, which was successfully applied by some of us to different compounds.39,52,96–98 The in vitro permeability (Pc) of selected hybrids (18, 19, and 23–27), the tricyclic ester 12, and 6-chlorotacrine through a lipid extract of porcine brain was determined by using phosphate buffered saline (PBS)/EtOH (80:20 or 70:30, depending on the solubility of compounds). At each solvent mixture, assay validation was made by comparing the experimental permeability with the reported values of 15 commercial drugs that gave a good lineal correlation: 

\[
P_c(\text{exp}) = 1.48P_c(\text{bibl}) + 1.91 (R^2 = 0.95) \quad \text{for PBS/EtOH (80:20)} \\
P_c(\text{exp}) = 1.99P_c(\text{bibl}) + 1.07 (R^2 = 0.92) \quad \text{for PBS/EtOH (70:30)}
\]

From these equations and taking into account the limits established by Di et al. for BBB permeation,95 we established that compounds with permeability values over 7.8 × 10−6 cm s⁻¹ (PBS/EtOH, 80:20) or 9.0 × 10−6 cm s⁻¹ (PBS/EtOH, 70:30) should cross the BBB. All tested hybrids showed permeability values over the above limits (Table 3; see also Table S5, Supporting Information), pointing out that they could cross the BBB and reach their pharmacological targets located in the CNS.

### Table 2. Aβ Aggregation and BACE-1 Inhibitory Activities of the Hydrochlorides of 6-Chlorotacrine and Tricyclic Ester 12. Propidium Iodide, and the Dihydropicrosides of the Pyrano[3,2-c]quinoline–6-Chlorotacrine Hybrids

<table>
<thead>
<tr>
<th>compd</th>
<th>AChE-induced Aβ1–40 aggregation (%)</th>
<th>Aβ1–42 self-induced aggregation (%)</th>
<th>BACE-1 activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-HCl</td>
<td>27.2 ± 7.1</td>
<td>16.4 ± 2.6</td>
<td>na</td>
</tr>
<tr>
<td>19-HCl</td>
<td>22.9 ± 3.2</td>
<td>28.6 ± 3.3</td>
<td>14.5 ± 5.7</td>
</tr>
<tr>
<td>20-HCl</td>
<td>28.6 ± 0.5</td>
<td>21.5 ± 1.8</td>
<td>18.5 ± 7.6</td>
</tr>
<tr>
<td>21-HCl</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>22-HCl</td>
<td>27.9 ± 5.2</td>
<td>20.5 ± 1.1</td>
<td>na</td>
</tr>
<tr>
<td>23-HCl</td>
<td>34.4 ± 1.0</td>
<td>45.3 ± 0.9</td>
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<td>24-HCl</td>
<td>37.9 ± 3.4</td>
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<td>25-2HCl</td>
<td>38.9 ± 2.6</td>
<td>30.8 ± 8.6</td>
<td>19.6 ± 5.1</td>
</tr>
<tr>
<td>26-2HCl</td>
<td>45.9 ± 2.8</td>
<td>49.1 ± 15.1</td>
<td>34.4 ± 3.6</td>
</tr>
<tr>
<td>27-2HCl</td>
<td>45.7 ± 0.3</td>
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<td>77.8 ± 6.4</td>
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<tr>
<td>12-HCl</td>
<td>10.5 ± 4.7</td>
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<td>na</td>
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<tr>
<td>propidium iodide</td>
<td>82.0 ± 2.5f</td>
<td>89.8 ± 0.9</td>
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<tr>
<td>6-chlorotacrine-HCl</td>
<td>8.5 ± 1.6h</td>
<td>7.1 ± 1.2</td>
<td>nah</td>
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*Values are expressed as mean ± standard error of the mean from two independent measurements, each performed in duplicate. A 100 μM concentration of the inhibitor was used. A 50 μM concentration of the inhibitor was used ([Aβ][I] = 1/1). A 2.5 μM concentration of the inhibitor was used. Not active. Not determined. Data from ref 88. Data from ref 31.

