Synthesis and multi-target biological profiling of a novel family of rhein derivatives as disease-modifying anti-Alzheimer agents

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ABSTRACT: We have synthesized a family of rhein–huprine hybrids to hit several key targets for Alzheimer’s disease. Biological screening performed *in vitro* and in *Escherichia coli* cells has shown that these hybrids exhibit potent inhibitory activities against human acetylcholinesterase butyrylcholinesterase, and BACE-1, dual A β 42 and tau anti-aggregating activity, and brain permeability. *Ex vivo* studies with the leads (+)- and (–)-**7e** in brain slices of C57bl6 mice have revealed that they efficiently protect against the A β -induced synaptic dysfunction , preventing the loss of synaptic proteins and/or have a positive effect on the induction of long term potentiation. *In vivo* studies in APP-PS1 transgenic mice treated i.p. for 4 weeks with (+)- and (–)-**7e** have shown a central soluble A β lowering effect, accompanied by an increase in the levels of mature amyloid precursor protein (APP). Thus, (+)- and (–)-**7e** emerge as very promising disease-modifying anti-Alzheimer drug candidates.

INTRODUCTION

Alzheimer's disease (AD), the most common type of dementia, is associated with ever increasing unacceptable personal and economic costs, owing to its high prevalence, mortality and, worryingly, to the elusiveness of efficacious drugs.¹

There has been a general consensus that β -amyloid peptide ($A\beta$) is the main culprit of a straightforward cascade process that begins with its increased formation by processing of the amyloid precursor protein (APP) by β -secretase (BACE-1) and γ -secretase, and aggregation into oligomers and fibrils. This follows with a series of downstream events including synaptic dysfunction, tau protein hyperphosphorylation and aggregation, neuroinflammation and oxidative stress, and eventually leads to neuronal death and neurotransmitter deficits.^{2,3} Because these deficits account for the cognitive and functional decline, which are the main diagnostic criteria for AD, the first and so far the sole marketed anti-Alzheimer drugs, namely the acetylcholinesterase (AChE) inhibitors tacrine, donepezil, rivastigmine and galantamine and the NMDA receptor antagonist memantine, were developed to hit those neurotransmitter deficits, particularly cholinergic and glutamatergic, but are regarded as merely symptomatic.

In the past years, there has been an intense research activity for developing drugs able to tackle the underlying mechanism of AD, by hitting $A\beta$ formation or aggregation or, to a minor extent, other downstream targets.⁴ However, despite the acute need and the hopes raised for the discovery of effective disease-modifying anti-Alzheimer drugs, a number of clinically advanced $A\beta$ -directed drug candidates have recently failed to show efficacy over placebo or they have been discontinued because of their toxicity. The efficacy issues of these drug candidates have been ascribed, at least partly, to the fact that AD pathology might not result from a straightforward process, but from a robust network of interconnected events, whereby $A\beta$

aggregation is not *the* cause but just *one* of the causes, together with tau hyperphosphorylation and aggregation, synaptic dysfunction or neuroinflammation.⁵ This novel conception of AD would allow explaining why modulation of a single target might be ineffective, whereas simultaneous modulation of several targets of the network, including A β , holds promise for deriving the eagerly awaited effective disease-modifying anti-Alzheimer drugs.^{6–10}

In the past years, many research groups have pursued the development of multi-target anti-Alzheimer drugs as an advantageous approach over multi-target multi-drug strategies (drug cocktails and fixed-dose combinations).^{11–15} Multi-target compounds have been usually designed to hit at least A β formation and aggregation and AChE activity,¹⁶ especially prompted by the finding that AChE can bind A β , thereby promoting its aggregation and increasing its neurotoxicity.^{17–19} The recognition site of A β within AChE is the so-called peripheral anionic site (PAS) and it is located at the entrance of a 20 Å deep narrow gorge that leads to the catalytic anionic site (CAS).²⁰ The design of inhibitors able to simultaneously reach both sites of AChE, i.e. dual binding site AChE inhibitors (AChEIs), emerged some years ago as a promising source of multi-target anti-Alzheimer compounds, inasmuch as they should be endowed at least with potent anticholinesterase and A β anti-aggregating effects.^{21–29} Moreover, the multi-target profile of these compounds can be enlarged by introducing pharmacophoric moieties that are also suitable for separate interactions with additional targets of interest other than AChE and A β . Two major challenges in the design and therapeutic potential of multi-target anti-Alzheimer agents are the choice of the targets to be hit and the potency of the compounds at the different targets. Indeed, it must be recognized that the timing of the different events in the progression of AD can be overlooked when selecting the biological targets to be hit. However, this issue will remain very difficult to be addressed until a precise description of the sequence of events that give rise

to the neuropathogenesis of AD becomes available. On the other hand, a multi-target action will be only effective when balanced activities are achieved at the different targets, which is also not an easy task.

Recently, the hydroxyanthraquinone derivatives emodin, **1**, and PHF016, **2** (Chart 1), have been shown to inhibit tau aggregation *in vitro* with IC₅₀ values in the low micromolar range.^{30,31} The structurally related compound rhein, **3** (Chart 1) is a natural product found in the traditional Chinese herbal medicine rhubarb, which is well tolerated in humans.³² In the form of a doubly acetylated prodrug, diacerein, it is used for the treatment of arthritis. Even though rhein has been reported to be essentially inactive as inhibitor of AChE^{33,34} and of A β aggregation,³⁵ its aromatic rings seem appropriate for establishing π -stacking interactions with the characteristic aromatic residue at the PAS of AChE, namely Trp286 (human AChE (hAChE) numbering). Moreover, its hydroxyanthraquinone scaffold might confer tau anti-aggregating properties to its derivatives. Thus, we inferred that combination in a hybrid of the hydroxyanthraquinone system of rhein with a moiety with high affinity for the CAS of AChE might result in a multi-target compound of interest for the treatment of AD, which should be endowed at least with AChE, A β aggregation and tau aggregation inhibitory activities.

Chart 1. Structures of tau aggregation inhibitors 1 and 2, rhein and huprine Y

Herein, we describe the synthesis, biological profiling, and investigation of the mechanism of action of a novel family of multi-target hybrid compounds, which are composed of a unit of rhein attached, through different linkers, to a unit of huprine Y, **4** (Chart 1), a high affinity AChE's CAS inhibitor, developed in our group some years ago.^{36–39} The biological profiling includes i) the *in vitro* evaluation of the inhibitory activities against hAChE, human butyrylcholinesterase (hBChE), hAChE-induced and self-induced A β aggregation, and human BACE-1 (hBACE-1), ii) the evaluation of the A β and tau anti-aggregating activities in intact *Escherichia coli* cells overexpressing these peptides, iii) the evaluation of the protective effects on synaptic integrity from A β -induced alterations by measuring the induction of long term potentiation and levels of synaptic proteins in hippocampal slices of 2-month-old C57bl6 mice, and iv) the assessment of their brain permeability using an artificial membrane model. Furthermore, kinetic and molecular modeling studies have been performed to shed light on the binding mode of these compounds with two of their main targets, namely AChE and BACE-1.

RESULTS AND DISCUSSION

Chemistry. The linkage between the huprine and rhein moieties was envisioned at one end through an N–C bond from the exocyclic amino group of huprine Y, as in a recently reported family of huprine-based dual binding site AChEI hybrids,⁴⁰ and at the other end through an amide bond from the carboxylic acid of rhein. Such linkages have proven to be chemically stable at physiological pH up to 4 days at 37 °C in a structurally related family of hybrid compounds.⁴¹ Chains from 5 to 11 methylene groups were considered for the linkers (in hybrids **7a–g**, Scheme 1), to afford suitable distances between huprine and rhein moieties to achieve a dual site binding to AChE and to explore the effect of the length of the linker in a target with a large binding site such as BACE-1. The incorporation of an aromatic ring within the linker, i.e. a 1,4-phenylene-bis(methylene) linker (in hybrid **7h**, Scheme 1), was also envisaged to explore potential additional interactions with the targets, i.e. interactions with midgorge residues of AChE or disruption of protein–protein interactions in the case of A β and tau aggregation.

Rhein–huprine hybrids **7a–h** were synthesized through a three- or four-step sequence from readily available racemic or enantiopure huprine Y, ($\pm$)-, (–)- or (+)-**4**,^{36–38} and commercial rhein, **3** (Scheme 1). Alkylation of racemic huprine Y with commercial or described ω -bromoalkanenitriles^{42–44} in the presence of KOH in DMSO afforded nitriles ($\pm$)-**5a–g** in moderate yields (25–87%). Nitrile ($\pm$)-**5h** was prepared in 72% overall yield by condensation of racemic huprine with 4-cyanobenzaldehyde followed by NaBH₃CN reduction of the resulting imine. LiAlH₄ reduction of nitriles ($\pm$)-**5a–h** gave in excellent yields amines ($\pm$)-**6a–h**, which were reacted with the mixed anhydride formed from rhein and ethyl chloroformate to afford in 9–27% yield the rhein–huprine hybrids ($\pm$)-**7a–h**.