### References

Publication Date (Web): August 7, 2009 | doi: 10.1021/jm900859q
We have synthesized a new series of pyrano[3,2-c]quinoline–6-chlorotacrine hybrids as a novel class of dual binding site AChEIs. Variation of the position of an amido group within the oligomethylene linker gives rise to two parallel isomeric series. In general, the new hybrids are potent AChEIs, with IC₅₀ values in the low nanomolar range and with slight differences between both series. The results indicate that linkage of a 5-phenylpyrano[3,2-c]quinoline moiety to a unit of the highly potent AChE active site inhibitor 6-chlorotacrine through an amido-containing tether does not result in an increased potency, as the most potent hybrids are equipotent to 6-chlorotacrine. Moreover, the AChE inhibitory potency of the hybrids shows a modest dependence on the length of the linker and the position of the amido group. Molecular modeling and kinetic studies have confirmed the dual site binding of these hybrids to hAChE. Apart from the characteristic interactions of the 6-chlorotacrine unit of these hybrids within the active site of AChE, the pyranoquinoline moiety is proposed to interact at the peripheral site forming a double near-parallel stacking with Trp286 and Tyr72, respectively, which are positioned in a way similar to the arrangement found in the complex of the enzyme with syn-TZ2PA6. The presence of a chlorine atom at position 6 of the tacrine unit, known to be detrimental for BChE inhibition, not unexpectedly accounts for the hAChE/hBChE selectivity of these hybrids.

Because of their dual binding site character, the new hybrids are able to inhibit the AChE-induced Aβ aggregation. Also, these compounds exhibit a significant ability to inhibit the self-induced Aβ₄₂ aggregation, and some of them can also inhibit BACE-1. In general, these effects seem to be sensitive to the position of the amido group of the linker, the hybrids of the second series being more potent than their counterparts of the first series. Moreover, in the second series the activities increase along with the tether length. Finally, these hybrids seem to be able to cross BBB. Overall, 27 emerges as a promising anti-Alzheimer drug candidate able to hit both AChE (catalytic and noncatalytic activities) and Aβ (aggregation and production) and, therefore, with potential symptomatic and disease-modifying effects.

**Experimental Section**

**Chemistry. General Methods.** Melting points were determined in open capillary tubes with a MFB 959010M Gallenkamp or a Büchi B-540 melting point apparatus. 300 MHz ¹H/ 75.5 MHz ¹³C NMR spectra, 400 MHz ¹H/100.6 MHz ¹³C NMR spectra, and 500 MHz ¹H NMR spectra were recorded on Varian Gemini 300, Varian Mercury 400, and Varian Inova 500 spectrometers, respectively. The chemical shifts are reported in ppm (δ scale) relative to internal tetramethylsilane, and coupling constants are reported in hertz (Hz). Assignments found in the NMR spectra of the new compounds have been carried out by comparison with the NMR data of 18, 27, and 6-chlorotacrine, as model compounds, which in turn were assigned on the basis of DEPT, COSY ¹H/¹H (standard procedures), and COSY ¹H/¹³C (gHSQC and gHMBC sequences) experiments. IR spectra were run on Perkin-Elmer Spectrum RX I or Thermo Nicolet Nexus spectrophotometers. Absorption values are expressed as wavenumbers (cm⁻¹); only significant absorption bands are given. Column chromatography was performed on silica gel 60 AC•C (35–70 mesh, SDS, ref 2000027). Thin-layer chromatography was performed with aluminum-backed sheets with silica gel 60 F₂₅₄ (Merck, ref 1.05554), and spots were visualized with UV light and 1% aqueous solution of KMnO₄. The analytical samples of all of the new hybrids that were subjected to pharmacological evaluation possess a purity of ≥95%, as evidenced by results of their elemental analyses.

(4aRS,5RS,10bRS)- and (4aRS,5SR,10bRS)-Ethyl 5-(4-Chlorophenyl)-3,4,5,6,10b-hexahydro-2H-pyran-3,2-cqquinoline-9-carboxylate (9). Y(OtBu)₃ (295 mg, 0.55 mmol, 0.2 equiv) was added to a solution of p-chlorobenzaldehyde, 7 (385 mg, 2.74 mmol, 1 equiv), and ethyl p-aminobenzoate, 5 (453 mg, 2.74 mmol, 1 equiv), in dry CH₂CN (20 mL). A solution of 3,4-dihydro-2H-pyran, 4 (250 mL, 231 mg, 2.74 mmol, 1 equiv), in dry CH₂CN (3 mL) was then added, and the reaction mixture was stirred at room temperature under inert atmosphere for 12 h. Saturated aqueous NaHCO₃ (20 mL) was added, and the resulting mixture was extracted with AcOEt (3 × 10 mL). The combined organic extracts were dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure to give an orange oil. IR spectra were run on Perkin-Elmer Spectrum RX I or Thermo Nicolet Nexus spectrophotometers. Absorption values are expressed as wavenumbers (cm⁻¹); only significant absorption bands are given. Column chromatography was performed on silica gel 60 AC•C (35–70 mesh, SDS, ref 2000027). Thin-layer chromatography was performed with aluminum-backed sheets with silica gel 60 F₂₅₄ (Merck, ref 1.05554), and spots were visualized with UV light and 1% aqueous solution of KMnO₄. The analytical samples of all of the new hybrids that were subjected to pharmacological evaluation possess a purity of ≥95%, as evidenced by results of their elemental analyses.

<table>
<thead>
<tr>
<th>compd</th>
<th>Pₑ (10⁻⁶ cm s⁻¹)</th>
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<tr>
<td>18-2HCl</td>
<td>14.9 ± 1.1</td>
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</tr>
<tr>
<td>19-2HCl</td>
<td>17.7 ± 0.2</td>
<td>CNS+</td>
</tr>
<tr>
<td>21-2HCl</td>
<td>15.1 ± 0.2</td>
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</tr>
<tr>
<td>24-2HCl</td>
<td>16.6 ± 0.4</td>
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</tr>
<tr>
<td>26-2HCl</td>
<td>13.2 ± 0.4</td>
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</tr>
<tr>
<td>26-2HCl</td>
<td>10.2 ± 0.3</td>
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</tr>
<tr>
<td>27-2HCl</td>
<td>10.1 ± 0.4</td>
<td>CNS+</td>
</tr>
<tr>
<td>12-</td>
<td>21.4 ± 0.6</td>
<td>CNS+</td>
</tr>
<tr>
<td>6-chlorotacrine · HCl</td>
<td>19.8 ± 0.4</td>
<td>CNS+</td>
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</tbody>
</table>

**Conclusion**

We have synthesized a new series of pyrano[3,2-c]quinoline–6-chlorotacrine hybrids as a novel class of dual binding site AChEIs. Variation of the position of an amido group within the oligomethylene linker gives rise to two parallel isomeric series. In general, the new hybrids are potent AChEIs, with IC₅₀ values in the low nanomolar range and with slight differences between both series. The results indicate that linkage of a 5-phenylpyrano[3,2-c]quinoline moiety to a unit of the highly potent AChE active site inhibitor 6-chlorotacrine through an amido-containing tether does not result in an increased potency, as the most potent hybrids are equipotent to 6-chlorotacrine. Moreover, the AChE inhibitory potency of the hybrids shows a modest dependence on the length of the linker and the position of the amido group. Molecular modeling and kinetic studies have confirmed the dual site binding of these hybrids to hAChE. Apart from the characteristic interactions of the 6-chlorotacrine unit of these hybrids within the active site of AChE, the pyranoquinoline moiety is proposed to interact at the peripheral site forming a double near-parallel stacking with Trp286 and Tyr72, respectively, which are positioned in a way similar to the arrangement found in the complex of the enzyme with syn-TZ2PA6. The presence of a chlorine atom at position 6 of the tacrine unit, known to be detrimental for BChE inhibition, not unexpectedly accounts for the hAChE/hBChE selectivity of these hybrids.

Because of their dual binding site character, the new hybrids are able to inhibit the AChE-induced Aβ₄₀ aggregation. Also, these compounds exhibit a significant ability to inhibit the self-induced Aβ₄₂ aggregation, and some of them can also inhibit BACE-1. In general, these effects seem to be sensitive to the position of the amido group of the linker, the hybrids of the second series being more potent than their counterparts of the first series. Moreover, in the second series the activities increase along with the tether length. Finally, these hybrids seem to be able to cross BBB. Overall, 27 emerges as a promising anti-Alzheimer drug candidate able to hit both AChE (catalytic and noncatalytic activities) and Aβ (aggregation and production) and, therefore, with potential symptomatic and disease-modifying effects.

Table 3. Permeability Results from the PAMPA-BBB Assay for Selected Pyrano[3,2-c]quinoline–6-Chlorotacrine Hybrids (Pₑ, 10⁻⁶ cm s⁻¹) With Their Predominant Penetration into the CNS.
2.09 (m, 2H, 3-H2), 2.76 (t, J = 6.3 Hz, 2H, 4-H2), 3.49 (q, J = 7.1 Hz, 2H, CH2-CH3), 4.60 (m, 2H, 2-H2), 7.60–7.70 (complex signal, 4H, 5-ArHpara), 8.04 (dd, J = 8.9 Hz, J′ = 0.5 Hz, 1H, 7-H1), 8.46 (dd, J = 8.9 Hz, J′ = 1.8 Hz, 1H, 8-H1), 8.93 (dd, J = 1.8 Hz, J′ = 0.5 Hz, 1H, 9-H1). 13C NMR (100.6 MHz, CD3OD) δ 13.4 (CH3, CH2-CH3), 20.3 (CH3, C4), 22.3 (CH3, C1), 62.0 (CH2, CH2=CH3), 70.3 (CH3, C2), 114.5 (C, C4a), 119.3 (C, C10a), 120.8 (CH, C10), 125.1 (C, C9), 129.4 (CH, C7), 130.1 (CH, C8), 130.3 (CH, 5-Ar-Cortho), 130.8 (CH, 5-Ar-Cmeta), 138.0 (C, 5-Ar-Cpara), 140.6 (C, C6a), 157.2 (C10b), 165.0 (C, C5), 166.3 (C, CO). HRMS calcd for (C5H18N3O3)H+ + H+ 368.1058, found 368.1056.