Scheme 1. Synthesis of the rhein–huprine hybrids **7a–h**

In the light of the biological results obtained for the racemic hybrids **7a–h**, we decided to synthesize the two enantiomers of the most interesting racemic hybrid, namely **7e**. Thus, enantiopure (–)- and (+)-**7e** were prepared from (–)- and (+)-**4** (>99% ee) using the same methodology.

The novel rhein–huprine hybrids were fully characterized in the form of hydrochloride salts through their spectroscopic data (IR, ¹H and ¹³C NMR), HRMS and elemental analysis. Hydrochloride salts were also used for the activity profile assessment.

***In Vitro* Biological Profiling of Racemic Rhein–Huprine Hybrids.** *Inhibition of human cholinesterases and molecular modeling study of hAChE inhibition.* Apart from AChE, BChE is another target of interest in the search for anti-Alzheimer drugs, inasmuch as this enzyme exerts a compensatory effect in response to a greatly decreased AChE activity in central nervous system (CNS) when AD progresses.⁴⁵ Accordingly, the inhibitory activity of the novel rhein–huprine hybrids and of the reference compounds rhein and huprine Y against human recombinant AChE (hAChE) and human serum BChE (hBChE) was evaluated by the method of Ellman *et al.*⁴⁶

All the racemic hybrids, (±)-**7a–h**, turned out to be potent inhibitors of hAChE, with IC₅₀ values in the low nanomolar range (Table 1), even though both the length of the linker and the presence of an aromatic ring in the linker seemed to have only a moderate effect on the hAChE inhibitory activity. Indeed, the inhibitory potency gradually decreased from the shortest pentamethylene-linked hybrid (±)-**7a** to the longest undecamethylene homologue, (±)-**7g** (16-fold less potent), whereas introduction of an aromatic ring within the linker seemed to be detrimental for hAChE inhibitory activity, hybrid (±)-**7h** being 12-fold less potent than hybrid (±)-**7b** with a similar tether length. Hybrid (±)-**7a**, the most potent hAChEI of the series (IC₅₀ 1.07 nM), was equipotent to the parent racemic huprine Y [(±)-**4**]. In agreement with the reported data,^{33,34} the parent rhein, **3**, was found essentially inactive against hAChE.

Table 1. Inhibitory Activities of the Hydrochlorides of Rhein–Huprine Hybrids and Reference Compounds toward AChE, BChE, BACE-1, and AChE-Induced and Self-Induced A β Aggregation and BBB Predicted Permeabilities^a

compd	hAChE IC ₅₀ (nM) ^b	hBChE IC ₅₀ (nM) ^b	hBACE-1 IC ₅₀ (nM) ^b	hAChE- induced A β 40 aggregation (% inhib.) ^c	self-induced A β 42 aggregation (% inhib.) ^d	P_e (10 ⁻⁶ cm s ⁻¹) ^e (Prediction)
($\pm$)- 7a	1.07 $\pm$ 0.05	950 $\pm$ 30	na ^f	45.0 $\pm$ 7.5	38.7 $\pm$ 5.0	20.0 $\pm$ 1.0 (CNS+)
($\pm$)- 7b	1.52 $\pm$ 0.08	1070 $\pm$ 40	na ^f	50.5 $\pm$ 8.7	40.8 $\pm$ 5.7	nd ^g
($\pm$)- 7c	3.18 $\pm$ 0.16	1460 $\pm$ 160	na ^f	52.5 $\pm$ 2.9	40.6 $\pm$ 1.9	27.5 $\pm$ 0.7 (CNS+)
($\pm$)- 7d	4.36 $\pm$ 0.22	350 $\pm$ 20	980 $\pm$ 170	44.7 $\pm$ 8.4	33.1 $\pm$ 5.4	22.4 $\pm$ 1.3 (CNS+)
($\pm$)- 7e	3.60 $\pm$ 0.21	620 $\pm$ 20	120 $\pm$ 90	48.7 $\pm$ 8.4	38.0 $\pm$ 4.6	21.5 $\pm$ 0.7 (CNS+)
(+)- 7e	2930 $\pm$ 285	265 $\pm$ 21	80 $\pm$ 10	36.9 $\pm$ 3.4	38.4 $\pm$ 5.5	nd ^g
(-)- 7e	2.39 $\pm$ 0.17	513 $\pm$ 58	80 $\pm$ 10	38.1 $\pm$ 0.7	43.2 $\pm$ 4.7	nd ^g
($\pm$)- 7f	7.61 $\pm$ 0.45	1100 $\pm$ 40	1190 $\pm$ 180	29.2 $\pm$ 2.4	40.9 $\pm$ 4.4	18.1 $\pm$ 0.7 (CNS+)
($\pm$)- 7g	17.4 $\pm$ 2.2	645 $\pm$ 67	1200 $\pm$ 150	38.2 $\pm$ 2.6	35.3 $\pm$ 4.0	16.4 $\pm$ 1.0 (CNS+)
($\pm$)- 7h	18.2 $\pm$ 2.2	510 $\pm$ 20	2020 $\pm$ 440	35.2 $\pm$ 1.8	32.4 $\pm$ 3.6	16.3 $\pm$ 2.5 (CNS+)
3	>10000	17000 $\pm$ 4220	na ^h	nd ^g	nd ^g	2.7 $\pm$ 0.1 (CNS+/-)
($\pm$)- 4	1.07 $\pm$ 0.05	181 $\pm$ 15	nd ^g	17.1 $\pm$ 4.5	nd ^g	23.8 $\pm$ 2.7 (CNS+)
(+)- 4	321 $\pm$ 16	170 $\pm$ 17	>5000 ⁱ	9.1 $\pm$ 3.6	13.2 $\pm$ 1.9	25.4 $\pm$ 2.9 (CNS+)
(-)- 4	0.74 $\pm$ 0.06	222 $\pm$ 17	>5000 ^j	24.7 $\pm$ 1.3	11.5 $\pm$ 5.2	23.6 $\pm$ 1.1 (CNS+)

^a Values are expressed as mean $\pm$ standard error of the mean (SEM) of at least three experiments (n=3), each performed in duplicate. ^b IC₅₀ inhibitory concentration (nM) of human recombinant AChE, human serum BChE, or human recombinant BACE-1. ^c % inhibition with inhibitor at 100 μ M, A β 40 at 230 μ M and hAChE at 2.3 μ M. ^d % inhibition with inhibitor at 10 μ M and A β 42 at 50 μ M ([A β]/[I]=5/1). ^e Permeability values from the PAMPA-BBB assay. ^f Not active at 1 μ M. ^g Not determined. ^h Not active. ⁱ 13.6 $\pm$ 2.3% inhibition at 5 μ M. ^j 14.0 $\pm$ 0.1% inhibition at 5 μ M.

The binding mode of the rhein–huprine hybrids to AChE was explored for compound **7h**, taking advantage of the reduced flexibility imposed by the aromatic ring. Docking studies showed that (–)-**7h** binds much more favorably than (+)-**7h**, as expected from the fact that the eutomer of huprines for AChE inhibition is the levorotatory (7*S*,11*S*)-enantiomer, (–)-**4**. The results show that (–)-**7h** may fit the hAChE gorge (Figure 1) placing i) the huprine moiety in an orientation that perfectly matches the arrangement of (–)-huprine X, the 9-ethyl-analog of (–)-huprine Y, in the CAS (PDB entry 1E66, Figure S1), and ii) the hydroxyanthraquinone system of rhein stacked between the aromatic rings of Trp286 and Tyr72 in the PAS, mimicking the arrangement of the tacrine unit of *bis*(7)-tacrine (PDB entry 2CKM, Figure S2).⁴⁷ Even though these findings support the ability of the hybrids to interact simultaneously at both CAS and PAS, the inhibitory potencies in Table 1 suggest that the rhein unit has a weak contribution to the binding, presumably due to the lack of a positive charge that would reinforce the stacking interaction, as compared with the inhibitory potency of *bis*(7)-tacrine (IC₅₀ hAChE 0.81 nM)⁴⁸ and huprine–tacrine hybrids (IC₅₀ hAChE up to 0.31 nM),⁴⁰ and to the lack of specific interactions formed by the polar groups of the hydroxyanthraquinone moiety at the PAS, thereby preventing a net gain in potency compared to huprine Y, (±)-**4**.

Figure 1. Binding mode of compound (–)-**7h** (shown as yellow sticks) in the AChE gorge.

All the rhein–huprine hybrids turned out to be selective for hAChE over hBChE inhibition, with selectivities in the range 28–888. However, these hybrids still exhibited a moderately potent hBChE inhibitory activity, with IC_{50} values in the submicromolar to low micromolar range (Table 1). The most potent hBChE inhibitor of the series, ($\pm$)-**7d** (IC_{50} = 350 nM), was slightly less potent than the parent huprine (2-fold) but 50-fold more potent than the parent rhein, **3**.