Methyl 3-[3,4-Dihydro-5-phenyl-2H-pyran-3,2-c]-quinolin-9-yl)propanoate (12). A. Synthesis of the Diastereomeric Mixture of Tetrahydroquinolines 10. To a solution of 3,4-dihydro-2H-pyran (508 μL, 4.68 mg, 5.58 mmol, 1 equiv), benzaldehyde (556 μL, 519 mg, 5.58 mmol, 1 equiv), and the aniline 6 (1.00 g, 5.58 mmol, 1 equiv) in CH2Cl2 (40 mL), 77% aqueous NH4OH (7.7 mL), and concentrated in vacuo to yield a residue (9.56 g) was obtained and subjected to column chromatography (35–70 μm silica gel, CH2Cl2/MeOH 25%/23% aqueous NH4OH mixtures). On elution with CH2Cl2/MeOH, 25% aqueous NH4OH, 99:10:05, bis-6-chlorotacrine 28 and the desired amine 17 was isolated as brown and yellowish oil, respectively.

For characterization purposes, analytical samples of the dihydrochlorides of 17 and 28 were prepared as follows: the amine 17 or the dimer 28 (1 mmol) were dissolved in MeOH (2–10 mL), the solution was filtered through a polytetrafluoroethylene (PTFE) 0.45 μm filter and treated with an excess of a methanolic solution of HCl (6–9 mmol), and the resulting solution was concentrated in vacuo to dryness. The solid was recrystallized from MeOH/AcOEt mixtures, triturated with Et2O, and dried at 65 °C/15 Torr for 4 days to give 17·2HCl or 28·2HCl as yellowish solids.

9-(6-Aminoethyl)amino]-6-chloro-1,2,3,4-tetrahydroacridine Dihydrochloride (17c·2HCl). From 15 (4.00 g, 15.9 mmol) and 1.6-diaminohexane, 16c (7.52 g, 64.8 mmol), a brown amine residue (7.30 g) was obtained and subjected to column chromatography (35–70 μm silica gel, CH2Cl2/MeOH 25%/50% aqueous NH4OH mixtures). On elution with CH2Cl2/MeOH, 25% aqueous NH4OH, 99:10.05, bis-6-chlorotacrine 28 (246 mg), a mixture 28c·17c in the approximate ratio of 20:80 (1 H NMR) (614 mg, 8% total yield of 28c), and amine 17c (3.61 g, 78% total yield) were consecutively isolated as brown-yellow oils: Rf17c = 0.39; Rf28c = 0.80. (CH3)2CH(MeOH)/25% aqueous NH4OH, 9:10:01.

17c·2HCl: mp 157–158 °C (MeOH); IR (KBr) ν 3500–2500 (max at 3425, 3205, 2414, N–H, N–H, and C=H st), 1630, 1573, and 1513 (ar–C–C and ar–C–Nst) cm–1; 1H NMR (300 MHz, CD3OD, preset/Watergate) δ 1.39–1.52 (complex signal, 4H, 3′-H1 and 4′-H2), 1.66 (tt, J ≈ 8.5 Hz, 2H, 7-H1), 1.80–1.98 (complex signal, 4H, 2-H2 and 3-H3), 2.69 (m, 2H, 1-H2), 2.90 (t, J = 7.5 Hz, 2H, 6-H2), 2.98 (m, 2H, 4-H2), 3.83 (t, J = 7.5 Hz, 2H, 1′-H2), 7.48 (dd, J = 9.3 Hz, J′ = 2.1 Hz, 1H, 7-H1), 7.75 (d, J = 2.1 Hz, 2H, 5-H2), 8.30 (d, J = 9.3 Hz, 1H, 8-H1). 13C NMR (75.4 MHz, CD3OD) δ 22.3 (CH2, C3), 23.2 (CH2, C2), 25.2 (CH2, C1), 27.1 (CH3, C3′ and C4′), 28.5 (CH3), 30.9 (CH3) and 31.4 (CH2) (C4', C2, and C5'), 40.6 (CH2, C6'), 49.1 (CH2, C1'), 114.3 (C, C9a), 116.5 (C, C8a), 121.3 (CH, C5), 126.1 (CH, C7), 128.0 (CH, C8), 138.5 (C, C6), 142.8 (C, C10a), 154.5 (C, C4a), 156.9 (C, C5), 160.2 (C, C5), 173.1 (C, COO). HRMS calcd for (C32H25N5O3·H2O)H+ + H+ 537.1871, found 537.1870.