Inhibition of AChE-induced A β aggregation. The occupancy of the AChE’s PAS by the rhein–huprine hybrids, predicted by docking simulations (see above), should result in a hindrance of the interaction of A β with AChE, and, hence, in a blockade of the A β proaggregating action of the enzyme. The inhibitory activity of these hybrids and the parent rhein and huprine Y against the AChE-induced aggregation of A β 40 was assessed using a thioflavin T-based fluorescent method.⁴⁹ Rhein–huprine hybrids exhibited a significant inhibitory activity against the AChE-

induced A β 40 aggregation, exhibiting percentages of inhibition ranging from 29 to 52% at a 100 μ M concentration, which are clearly superior to that found for the AChE's CAS inhibitor huprine Y, **4** (Table 1). These activities are similar to those reported for other families of dual binding site AChEIs,^{40,50–55} even though more potent inhibitors of the AChE-induced A β 40 aggregation have been described.^{56,57} In agreement with the hypothesis that both inhibitory activities are related to some extent to the ability of interacting with the AChE's PAS, the inhibitory activities of the rhein–huprine hybrids against the AChE-induced A β 40 aggregation roughly matches the SAR trends for hAChE inhibition, i.e. the shorter homologues are the most potent and the presence of a benzene ring in the linker is slightly detrimental for the activity.

Inhibition of self-induced A β aggregation. The inhibitory activity of the rhein–huprine hybrids against the spontaneous aggregation of A β 42 was determined *in vitro* using a thioflavin T-based fluorometric assay.⁵⁸ These compounds exhibit a significant A β 42 anti-aggregating activity, displaying percentages of inhibition in the narrow range 32–41% when tested at a concentration equal to 1/5 of that of A β 42 ([I] = 10 μ M, [A β 42] = 50 μ M), they being clearly more potent than the parent huprine Y (Table 1). Of note, rhein could not be evaluated because of solubility problems under the assay conditions.

Inhibition of human BACE-1 and molecular modeling study. BACE-1 catalyzes the first and rate-limiting step of the biosynthesis of A β from APP, thereby constituting a prime target in the search for disease-modifying anti-Alzheimer drugs.^{59,60} Strikingly, a number of dual binding site AChEIs have been found to be potent or moderately potent inhibitors of BACE-1, even if they were not purported to display this activity.⁶¹ In most cases, these compounds inhibit BACE-1 with low micromolar IC₅₀ values, as it happens with the prototypic dual binding site AChEI *bis*(7)-tacrine (IC₅₀ 7.5 μ M)⁶² or with huprine–tacrine heterodimers (IC₅₀ 4.9–7.3 μ M).⁴⁰

However, another dual binding site AChEI with more potent BACE-1 inhibitory activity, i.e. memoquin (IC_{50} 108 nM),⁵⁶ has also been reported.

In the light of these results, the novel racemic rhein–huprine hybrids were tested *in vitro* against human recombinant BACE-1 using a FRET assay.⁶³ Results clearly showed the importance of the linker chain length. The pentamethylene- to heptamethylene-linked hybrids ($\pm$)-**7a–c** were inactive for BACE-1 inhibition at a concentration of 1 μ M, whereas the longer homologues ($\pm$)-**7d**, ($\pm$)-**7f**, and ($\pm$)-**7g**, as well as ($\pm$)-**7h**, containing an aromatic ring in the linker, exhibited a moderately potent BACE-1 inhibitory activity, with IC_{50} values around 1–2 μ M (Table 1). Far beyond our expectations, the BACE-1 inhibitory activity in this series peaked for the nonamethylene-linked hybrid ($\pm$)-**7e**, with a remarkable IC_{50} value of 120 nM. Thus, ($\pm$)-**7e** was roughly equipotent to memoquin,⁵⁶ about 50-fold more potent than *bis*(7)-tacrine⁶² and other huprine-based hybrids.⁴⁰ Very interestingly, the BACE-1 inhibitory activity of ($\pm$)-**7e** seems to be slightly higher than that of the Lilly's BACE-1 inhibitor LY2811376, a very promising anti-Alzheimer drug candidate whose Phase I clinical trials were recently discontinued due to toxicity issues unrelated to BACE-1 inhibition,⁵⁹ even though a head-to-head test in the same assay conditions would be required to confirm that point. Thus, the combination of huprine Y and rhein in a new hybrid molecule with a proper linker chain led to a significant enlargement of the activity profile, namely the strong inhibitory activity on BACE-1 that is not present in any of the parent compounds.

The preceding findings made it worth to determine the binding mode of the hybrids to BACE-1. In a first step, this was accomplished by examining the druggable pockets present in the enzyme in order to determine their ability to accommodate the huprine and hydroxyanthraquinone moieties (see Experimental Section). Among the seven druggable pockets

shared by the four X-ray structures explored with the MDpocket program (PDB entries 1M4H, 1SGZ, 2OHL and 3CIB), only two of them, namely BS1, which encompasses the catalytic site, and BS2, which includes subsites P5–P7 (Figure S3, Supporting Information), were found to be suitable for binding of the huprine and hydroxyanthraquinone moieties present in hybrids. Furthermore, the distance between the centroids of the most populated clusters of huprine and hydroxyanthraquinone in these pockets was comprised between 7 and 11 Å, thus satisfying the geometrical criteria required for the tether in the rhein–huprine hybrids. Accordingly, docking of (+)-**7h** and (–)-**7h** was performed by exploring the volume defined by both BS1 and BS2 sites. Even though this compound is not the most potent within the series, it was chosen due to the limited number of rotatable bonds, which thus makes it easier to perform a more exhaustive exploration of the binding mode and examine the potential interaction at the two pockets identified separately for huprine and hydroxyanthraquinone moieties. Calculations led to a clear binding mode, in which the huprine moiety is accommodated at the BS1 pocket, and the hydroxyanthraquinone moiety fills the BS2 site. The structural integrity of this binding mode was supported by the analysis of four independent 50 ns molecular dynamics simulations run for both (+)-**7h** and (–)-**7h** bound to the enzyme (Figure 2). The huprine moiety remains tightly bound to BS1 in all cases, as expected from the electrostatic stabilization between the protonated aminoquinoline system and the catalytic dyad (Asp32 and Asp228), whereas the bicyclic system of the huprine moiety fills the hydrophobic pocket formed by residues Leu30, Phe108, Ile110 and Ile118 from subsites P2 and P3. On the other hand, the hydroxyanthraquinone moiety in (+)-**7h** exhibits a common binding mode, which involves the electrostatic interaction with Lys321, hydrogen bonds with the backbone of Phe322 and the side chain of Asn233, and hydrophobic contacts with Val309. For (–)-**7h**, the results show that, besides the preceding binding mode, the

hydroxyanthraquinone moiety can adopt an alternative arrangement, which mainly involves the direct electrostatic interaction with Arg307. On the basis of these findings, it is reasonable to expect that the larger flexibility afforded by the linker in **7e** will facilitate a proper accommodation to both BS1 and BS2 in BACE-1, thus explaining the increase in BACE-1 inhibitory activity compared to **7h**. Overall, this analysis suggests that BS2 may be exploited to find novel moieties leading to enhanced binding affinity and hence to hybrid compounds with more potent inhibitory activity against BACE-1.

Figure 2. Superposition of the last snapshots sampled in four independent MD simulations of hybrid compounds (A) (+)-**7h** (shown as orange sticks) and (B) (-)-**7h** (shown as yellow sticks) bound to BACE-1 (shown as cyan cartoon). Selected residues that mediate the binding of the hybrids are shown as sticks, and the “flap” loop is shown in blue.

***In Vitro* Biological Profiling of Enantiopure Rhein–Huprine Hybrids (+)- and (-)-7e.**

After the *in vitro* biological profiling of the racemic rhein–huprine hybrids, ($\pm$)-**7e** emerged as the most interesting member of the series, mainly by virtue of its outstanding BACE-1 inhibitory potency, but also because it was one of the most potent compounds of the series for hAChE, hBChE, AChE-induced and self-induced A β aggregation. To further optimize the lead of the series, the two enantiomers of **7e** were synthesized separately (see above) and subjected to *in vitro* biological profiling with the aim of unveiling potential enantioselective interactions with the different targets.

In agreement with the stereoselective interaction of the parent huprine Y, the levorotatory rhein–huprine hybrid (-)-**7e** was far more potent hAChE inhibitor than its enantiomer (+)-**7e**, with an enantioselectivity (1200-fold) somewhat more pronounced than that found for the parent huprine Y (430-fold) (Table 1), in agreement with the docking calculations (Figure 1). To further confirm the inhibition mechanism, a kinetic study of the interaction of (-)-**7e** with hAChE was also carried out. The mode of inhibition of (-)-**7e** was determined with the aid of Lineweaver-Burk double reciprocal plot. The interception of the lines in the Lineweaver-Burk plot above the x-axis (Figure 3) demonstrated that (-)-**7e** acts as a mixed-type inhibitor of hAChE.

The inhibitor dissociation constants K_i and K'_i , which denote the dissociation constant for the enzyme–inhibitor and enzyme–substrate–inhibitor complexes respectively (see Experimental Section), were estimated and resulted to be 2.65 and 3.41 nM, respectively.