General Procedure for the Reaction of 6,9-Dichloro-1,2,3,4-tetrahydroacridine, 15, with α,ω-Alkaneamides. A mixture of 15 (1 mmol) and an excess of the diamine 16 (4 mmol) in 1-pentanol (1.3 mL) was heated under reflux with magnetic stirring for 18 h. The resulting mixture was cooled to room temperature, diluted with CH2Cl2 (5 mL), and washed successively with aqueous 2 N NaOH (3 × 3 mL) and water (2 × 3 mL). The organic phase was dried with anhydrous Na2SO4 and concentrated in vacuo to give a brown oily residue, from which the pentanol and the excess of diamine were removed by distillation at 100°C/1 Torr and 140°C/1 Torr, respectively. The distillation residue was taken in CH2Cl2 (1.3 mL), filtered, and concentrated in vacuo to give a brown oily residue which was subjected to column chromatography (35–70 μm silica gel, CH2Cl2/MeOH 25%/50% aqueous NH4OH or AcOEt/MeOH/ET3N mixtures as eluent) to afford, separately, bis-6-chlorotacrine 28 and the desired amine 17 as a brown and yellowish oil, respectively.
25\% aqueous NH$_4$OH, 96:4:0.1 to 85:15:0.2, bis(8)-6-chloroacridine, 28e (2.11 g, 46\% yield) and amine 17e (2.00 g, 35\% yield) were consecutively isolated as brown-yellow oils: R$_f$ (MeOH/AcOEt, 1:1) = 0.33; R$_f$ (MeOH/CH$_2$Cl$_2$) = 0.88 (MCI silica gel, hexane/MeOH/N$_2$O$_4$/AcOEt, 10:0:1).

$^{13}$C NMR (300 MHz, CD$_2$OD) $\delta$ 1.24–1.44 (complex signal, 8H, 3'-H$_2$, 4'-H$_2$, 5'-H$_2$, and 6'-H$_2$), 1.63 (t, $J\approx 7.5$ Hz, 2H, 7'-H$_2$), 1.71 (tt, $J\approx 7.5$ Hz, 2H, 2'-H$_2$), 1.84–1.94 (complex signal, 4H, 2H, 2'-H$_2$, and 3'-H$_2$), 2.66 (m, 2H, 1'-H$_2$), 2.89 (t, $J= 7.5$ Hz, 2H, 8'-H$_2$), 2.94 (m, 2H, 4H, 3'-H$_2$), 3.69 (t, $J= 7.0$ Hz, 2H, 3(6)-H$_2$), 7.54 (dd, $J= 9.3$ Hz, $J\approx 2.1$ Hz, 2H, 7(8)-H$_2$), 7.77 (d, $J\approx 2.1$ Hz, 2H, 5(6)-H$_2$), 8.37 (d, $J= 9.3$ Hz, 2H, 2H-CH$_2$), $^{13}$C NMR (75.4 MHz, CD$_2$OD) $\delta$ 21.8 (CH$_2$, C2), 22.9 (CH$_2$, C2), 24.8 (CH$_2$, C1), 27.6 (CH$_2$, C3), 29.4 (CH$_2$, C4), 30.2 (CH$_2$), and 31.3 (CH$_2$) (C2' and C4'), 49.2 (CH$_2$, C1'), 113.2 (C, C9a), 115.3 (C, C8a), 119.0 (CH, C5), 126.6 (CH, C7), 128.7 (CH, C8), 139.9 (C, C6), 140.3 (C, C10a), 152.0 (C, C4a), 157.5 (C, C9).

**General Procedure for the Preparation of Amines 18–27 from Esters 11 or 12 and Amines 17.** A.1. Hydrolysis of Estrer 11. A solution of ester 11 (1 mmol) and aqueous 5 N NaOH (1.2 mL, 6 equiv) in MeOH (56 mL) was heated under reflux with magnetic stirring for 16 h. The resulting mixture was cooled to room temperature and evaporated in vacuo. The obtained solid residue was treated with MeOH (18 mL) and the organic extract was evaporated at reduced pressure to give the hydrochloride of the corresponding carboxylic acid, 13, as a white solid, which was used in the next step without further purification.

A.2. Hydrolysis of Ester 12. A solution of ester 12 (1 mmol) and KOH pellets (240 mg of 85\% purity reagent, 3.6 equiv) in MeOH (25 mL) was heated under reflux with magnetic stirring for 24 h. The resulting mixture was cooled to room temperature and evaporated in vacuo. The obtained solid residue was treated with a solution of HCl in Et$_2$O (20 equiv) with stirring for 30 min, and the suspension was evaporated in vacuo to give the hydrochloride of the corresponding carboxylic acid, 14, as a white solid, which was used in the next step without further purification.

B. Reaction of Carboxylic Acids 13 or 14 with Amines 17. To a cold solution (0 \^C, ice-water bath) of 13 or 14 (crude product arising from 1 mmol of the starting ester 11 or 12) and anhydrous Et$_3$N (2.2 mmol) in anhydrous CH$_2$Cl$_2$ (50 mL), ethyl chloroformate (1 mmol) was added and the mixture was thoroughly stirred at 0 \^C for 30 min. To the resulting solution, a cold solution (0 \^C, ice-water bath) of amine 17 (1 mmol) in anhydrous CH$_2$Cl$_2$ (30 mL) was added, and the reaction mixture was stirred at room temperature for 64–72 h and treated with 10\% aqueous Na$_2$CO$_3$ until pH 10 (95 mL). The organic phase was separated, and the aqueous one was extracted with CH$_2$Cl$_2$ (3 \times 40 mL). The organic phase and combined extracts were washed with water (2 \times 80 mL), dried with anhydrous Na$_2$SO$_4$, and evaporated at reduced pressure to give a yellow oily residue, which was subjected to column chromatography (35–70 \^m silica gel, hexane/AcOEt/Et$_3$N or heptane/AcOEt/Et$_3$N mixtures), followed by preparative TLC (10\% aqueous Na$_2$CO$_3$).
the GraphPad Prism program package (GraphPad Software; San Diego, CA), which gave estimates of the IC_{50} (concentration of drug producing 50% of enzyme activity inhibition). Results are expressed as the mean ± SEM of at least four experiments performed in triplicate. DTNB, acetylthiocholine, butyrylthiocholine, and enzymes were purchased from Sigma, and eserine was purchased from Fluka.

**Kinetic Analysis of AChE Inhibition.** To obtain estimates of the mechanism of action of AChE, reciprocal plots of 1/velocity versus 1/substrate were constructed at relatively low concentration of substrate (0.56–0.11 mM) by using Ellman’s method and human recombinant AChE (Sigma, Milan, Italy). Three concentrations of AChE were selected for this study: 1.25, 1.88, and 3.75 nM. The plots were assessed by a weighted least-squares analysis that assumed the variance of the velocity (v) to be a constant percentage of v for the entire data set. Data analysis was performed with GraphPad Prism 4.03 software (GraphPad Software Inc.).

Slopes of the obtained reciprocal plots were then plotted against AChE concentration in a similar weighted analysis, and K_{i} was determined as the intercept on the negative x-axis.

**AChE-Induced Aβ_{1–40} Aggregation Inhibition Assay.** Thioflavin T (Basic Yellow 1), human recombinant AChE, phosphatidyl powder, 1.1:1.3:3.5-hexadecanol-2-propanol (HFIP), were purchased from Sigma Chemicals. Absolute DMSO over molecular sieves was from Fluka. Water was deionized and doubly distilled. Aβ_{1–40}, supplied as trifluoroacetate salt, was purchased from Bachem AG (Bubendorf, Switzerland). Aβ_{1–40} (2 mg mL^{-1}) was dissolved in HFIP and lyophilized. The 1 mM solutions of tested inhibitors were prepared by dissolving in MeOH.

Aliquots of 2 μL Aβ_{1–40} peptide, lyophilized from 2 mg mL^{-1} HFIP solution and dissolved in DMSO, were incubated for 24 h at room temperature in 0.215 M sodium phosphate buffer (pH 8.0) at a final concentration of 230 μM. For co-incubation experiments aliquots (16 μL) of hAChE (final concentration 2.30 μM, Aβ/hAChE molar ratio 100:1) and AChE in the presence of 2 μL of the tested inhibitor (final inhibitor concentration 100 μM) in 0.215 M sodium phosphate buffer, pH 8.0, solution were added. Blanks containing Aβ_{1–40} alone, human recombinant AChE alone, and Aβ_{1–40} plus tested inhibitors in 0.215 M sodium phosphate buffer (pH 8.0) were prepared. The final volume of each vial was 20 μL. Each assay was run in duplicate. To quantify amyloid fibril formation, the thioflavin T fluorescence method was then applied.88 The fluorescence intensities due to β-sheet conformation were monitored for 300 s at λ_{ex} = 490 nm (λ_{em} = 466 nm). The percent inhibition of the AChE-induced aggregation due to the presence of the tested compound was calculated by the following expression:

\[
\% \text{ inhibition} = \frac{(I_{Fo} - I_{Fi})}{I_{Fo}} \times 100
\]

where \(I_{Fo}\) and \(I_{Fi}\) are the fluorescence intensities obtained for Aβ_{1–40} and the presence of test compound, respectively. The % inhibition due to the absence of inhibitor, respectively, minus the fluorescence intensities due to the respective blanks.