Figure 3. Kinetic study on the mechanism of AChE inhibition by (–)-**7e**. Overlaid Lineweaver–Burk reciprocal plots of AChE initial velocity at increasing substrate concentration (ATCh, 0.56–0.11 mM) in the absence and in the presence (0.84–3.65 nM) of (–)-**7e** are shown. Lines were derived from a weighted least-squares analysis of the data points.

Contrary to the inhibition of hAChE by the enantiomers of huprines, for hBChE inhibition the eutomer is the dextrorotatory (7*R*,11*R*)-enantiomer, (+)-**4**. Thus, not unexpectedly, for hBChE inhibition, the rhein–huprine hybrid (+)-**7e** was 2-fold more potent than (–)-**7e**, the enantioselectivity being quite similar to that found for huprine Y (1.3-fold) (Table 1).

In contrast to hAChE and hBChE inhibition and in agreement with the idea that the enantioselective inhibition of the cholinesterase catalytic activity is related to the interaction with CAS, no enantioselectivity was found in the inhibition by (+)- and (–)-**7e** of the AChE-induced aggregation of Aβ40 (37–38% inhibition at 100 μM). Similarly, no differences were highlighted

for the inhibition of the self-induced aggregation of A β 42 (38–43% inhibition at 10 μ M), and hBACE-1 activity (IC₅₀ 80 nM in both enantiomers) by (+)- and (–)-**7e**.

***In Vitro* Blood–Brain Barrier Permeation Assay.** CNS drugs must be able to efficiently cross the blood–brain barrier (BBB). However, cell permeability and brain penetration constitute a major hurdle in the development of some classes of anti-Alzheimer drugs, particularly in BACE-1 inhibitors and in A β and tau aggregation inhibitors.^{4,31,60,64} Moreover, compounds designed by combination of two pharmacophoric moieties, such as the novel rhein–huprine hybrids, usually have molecular weights over 500, which might challenge their ability to cross cell membranes.^{65,66} However, different families of anti-Alzheimer hybrid compounds with molecular weights over 500 have shown good oral availability and/or brain permeability in *ex vivo* and *in vivo* studies in mice.^{12,40,67,68} Even though the potential of a CNS drug to enter into the brain also depends on a low P-glycoprotein efflux liability,^{69,70} a good brain permeability is a necessary requirement. In this work, the brain permeability of the novel rhein–huprine hybrids has been predicted through the widely known parallel artificial membrane permeation assay (PAMPA-BBB).⁷¹ The *in vitro* permeability (P_e) of racemic rhein–huprine hybrids through a lipid extract of porcine brain was determined using phosphate-buffered saline (PBS)/EtOH 70:30. Assay validation was carried out by comparing the experimental and literature permeability values of 14 commercial drugs (Table S1, Supporting Information), which gave a good linear correlation: P_e (exp) = 1.4525 P_e (lit) – 0.4926 (R^2 = 0.9199). Using this equation and the limits established by Di et al. for BBB permeation,⁷¹ the following ranges of permeability were established: P_e (10^{-6} cm s⁻¹) > 5.3 for compounds with high BBB permeation (CNS+); P_e (10^{-6} cm s⁻¹) < 2.4 for compounds with low BBB permeation (CNS–); and 5.3 > P_e (10^{-6} cm s⁻¹) > 2.4 for compounds with uncertain BBB permeation (CNS+/-). All the tested

rhein–huprine hybrids were predicted to be able to cross BBB, their measured P_e values being far above the threshold for high BBB permeation (Table 1), as it is also the case for the parent huprine Y, ($\pm$)-**4**, but not for rhein, **3**, for which an uncertain BBB permeability was predicted. Indeed, rhein is therapeutically used against arthritis in the form of the more lipophilic and cell permeable doubly acetylated prodrug diacerein.

Inhibition of A β 42 and Tau Aggregation in Intact *Escherichia coli* Cells. The aggregation of two amyloidogenic proteins, namely A β and tau, plays a pivotal role in the pathological network of AD. Protein aggregation also occurs during the production of heterologous proteins in bacteria, leading to the formation of insoluble inclusion bodies (IBs). Because IBs contain highly ordered amyloid-like structures and their formation seems to share mechanistic features with amyloid self-assembly, they have been recently proposed as a model to study amyloid aggregation.^{72,73} We have recently developed a methodology that allows a fast, simple, and inexpensive evaluation of the anti-aggregating activity of putative inhibitors, which is based on the *in vivo* staining with thioflavin S of IBs in intact *E. coli* cells that overexpress a given amyloidogenic protein in the presence and absence of the inhibitors, and monitoring of the corresponding changes in the fluorescence of thioflavin S.⁷⁴ The applicability of this method to the screening of both A β 42 and tau aggregation inhibitors has been recently demonstrated.⁷⁴ Worthy of note, the A β 42 anti-aggregating activities determined through this method for a number of known active and inactive inhibitors were very similar to those previously reported in *in vitro* assays using synthetic peptides, thereby validating this methodology.⁷⁴ A potential limitation of this method lies in the fact that only compounds that are membrane permeable can be detected, whereas potential hits that are active as aggregation inhibitors but unable to cross biological membranes would remain undetected. In the light of the results obtained in the

PAMPA-BBB assays this should not be an issue for the novel rhein–huprine hybrids, which were tested for their ability to inhibit both A β 42 and tau aggregation in intact *E. coli* cells (Table 2).

Table 2. Inhibitory Activities of the Hydrochlorides of Rhein–Huprine Hybrids and Reference Compounds Toward A β 42 and Tau Aggregation in Intact *E. coli* Cells^a

compd	A β 42 aggregation (% inhibition) ^b	tau aggregation (% inhibition) ^b
($\pm$)- 7a	68.0 $\pm$ 0.8	43.7 $\pm$ 1.1
($\pm$)- 7b	68.4 $\pm$ 8.9	30.9 $\pm$ 3.1
($\pm$)- 7c	63.2 $\pm$ 9.8	52.0 $\pm$ 1.1
($\pm$)- 7d	50.2 $\pm$ 5.6	29.2 $\pm$ 1.3
($\pm$)- 7e	47.9 $\pm$ 14.5	29.6 $\pm$ 8.5
(+)- 7e	58.6 $\pm$ 7.1	24.9 $\pm$ 0.4
(-)- 7e	47.2 $\pm$ 5.1	34.3 $\pm$ 0.1
($\pm$)- 7f	43.3 $\pm$ 4.1	57.1 $\pm$ 1.9
($\pm$)- 7g	40.7 $\pm$ 2.2	46.3 $\pm$ 0.7
($\pm$)- 7h	57.9 $\pm$ 0.0	23.1 $\pm$ 5.1
3	49.9 $\pm$ 6.4	40.8 $\pm$ 0.7
($\pm$)- 4	na ^c	na ^c

^a Values are expressed as mean $\pm$ standard error of the mean (SEM) of four independent experiments (n=4). ^b % inhibition with inhibitor at 10 μ M. ^c Not active.

All the rhein–huprine hybrids exhibited a moderately potent A β 42 anti-aggregating activity in *E. coli* cells, with percentages of inhibition in the range 41–68% at 10 μ M, and, hence, with IC₅₀ values below 10 μ M in most cases (Table 2). Some clear SAR trends became apparent, namely a higher A β 42 anti-aggregating activity for the hybrids with the shortest oligomethylene linkers

and for the dextrorotatory over the levorotatory enantiomer of **7e**. The presence of an aromatic ring within the linker seemed not to be detrimental for A β 42 anti-aggregating activity, hybrid ($\pm$)-**7h** displaying a notable 58% inhibition at 10 μ M, albeit slightly lower than that of the oligomethylene-linked hybrid of similar tether length, ($\pm$)-**7b**. Interestingly, the A β 42 anti-aggregating activities measured for the rhein–huprine hybrids in intact *E. coli* cells were of similar magnitude than those determined in the *in vitro* assay, albeit somewhat higher in most cases. Regarding the parent compounds, huprine Y was found to be essentially inactive in *E. coli* as it was in the *in vitro* assay, whereas rhein, which could not be evaluated *in vitro* for solubility problems under the assay conditions, was found to exhibit an interesting 50% inhibition at 10 μ M in *E. coli* cells.

In agreement with our design strategy, the parent hydroxyanthraquinone derivative rhein was found to be active as inhibitor of tau aggregation in intact *E. coli* cells (41% inhibition at 10 μ M), and this was also the case for the novel rhein–huprine hybrids (23–57% inhibition at 10 μ M), but not for the parent huprine Y, which was inactive, as expected (Table 2). For the inhibitory activity on tau aggregation, the presence of an aromatic ring within the linker seemed to be detrimental and the levorotatory enantiomer of **7e** was more potent than (+)-**7e**. However, a clear SAR trend regarding the length of the linker could not be derived.

The dual A β 42 and tau anti-aggregating action of the novel rhein–huprine hybrids seems very promising for anti-Alzheimer drug candidates. These results are also important in the context of the treatment of amyloidoses in general, insofar as they support the increasingly accepted notion that all diseases that involve the pathological aggregation of a particular protein might share common mechanisms and common therapeutic interventions.^{75,76} Indeed, other examples of

compounds able to inhibit the aggregation of several amyloidogenic proteins have been reported.^{40,75,77}

Protection Against the Synaptic Failure Induced by A β Oligomers in Hippocampal Slices of C57bl6 Mice. In the light of the outstanding multi-target pharmacological profile found *in vitro* and in *E. coli* cells for the enantiopure rhein–huprine hybrids (+)- and (–)-**7e**, involving the modulation of crucial targets of the pathological network of AD, these compounds were selected as leads for further pharmacological characterization.

Even though all aggregated forms of A β are likely pathological,⁷⁸ it has been reported that A β oligomers (A β -o) cause an impairment of synaptic functions observed in AD patients and mouse models of AD.⁷⁹ Because the target of A β -o is located at the postsynaptic region,^{80,81} processes associated to synaptic plasticity as long-term potentiation (LTP) are critically altered, leading to diminished synaptic strength and efficacy. These dysfunctions are correlated with reduction in the levels of synaptic proteins that participate in events associated with learning and memory such as glutamatergic receptors and scaffold proteins.⁸² To further assess the disease-modifying effects of the rhein–huprine hybrids (+)- and (–)-**7e**, their potential protective effect on synaptic integrity was assessed in hippocampal slices of 2-month-old C57bl6 male mice incubated with A β 42 oligomers.

Electrophysiological Assays. Firstly, electrophysiological registers of the hippocampal slices were used to evaluate synaptic transmission through the LTP. Hippocampal slices were incubated with A β 42 oligomers (1 μ M) 10 min prior and 10 min after LTP induction. No induction of LTP was observed in hippocampal slices incubated either with A β 42 oligomers alone or with A β 42 oligomers and (–)-**7e** (10 μ M) (Figure 4A, white circles and light grey circles, respectively). On the contrary, treatment with (+)-**7e** (10 μ M) led to a 200% LTP

induction (Figure 4A, dark grey circles), similarly to the effect found in the control slices treated only with artificial cerebrospinal fluid (ACSF). In Figure 4B, the quantification at min 60 of the field excitatory postsynaptic potentials (fEPSP) are shown. Treatment with (+)-**7e** led to a 200% magnitude response when compared to slices treated either with A β 42 oligomers alone or with A β 42 oligomers and (-)-**7e**. These results indicate that (+)-**7e** protects against the synaptic failure induced by acute treatments with A β 42 oligomers, within the context of LTP induction.

Figure 4. Electrophysiological assays in hippocampal slices of 2-month-old C57bl6 mice incubated with A β oligomers (1 μ M) and (+)- or (-)-**7e** (10 μ M). (A) Electrophysiological registers. (B) Quantification of field excitatory postsynaptic potentials (fEPSP).

Synaptic Protein Levels. Changes in the synaptic protein levels of hippocampal slices of 2-month-old C57bl6 mice treated with A β 42 oligomers and different concentrations of (+)- and (-)-**7e** (1–100 μ M) for 1 h were studied by densitometric analysis of western blots gels. Treatment of the hippocampal slices with A β 42 oligomers (Figure 5, black bars) produced a decrease in the levels of the postsynaptic proteins GluA2 (a subunit of the AMPA receptor) and PSD95 (postsynaptic density protein 95, a scaffold protein within the postsynaptic density), and of the

presynaptic proteins synapsin (SYN) and the vesicular glutamate transporter 1 (VGlut1) in comparison with control slices (Figure 5, white bars), whereas the levels of the presynaptic protein synaptophysin (SYP) remained unchanged. In hippocampal slices co-treated with (–)-**7e** (Figure 5A) a neuroprotective effect was observed. Evident increases in the levels of GluA2 and PSD95 were observed at 10 and 100 μ M, and in the levels of VGlut1 for every used concentration of (–)-**7e**, whereas increases in the levels of SYN and SYP were found only at the highest assayed concentration or were not observed. Co-treatment with (+)-**7e** produced increases, albeit more modest, in the levels of GluA2, PSD95 and SYN with every used concentration, whereas it had no effect on the levels of VGlut1 and SYP. Thus, both (+)- and (–)-**7e** exert a protective effect on synaptic proteins related with the stability of the synapses and the synaptic plasticity in the hippocampus.

Prevention of the A β -o-induced loss of synaptic proteins by treatment of hippocampal slices of 2-month-old C57bl6 mice with (+)-**7e** is in agreement with the results found in the electrophysiological assays and with the anti-amyloid activities found in the *in vitro* and in *E. coli* assays, namely its potent BACE-1 and A β aggregation inhibitory activities. Thus, the protective effects of (+)-**7e** and also (–)-**7e** on synaptic integrity might be ascribed to a reduction of the amount of A β aggregated species or to a reduction of A β production through the inhibition of APP processing, although participation of alternative or additional mechanisms can not be ruled out. For example, it has been reported that the increased levels of neurotransmitter acetylcholine due to AChE inhibition may promote changes in synaptic plasticity improving LTP.^{83,84} Indeed, (–)-**7e**, the eutomer for hAChE inhibition, seems to be more potent regarding the preservation of synaptic proteins, even though it does not improve LTP. Different mechanisms are likely involved in the neuroprotective effect shown by (+)- and (–)-**7e**.

A

B

Figure 5. Synaptic protein levels of hippocampal slices of 2-month-old C57bl6 mice incubated with A β oligomers and (-)-7e (A) or (+)-7e (B).

***In Vivo* A β Lowering Effect.** Among the different interesting biological activities found *in vitro*, in *E. coli*, and *ex vivo* for the enantiopure rhein–huprine hybrids (+)-**7e** and (–)-**7e**, likely the most notable effect is the potent inhibition of BACE-1 (IC₅₀ 80 nM). To gain further insight into the relevance of this activity, we have determined the *in vivo* effects of (+)-**7e** and (–)-**7e** on the level of total soluble A β oligomers, thought to be involved in synapse destruction and memory impairment in AD.⁸⁵ To this end, we used a well-established animal model of AD, namely the double transgenic mice APP-PS1 (see Experimental Section). This aggressive model of AD has been characterized both in the generation of senile plaques in the brain and in their behavioral changes,⁸⁶ describing the age at which these animals exhibit early senile plaques and cognitive impairment from 7 months of age, which occur mainly in the cortex and hippocampus.⁸⁶ 6- and 10-month-old APP-PS1 mice were treated with 2 mg/kg of (+)-**7e** or (–)-**7e** intraperitoneally three times per week for 4 weeks to evaluate the effects of these compounds on the production and aggregation of A β species in two different stages of the AD model; initial and severe, respectively. The soluble protein fraction from hippocampus homogenates of control and treated APP-PS1 mice was separated in tris-tricine gels, each lane being a different mouse, and specific mouse anti- β peptide 6E10 antibody was used, followed by immunoblot analysis.

Young (7-month-old after the treatment; n=3 control; n=3 (+)-**7e** treated; and n=2 (–)-**7e** treated APP-PS1 mice) and aged (11-month-old after the treatment; n=3 control; n=3 (+)-**7e** treated; and n=3 (–)-**7e** treated APP-PS1 mice) APP-PS1 mice exhibit several forms of A β peptides, including hexamers (ca. 24 kDa) and higher levels of A β dodecamers (ca. 56 kDa). Interestingly, young and aged APP-PS1 mice treated with (+)-**7e** showed a significant decrease in the levels of dodecamers, whereas only in young mice a reduction in the levels of hexamers was observed (Figures 6 and 7). In addition, young APP-PS1 mice treated with (–)-**7e** showed a

tendency toward decreased levels of both oligomeric species, whereas aged APP-PS1 mice treated with (–)-**7e** showed a diminution in the levels of dodecamers, without altering the levels of hexamers (Figures 6 and 7). The results observed in 11-month-old mice might be due to the high load of A β present in aging APP-PS1 mice. However the reduction in the levels of dodecamers, considered to be synaptotoxic, is very important, because it might be indicative of a reversion in the neurodegenerative process observed in the animal. More studies are necessary to corroborate this possibility. Together, these results suggest that rhein–huprine hybrids (+)-**7e** and (–)-**7e** either could favor the formation of senile plaques and the decrease of the soluble A β forms or prevent the APP processing and the subsequent aggregation, through inhibition of BACE-1. To corroborate the last hypothesis, the levels of APP protein were analyzed in the same gel described before where each lane represents an independent animal. The results showed that in young and aged APP-PS1 mice treated with (+)-**7e** and (–)-**7e** the levels of mature APP (ca. 100 KDa) are increased (Figures 6 and 7), indicating that these rhein–huprine hybrids are able to prevent APP processing, A β production and aggregation.

Figure 6. Soluble A β and APP protein levels of hippocampal samples from 7-month-old APP-PS1 mice treated with (-)-7e or (+)-7e for 4 weeks. Lanes 1-3 show hippocampus lysates from control mice (n=3), lanes 4-6 show hippocampus lysates from (+)-7e treated mice (n=3) and lanes 7-8 show hippocampus lysates from (-)-7e treated mice (n=2), each lane being an independent animal.