**Aβ_{1–42} Self-Aggregation Inhibition Assay.** As reported in a previously published protocol.89 HFIP pretreated Aβ_{1–42} samples (Bachem AG, Switzerland) were solubilized with a CH3CN/NaCl/Na2CO3/NaOH (48.4:48.4:3.2) mixture. Experiments were performed by incubating the peptide in 10 mM phosphate buffer (pH 8.0) containing 10 mM NaCl at 30 °C for 24 h (final Aβ concentration 50 μM) with and without inhibitor (50 μM, Aβ/inhibitor = 1/1). Blanks containing the tested inhibitors were also prepared and tested. To quantify amyloid fibrils formation, the thioflavin T fluorescence method was used.89 After incubation, samples were diluted to a final volume of 2.0 mL with 50 mM glycine–NaOH buffer (pH 8.5) containing 1.5 μM thioflavin T. A 300 s time scan of fluorescence intensity was carried out (λ_{ex} = 446 nm; λ_{em} = 490 nm, FP-6200 fluorometer, Jasco Europe), and values at plateau were averaged after subtracting the background fluorescence of 1.5 μM thioflavin T solution. The fluorescence intensities were compared, and the percent inhibition due to the presence of the inhibitor was calculated by the following formula 100 – [(IF_{Fo}/IF_{Fi}) \times 100] where IF_{Fi} and IF_{Fo} are the fluorescence intensities obtained for Aβ_{1–42} in the presence and in the absence of inhibitor, respectively.

**β-Secretase (BACE-1) Inhibition Assay.** β-Secretase (BACE-1, Sigma) inhibition studies were performed by employing a peptide mimicking APP sequence as substrate (M-2420, Bachem). The following procedure was employed: an amount of 5 μL of test compound (or DMSO) was preincubated with 175 μL of the enzyme (c~17.2 nM) for 1 h at room temperature. The substrate (3 μM) was then added and left to react for 15 min. The fluorescence signal was read at λ_{ex} = 405 nm (λ_{em} = 420 nm). The fluorescence intensities with and without inhibitor were compared, and the percent inhibition due to the presence of test compounds was calculated. The % inhibition due to the presence of increasing test compound concentration was calculated by the following expression: 100 – [(IF_{Fo}/IF_{Fi}) \times 100], where IF_{Fi} and IF_{Fo} are the fluorescence intensities obtained for BACE-1 in the presence and in the absence of inhibitor, respectively. Inhibition curve was obtained for 27 by plotting the % inhibition versus the logarithm of inhibitor concentration in the assay sample. The linear regression parameters were determined and the IC_{50} was extrapolated, when possible (GraphPad Prism 4.0, GraphPad Software Inc.).

To demonstrate inhibition of BACE-1 activity, a peptido-mimetic inhibitor (β-secretase inhibitor IV, Calbiochem) was serially diluted into the reactions’ wells (IC_{50} = 0.013 μM).
Molecular Modeling. Docking was performed with the program rDock, which is an extension of the program Ribodock, using an empirical scoring function calibrated on the basis of protein–ligand complexes. The reliability of rDock was assessed by docking a set of known dual binding site AChEIs taking advantage of the X-ray crystallographic structures of their complexes with AChE (PDB entries 1Q83, 1Q84, 1ODC, 1ZGB, 1ZGC, 2CKM, and 2CMF), which was also chosen to identify three different orientations of the indole ring of Trp286 in the peripheral binding site in hAChE (see Molecular Modeling Studies and also Tables S2 and S3 in Supporting Information).

The docking of compounds 20, 25, and 27 in hAChE was then explored using the three structural models of the target hAChE (named A, B, and C). Structural water molecules that mediate relevant interactions between the tacrine moiety and the enzyme were retained in the target models. The docking volume was defined as the space within 10 Å of the ligands spanning both catalytic and peripheral binding sites. The ligands were considered in their monocationic form on the basis of the pKa estimated using ACD software. The structure of the ligands was initially energy minimized at the AM1 level using Gaussian 03. Suitable restraints were introduced to position the tacrine moiety of the hybrids in the catalytic site, as inspection of the X-ray crystallographic structures of several tacrine-based dual binding site AChEIs reveals that the tacrine unit shares a common binding mode, which in turn mimics the pose found for AChE complexes with tacrine and huprine X (see text). This allowed us to focus the sampling effort on the orientation of the pyrano[3,2-\text{c}]quinoline unit of the hybrids at the peripheral binding site and the linker along the gorge. Each compound was subjected to 100 docking runs, and the output docking modes were analyzed by visual inspection in conjunction with the docking scores.