Figure 7. Soluble A β and APP protein levels of hippocampal samples from 11-month-old APP-PS1 mice treated with (-)-7e or (+)-7e for 4 weeks. Lanes 1-3 show hippocampus lysates from control mice (n=3), lanes 4-6 show hippocampus lysates from (+)-7e treated mice (n=3) and lanes 7-9 show hippocampus lysates from (-)-7e treated mice (n=3), each lane being an independent animal.

CONCLUSIONS

We have synthesized a series of racemic and enantiopure hybrid compounds that combine a unit of the hydroxyanthraquinone drug rhein and a unit of the potent AChE's CAS inhibitor huprine Y, connected through penta- to undeca-methylene or 1,4-phenylene-bis(methylene)

linkers. These hybrids were purportedly designed to exhibit a multi-target profile of interest for the efficient management of AD, which included the inhibitory actions expected for dual binding site inhibitors of AChE against the catalytic and the A β -proaggregating activities of this enzyme and the tau anti-aggregating effect known for some hydroxyanthraquinone compounds similar to rhein. In agreement with the design rationale, the novel rhein–huprine hybrids are endowed with very potent hAChE inhibitory activity and moderately potent inhibitory activities against hAChE-induced and self-induced aggregation of A β and against hBChE. Far beyond our expectations, some rhein–huprine hybrids additionally display a very potent BACE-1 inhibitory activity. Of note, these hybrids have been predicted to be able to cross BBB using an artificial membrane model, likely having the ability to exert the same actions *in vivo*. Both cell permeability and the A β anti-aggregating activity found *in vitro* for the rhein–huprine hybrids, as well as their expected tau anti-aggregating activity, have been confirmed by some assays based on the direct thioflavin S staining of IBs in intact *E. coli* cells that were genetically altered to overexpress A β 42 and tau protein. On the basis of the biological profiling *in vitro* and in *E. coli*, (+)- and (–)-7e have been selected as lead rhein–huprine hybrids and their protective effects against the synaptic failure induced by A β oligomers in hippocampal slices of 2-month-old C57bl6 mice have been assessed. These studies have shown that both hybrids prevent the loss of synaptic proteins. Additionally, the beneficial effects of (+)-7e on synaptic integrity were apparent in the context of LTP induction. Finally, *in vivo* experiments with transgenic APP-PS1 mice have shown that (+)- and (–)-7e are able to lower the levels of hippocampal total soluble A β and increase the levels of APP both in initial and advanced stages of this AD model, thus suggesting a reduction of APP processing, as expected from their potent BACE-1 inhibitory activity. Overall, the novel rhein–huprine hybrids (+)- and (–)-7e emerge as very promising

multi-target anti-Alzheimer drug candidates with potential to positively modify the underlying mechanisms of this disease.

EXPERIMENTAL SECTION

Chemistry. Melting points were determined in open capillary tubes with a MFB 595010M Gallenkamp melting point apparatus. 400 MHz ^1H /100.6 MHz ^{13}C NMR spectra, and 500 MHz ^1H /125.7 MHz ^{13}C NMR spectra were recorded on 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 given for the NMR spectra of the new compounds have been carried out on the basis of DEPT, COSY $^1\text{H}/^1\text{H}$ (standard procedures), and COSY $^1\text{H}/^{13}\text{C}$ (gHSQC or gHMBC sequences) experiments. The *syn* (*anti*) notation of the protons at position 13 of the huprine moiety of the hybrids means that the corresponding proton at position 13 is on the same (different) side of the quinoline moiety with respect to the cyclohexene ring. IR spectra were run on a Perkin-Elmer Spectrum RX I spectrophotometer. Absorption values are expressed as wave-numbers (cm^{-1}); only significant absorption bands are given. 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. Column chromatography was performed on silica gel 60 AC.C (40–60 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 KMnO_4 . NMR spectra of all of the new compounds were performed at the Centres Científics i Tecnològics of the University of Barcelona (CCiTUB), while elemental analyses and high resolution mass spectra were carried out at the Microanalysis Service of the IIQAB (CSIC,

Barcelona, Spain) with a Carlo Erba 1106 analyzer, and at the CCiTUB with a LC/MSD TOF Agilent Technologies spectrometer, respectively. The synthetic procedures for the preparation of the intermediate nitriles **5a–h** and amines **5a–h** are exemplified through the synthesis of **5a** and **6a**. The synthesis of the rest of nitriles and amines is included in the Supporting Information. The analytical samples of all of the rhein–huprine hybrids which were subjected to pharmacological evaluation possess a purity $\geq 95\%$ as evidenced by their elemental analyses.

($\pm$)-5-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]pentanenitrile [($\pm$)-5a**].** A suspension of ($\pm$)-huprine Y, ($\pm$)-**4** (300 mg, 1.06 mmol) and finely powdered KOH (85% purity, 139 mg, 2.11 mmol), and 4 Å molecular sieves in anhyd DMSO (6 mL) was stirred, heating every 10 min approximately with a heat gun for 1 h and at rt one additional hour, and then treated with 5-bromovaleronitrile (0.15 mL, 208 mg, 1.28 mmol). The reaction mixture was stirred at rt overnight, diluted with 5N NaOH (25 mL) and extracted with EtOAc (3×40 mL). The combined organic extracts were washed with H₂O (3×50 mL), dried over anhyd Na₂SO₄, and evaporated under reduced pressure to give a yellow oil (469 mg), which was purified by column chromatography (40–60 µm silica gel, CH₂Cl₂/MeOH/50% aq. NH₄OH mixtures, gradient elution). On elution with CH₂Cl₂/50% aq. NH₄OH 100:0.2, the *N,N*-dialkylated derivative ($\pm$)-**8a** (78 mg, 16% yield) was isolated as a yellow oil. On elution with CH₂Cl₂/MeOH/50% aq. NH₄OH 99:1:0.2, nitrile ($\pm$)-**5a** (273 mg, 70% yield) was isolated as a beige solid; $R_{f[(\pm)\text{-}5a]}$ 0.50; $R_{f[(\pm)\text{-}8a]}$ 0.77 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

A solution of ($\pm$)-**5a** (52 mg, 0.14 mmol) in CH₂Cl₂ (4.5 mL) was filtered through a 0.2 µm PTFE filter, treated with methanolic HCl (1.40N, 0.30 mL) and evaporated under reduced pressure. The resulting solid was washed with pentane (3×2 mL) to give, after drying at 65 °C/2 Torr for 48 h, ($\pm$)-**5a**·HCl (58 mg) as a yellow solid: mp 181–183 °C (CH₂Cl₂/MeOH 94:6); IR

(KBr) ν 3500–2500 (max at 3364, 3254, 3049, 3014, 2926, 2885, 2651, N-H, N⁺-H and C-H st), 2243 (CN st), 1635, 1629, 1602, 1582, 1570, 1562 (ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 1.59 (s, 3H, 9'-CH₃), 1.79 (tt, $J \approx J' \approx 7.2$ Hz, 2H, 3-H₂), 1.93 (d, $J=17.6$ Hz, 1H, 10'-H_{endo}), superimposed in part 1.92–1.98 (m, 1H, 13'-H_{syn}), 2.01 (tt, $J \approx J' \approx 7.2$ Hz, 2H, 4-H₂), 2.09 (dm, $J=12.8$ Hz, 1H, 13'-H_{anti}), 2.56 (t, $J=7.2$ Hz, 2H, 2-H₂), superimposed in part 2.53–2.60 (m, 1H, 10'-H_{exo}), 2.77 (m, 1H, 7'-H), 2.88 (ddd, $J=18.0$ Hz, $J'=J''=1.6$ Hz, 1H, 6'-H_{endo}), 3.22 (dd, $J=18.0$ Hz, $J'=5.2$ Hz, 1H, 6'-H_{exo}), 3.47 (m, 1H, 11'-H), 4.03 (t, $J=7.6$ Hz, 2H, 5-H₂), 4.85 (s, NH and ⁺NH), 5.59 (br d, $J=4.4$ Hz, 1H, 8'-H), 7.57 (dd, $J=9.2$ Hz, $J'=2.0$ Hz, 1H, 2'-H), 7.78 (d, $J \approx 2.0$ Hz, 1H, 4'-H), 8.40 (d, $J \approx 9.2$ Hz, 1H, 1'-H); ¹³C NMR (100.6 MHz, CD₃OD) δ 17.1 (CH₂, C2), 23.4 (CH₃, 9'-CH₃), 23.9 (CH₂, C3), 27.3 (CH, C11'), 27.9 (CH, C7'), 29.3 (CH₂, C13'), 30.3 (CH₂, C4), 36.1 (CH₂), 36.2 (CH₂) (C6', C10'), 48.8 (CH₂, C5), 115.8 (C, C12a'), 117.9 (C, C11a'), 119.2 (CH, C4'), 120.9 (C, CN), 125.1 (CH, C8'), 126.8 (CH, C2'), 129.4 (CH, C1'), 134.6 (C, C9'), 140.3 (C, C3'), 141.0 (C, C4a'), 151.5 (C, C5a'), 157.0 (C, C12'). HRMS (ESI) calcd for (C₂₂H₂₄³⁵ClN₃ + H⁺): 366.1732, found 366.1729.

A solution of (±)-**8a** (78 mg, 0.17 mmol) in CH₂Cl₂ (4.5 mL) was filtered through a 0.2 μ m PTFE filter, treated with methanolic HCl (0.65N, 0.64 mL) and evaporated under reduced pressure. The resulting solid was washed with pentane (3×2 mL) to give, after drying at 65 °C/2 Torr for 48 h, (±)-**8a**·HCl (80 mg) as a yellow solid: mp 196–198 °C (CH₂Cl₂/MeOH 88:12); IR (KBr) ν 3500–2450 (max at 3430, 3044, 2919, 2875, 2464, N⁺-H and C-H st), 2243 (CN st), 1632, 1604, 1574 (ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 1.59 (s, 3H, 9'-CH₃), 1.65 (tt, $J \approx J' \approx 7.2$ Hz, 4H, 3-H₂), 1.72–1.98 (complex signal, 4H, 4-H₂), 2.00 (br d, $J=17.6$ Hz, 1H, 10'-H_{endo}), superimposed in part 2.06 (dm, $J \approx 12.8$ Hz, 1H, 13'-H_{syn}), 2.12 (dm, $J=12.8$ Hz, 1H, 13'-H_{anti}), 2.48 (t, $J=7.2$ Hz, 4H, 2-H₂), 2.69 (dd, $J=17.6$ Hz, $J'=5.6$ Hz, 1H, 10'-H_{exo}),

2.82 (m, 1H, 7'-H), 3.17 (ddd, $J=18.0$ Hz, $J'\approx J''\approx 1.8$ Hz, 1H, 6'-H_{endo}), 3.39 (dd, $J=18.0$ Hz, $J'=5.6$ Hz, 1H, 6'-H_{exo}), superimposed in part 3.73 (m, 1H, 11'-H), 3.73 (ddd, $J=13.6$ Hz, $J'=9.6$ Hz, $J''=5.6$ Hz, 2H) and 3.80 (ddd, $J=13.6$ Hz, $J'=9.2$ Hz, $J''=6.0$ Hz, 2H) (5-H₂), 4.84 (s, ⁺NH), 5.68 (br d, $J=4.4$ Hz, 1H, 8'-H), 7.72 (dd, $J=9.2$ Hz, $J'=2.4$ Hz, 1H, 2'-H), 7.99 (d, $J=2.4$ Hz, 1H, 4'-H), 8.26 (d, $J=9.2$ Hz, 1H, 1'-H); ¹³C NMR (100.6 MHz, CD₃OD) δ 17.1 (CH₂, C2), 23.3 (CH₃, 9'-CH₃), 23.9 (CH₂, C3), 27.6 (CH, C7'), 28.6 (CH₂, C13'), 28.9 (CH₂, C4), 29.7 (CH, C11'), 37.7 (CH₂, C6'), 38.7 (CH₂, C10'), 55.1 (CH₂, C5), 120.0 (CH, C4'), 120.9 (C, CN), 125.5 (C, C12a'), 125.7 (CH, C8'), 192.2 (CH, C2'), 130.1 (CH, C1'), 132.5 (C, C11a'), 134.3 (C, C9'), 140.36 (C), 140.43 (C) (C3', C4a'), 157.6 (C, C5a'), 163.9 (C, C12'); HRMS (ESI) calcd for (C₂₇H₃₁³⁵ClN₄ + H⁺) 447.2310, found 447.2309.

(±)-N-(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[b]quinolin-12-yl)pentane-1,5-diamine [(±)-6a]. To a suspension of LiAlH₄ (0.17 g, 4.47 mmol) in anhyd Et₂O (63 mL), nitrile (±)-5a (0.76 g, 2.08 mmol) was added, and the reaction mixture was stirred at rt overnight. The resulting mixture was treated with wet Et₂O (200 mL) and evaporated under reduced pressure. The solid was suspended in CH₂Cl₂, filtered through Celite[®] and evaporated under reduced pressure, to give amine (±)-6a (750 mg, 98% yield) as a yellow oil; *R_f* 0.03 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

A solution of (±)-6a (50 mg, 0.14 mmol) in CH₂Cl₂ (3.5 mL) was filtered through a 0.2 μ m PTFE filter, treated with methanolic HCl (1.4N, 0.87 mL), and evaporated under reduced pressure. The resulting solid was washed with pentane (3×2 mL) to give, after drying at 65 °C/2 Torr for 48 h, (±)-6a·2HCl (54 mg) as a yellow solid: mp 193–194 °C (CH₂Cl₂/MeOH 80:20); IR (KBr) ν 3500–2500 (max at 3396, 3253, 2925, 2856, N-H, N⁺-H and C-H st), 1628, 1583, 1570, 1558 (ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 1.55 (tt, $J\approx J'\approx 7.6$ Hz, 2H, 3-

H₂), 1.59 (s, 3H, 9'-CH₃), 1.76 (m, 2H, 4-H₂), 1.90–2.02 (complex signal, 4H, 10'-H_{endo}, 13'-H_{syn}, 2-H₂), 2.09 (dm, $J=12.8$ Hz, 1H, 13'-H_{anti}), 2.57 (ddm, $J=17.6$ Hz, $J'=6.0$ Hz, 1H, 10'-H_{exo}), 2.78 (m, 1H, 7'-H), 2.87 (br d, $J=18.0$ Hz, 1H, 6'-H_{endo}), 2.97 (t, $J=7.6$ Hz, 2H, 5-H₂), 3.21 (dd, $J=18.0$ Hz, $J'=5.6$ Hz, 1H, 6'-H_{exo}), 3.49 (m, 1H, 11'-H), 4.01 (t, $J\approx 7.6$ Hz, 2H, 1-H₂), 4.85 (s, NH and ⁺NH), 5.59 (br d, $J=4.4$ Hz, 1H, 8'-H), 7.57 (dd, $J=9.2$ Hz, $J'=2.0$ Hz, 1H, 2'-H), 7.78 (d, $J=2.0$ Hz, 1H, 4'-H), 8.41 (d, $J=9.2$ Hz, 1H, 1'-H); ¹³C NMR (100.6 MHz, CD₃OD) δ 23.5 (CH₃, 9'-CH₃), 24.7 (CH₂, C3), 27.3 (CH, C11'), 27.9 (CH, C7'), 28.2 (CH₂, C4), 29.3 (CH₂, C13'), 30.8 (CH₂, C2), 36.0 (CH₂), 36.2 (CH₂) (C6', C10'), 40.5 (CH₂, C5), 49.4 (CH₂, C1), 115.7 (C, C12a'), 117.8 (C, C11a'), 119.2 (CH, C4'), 125.1 (CH, C8'), 126.8 (CH, C2'), 129.4 (CH, C1'), 134.6 (C, C9'), 140.3 (C, C3'), 141.0 (C, C4a'), 151.4 (C, C5a'), 156.9 (C, C12'); HRMS (ESI) calcd for (C₂₂H₂₈³⁵ClN₃ + H⁺) 370.2045, found 370.2041.

(±)-N-{5-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]pentyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide [(±)-**7a**]. A suspension of rhein, **3** (477 mg, 1.68 mmol), in anhyd CH₂Cl₂ (6 mL) was cooled to 0 °C with an ice bath, and treated dropwise with freshly distilled Et₃N (0.47 mL, 341 mg, 3.38 mmol) and ClCO₂Et (0.16 mL, 182 mg, 1.68 mmol). The resulting solution was stirred at 0 °C for 30 min and treated with a solution of amine (±)-**6a** (621 mg, 1.68 mmol) in anhyd CH₂Cl₂ (6 mL). The reaction mixture was stirred at rt for 3 days, diluted with 10% aq. Na₂CO₃ (50 mL), the phases were separated and the aqueous phase was extracted with CH₂Cl₂ (3×35 mL). The combined organic extracts were dried over anhyd Na₂SO₄ and evaporated under reduced pressure, to give a red solid (770 mg), which was purified by column chromatography (40–60 μ m silica gel, CH₂Cl₂/MeOH/50% aq. NH₄OH mixtures, gradient elution). On elution with

CH₂Cl₂/MeOH/50% aq. NH₄OH 99:1:0.2, hybrid (±)-**7a** (140 mg, 13% yield) was isolated as a red solid; *R_f* 0.45 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