The ligand–protein poses were reranked using the MM–PBSA approach (Table S4, Supporting Information). An energy minimization of the complexes was first carried out in order to reduce steric clashes that might arise from docking calculations. All minimizations and MM–PBSA calculations were performed with the parm99 force field of the Amber-9 package. All minimizations and MM-PBSA calculations were performed with the parm99 force field of the Amber-9 package.

The relative binding affinities of the best poses of the ligands were determined by using eq 1.

$$\Delta G_{\text{binding}} = \Delta G_{\text{MM}} + \Delta G_{\text{sol}} + \Delta G_{\text{nonpolar}} - T\Delta S$$

The partial atomic charges for the compounds were derived using the RESP protocol by fitting to the molecular electrostatic potential calculated at the HF/6-31G* level with Gaussian 03. The internal conformational energy (\(\Delta G_{\text{MM}}\)) was determined using the standard formalism and parameters implemented in AMBER. The electrostatic contribution (\(\Delta G_{\text{elec}}\)) was computed using a dielectric constant of 78.4 for the aqueous environment, while values of 2 and 4 were considered for the ligand–enzyme complex. The electrostatic potentials were calculated using a grid spacing of 0.25 Å. The interior of the solutes was defined as the volume inaccessible to a solvent probe sphere of radius 1.4 Å.

The nonpolar contribution (\(\Delta G_{\text{nonpolar}}\)) was calculated using a linear dependence with the solvent-accessible surface. Finally, entropy changes upon complexation were assumed to cancel out in the comparison of the binding affinities of 20 and 25, as the number of rotatable bonds is the same in both compounds. However, since the spacer of 27 is larger by two methylene units, its binding affinity was corrected for the entropic penalty associated with the freezing of the two extra internal degrees of freedom using an empirical correction of 0.6 kcal/mol per rotatable bond.

Finally, molecular dynamics simulations were run to further predict the binding mode of huprines to AChE. Briefly, the enzyme was immersed in a pre-equilibrated box of TIP3P water molecules. The final systems contained the protein–ligand complex and around 16 000 water molecules (about 57 000 atoms). After thermalization at 298 K, a series of 10 ns trajectories were sampled for the different compounds in the receptor–ligand complex. The system was simulated in the NPT ensemble using periodic boundary conditions and Ewald sums for treating long-range electrostatic interactions (with the default Amber-9 parameters). All simulations were performed with the parm99 force field of the Amber-9 package. The structural analysis was performed using in-house software and standard codes (PTRAJ module) of Amber-9.

In Vitro BBB Permeation Assay. Prediction of the brain penetration was evaluated using a parallel artificial membrane permeation assay (PAMPA) in a similar manner as described previously. Commercial drugs, phosphate buffered saline solution at pH 7.4 (PBS), and dodecane were purchased from Sigma, Aldrich, Acros, and Fluka. Millex filter units (PVDF membrane, diameter 25 mm, pore size 0.45 μm) were acquired from Millipore. The porcine brain lipid (PBL) was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μm), and the acceptor microplate was an indented 96-well plate, both from Millipore. The acceptor 96-well microplate was filled with 180 μL of PBS/EtOH (80:20 or 70:30), and the filter surface of the donor microplate was impregnated with 4 μL of PBL in dodecane (20 mg mL⁻¹). Compounds were dissolved in PBS/EtOH (80:20 or 70:30) at 1 mg mL⁻¹, filtered through a Millex filter, and then added to the donor wells (180 μL). The donor filter plate was carefully put on the acceptor plate to form a sandwich, which was left undisturbed for 120 min at 25°C. After incubation, the donor plate was carefully removed and the concentration of compounds in the acceptor wells was determined by UV spectroscopy. Every sample was analyzed at five wavelengths, in four wells, and at least in three independent runs, and the results are given as the mean ± standard deviation.

In each experiment, 15 quality control standards of known BBB permeability were included to validate the analysis set.

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Supporting Information Available: Experimental procedures, spectral and analytical data of synthesized compounds (except for 9–14, 17c,e, and 28c,e, 18, and 27, herein described), solution stability study with 23; table with pKa values of selected compounds; tables and figures with additional data on docking and molecular dynamics; PAMPA-BBB studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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