A solution of hybrid (±)-**7a** (79 mg, 0.12 mmol) in CH₂Cl₂ (4 mL) was filtered through a 0.2 μm PTFE filter, treated with methanolic HCl (1.4N, 0.26 mL) and evaporated under reduced pressure. The resulting solid was washed with pentane (3×2 mL) to give, after drying at 65 °C/2 Torr for 48 h, (±)-**7a**·HCl (80 mg) as a yellow solid: mp 213–215 °C (CH₂Cl₂/MeOH 94:6); IR (KBr) ν 3500–2500 (max at 3224, 3047, 3005, 2923, 2852, O-H, N-H, N⁺-H and C-H st), 1669, 1628, 1582, 1565 (C=O, ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 1.54–1.64 (complex signal, 2H, 3'-H₂), superimposed in part 1.59 (s, 3H, 9''-CH₃), 1.77 (tt, *J*≈*J'*≈7.0 Hz, 2H, 2'-H₂), superimposed in part 1.90–2.00 (complex signal, 3H, 4'-H₂, 13''-H_{syn}), 1.94 (d, *J*≈17.0 Hz, 1H, 10''-H_{endo}), 2.08 (br d, *J*=12.5 Hz, 1H, 13''-H_{anti}), 2.56 (br dd, *J*=17.0 Hz, *J'*=4.0 Hz, 1H, 10''-H_{exo}), 2.77 (m, 1H, 7''-H), 2.84 (d, *J*=17.5 Hz, 1H, 6''-H_{endo}), 3.18 (dd, *J*=17.5 Hz, *J'*=5.5 Hz, 1H, 6''-H_{exo}), 3.42–3.52 (complex signal, 3H, 1'-H₂, 11''-H), 4.00 (dt, *J*=*J'*=7.5 Hz, 2H, 5'-H₂), 4.87 (s, NH, ⁺NH and OH), 5.58 (br d, *J*=4.5 Hz, 1H, 8''-H), 7.35 (dd, *J*=7.5 Hz, *J'*=2.0 Hz, 1H, 6-H), 7.47 (br d, *J*=8.5 Hz, 1H, 2''-H), 7.62 (br s, 2H, 3-H, 4''-H), 7.76 (d, *J*=7.5 Hz, 1H, 8-H), 7.78 (dd, *J*=*J'*=7.5 Hz, 1H, 7-H), 8.04 (s, 1H, 1-H), 8.34 (d, *J*=8.5 Hz, 1H, 1''-H); ¹³C NMR (125.7 MHz, CD₃OD) δ 23.5 (CH₃, 9''-CH₃), 25.1 (CH₂, C3'), 27.3 (CH, C11''), 27.9 (CH, C7''), 29.3 (CH₂, C13''), 29.9 (CH₂, C2'), 31.0 (CH₂, C4'), 36.1 (CH₂, C6''), 36.2 (CH₂, C10''), 40.7 (CH₂, C1'), 49.5 (CH₂, C5'), 115.7 (C, C12a''), 117.0 (C, C10a), 117.6 (C, C11a''), 118.6 (C, C4a), 118.8 (CH, C1), 119.1 (CH, C4''), 120.9 (CH, C8), 123.7 (CH, C3), 125.1 (CH, C8''), 125.8 (CH, C6), 126.7 (CH, C2''), 129.3 (CH, C1''), 134.6 (C, C9''), 134.8 (C, C8a), 135.2 (C, C9a), 138.8 (CH, C7), 140.2 (C, C3''), 140.9 (C, C4a''), 143.4 (C, C2), 151.4 (C, C5a''), 156.7 (C, C12''), 163.4 (C, C4), 163.7 (C, C5), 167.3 (C, CONH), 182.2 (C, C9), 193.7 (C, C10);

HRMS (ESI) calcd for $(C_{37}H_{34}^{35}ClN_3O_5 + H^+)$ 636.2260, found 636.2255. Anal. $(C_{37}H_{34}ClN_3O_5 \cdot HCl \cdot 1.5H_2O)$ C, H, N, Cl.

(±)-N-{6-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]hexyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide

[(±)-7b]. It was prepared as described for (±)-7a. From rehn, **3** (1.19 g, 4.18 mmol), and amine (±)-6b (1.60 g, 4.18 mmol), a red solid (2.00 g) was obtained and purified by column chromatography (40–60 μ m silica gel, hexane/EtOAc/MeOH/Et₃N mixtures, gradient elution). On elution with hexane/EtOAc/MeOH/Et₃N 10:90:0:0.2 to 0:80:20:0.2, hybrid (±)-7b (647 mg, 24% yield) was isolated as a red solid; R_f 0.54 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

(±)-7b·HCl: mp 203–205 °C (CH₂Cl₂/MeOH 89:11); IR (KBr) ν 3500–2500 (max at 3228, 3042, 2927, 2859, 2722, O-H, N-H, N⁺-H and C-H st), 1627, 1586, 1570 (C=O, ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 1.49–1.56 (complex signal, 4H, 3'-H₂, 4'-H₂), 1.59 (s, 3H, 9''-CH₃), 1.70 (tt, $J \approx J' \approx 6.7$ Hz, 2H, 2'-H₂), superimposed in part 1.87–1.94 (complex signal, 3H, 5'-H₂, 13''-H_{syn}), superimposed in part 1.94 (br d, $J = 17.5$ Hz, 1H, 10''-H_{endo}), 2.07 (dm, $J = 12.0$ Hz, 1H, 13''-H_{anti}), 2.54 (br dd, $J \approx 17.5$ Hz, $J' \approx 3.5$ Hz, 1H, 10''-H_{exo}), 2.77 (m, 1H, 7''-H), 2.82 (br d, $J = 18.0$ Hz, 1H, 6''-H_{endo}), 3.17 (dd, $J = 18.0$ Hz, $J' = 5.0$ Hz, 1H, 6''-H_{exo}), 3.39–3.49 (complex signal, 3H, 1'-H₂, 11''-H), 3.96 (dt, $J = J' = 6.7$ Hz, 2H, 6'-H₂), 4.84 (s, NH, ⁺NH and OH), 5.58 (br d, $J = 5.0$ Hz, 1H, 8''-H), 7.35 (dd, $J = 7.5$ Hz, $J' = 1.5$ Hz, 1H, 6-H), 7.46 (d, $J \approx 9.0$ Hz, 1H, 2''-H), 7.60 (s, 1H, 4''-H), 7.64 (s, 1H, 3-H), superimposed in part 7.75 (d, $J = 8.0$ Hz, 1H, 8-H), superimposed in part 7.77 (dd, $J \approx J' \approx 8.0$ Hz, 1H, 7-H), 8.07 (s, 1H, 1-H), 8.32 (d, $J \approx 9.0$ Hz, 1H, 1''-H); ¹³C NMR (125.7 MHz, CD₃OD) δ 23.4 (CH₃, 9''-CH₃), 27.2 (CH, C11''), 27.3 (2CH₂, C3', C4'), 27.9 (CH, C7''), 29.3 (CH₂, C13''), 30.0 (CH₂, C2'), 31.1 (CH₂, C5'), 36.1 (2CH₂, C6'', C10''), 40.7 (CH₂, C1'), 49.5 (CH₂, C6'), 115.7 (C, C12a''), 117.0 (C, C10a),

117.7 (C, C11a''), 118.6 (C, C4a), 118.8 (CH, C1), 119.1 (CH, C4''), 120.9 (CH, C8), 123.7 (CH, C3), 125.1 (CH, C8''), 125.8 (CH, C6), 126.6 (CH, C2''), 129.4 (CH, C1''), 134.6 (C, C9''), 134.8 (C, C8a), 135.3 (C, C9a), 138.8 (CH, C7), 140.2 (C, C3''), 141.0 (C, C4a''), 143.6 (C, C2), 151.3 (C, C5a''), 156.8 (C, C12''), 163.4 (C, C4), 163.8 (C, C5), 167.3 (C, CONH), 182.2 (C, C9), 193.8 (C, C10); HRMS (ESI) calcd for (C₃₈H₃₆³⁵ClN₃O₅ + H⁺) 650.2416, found 650.2414. Anal. (C₃₈H₃₆ClN₃O₅·HCl·1.25H₂O) C, H, N, Cl.

(±)-N-{7-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]heptyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide [(±)-**7c**]. It was prepared as described for (±)-**7a**. From rhein, **3** (1.43 g, 5.04 mmol), and amine (±)-**6c** (2.00 g, 5.04 mmol), a red solid (2.20 g) was obtained and purified by column chromatography (40–60 μm silica gel, hexane/EtOAc/MeOH/Et₃N mixtures, gradient elution). On elution with hexane/EtOAc/MeOH/Et₃N 40:60:0:0.2 to 0:80:20:0.2, hybrid (±)-**7c** (864 mg, 26% yield) was isolated as a red solid; *R_f* 0.48 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

(±)-7c·HCl: mp 202–203 °C (EtOAc/CH₂Cl₂/MeOH 75:19:6); IR (KBr) ν 3500–2500 (max at 3048, 2926, 2855, 2742, O-H, N-H, N⁺-H and C-H st), 1654, 1628, 1593, 1583, 1569, 1546 (C=O, ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 1.49 (complex signal, 6H, 3'-H₂, 4'-H₂, 5'-H₂), 1.58 (s, 3H, 9''-CH₃), 1.69 (tt, *J*≈*J'*≈6.5 Hz, 2H, 2'-H₂), superimposed in part 1.87–1.94 (complex signal, 3H, 6'-H₂, 13''-H_{syn}), superimposed in part 1.94 (br d, *J*≈18.0 Hz, 1H, 10''-H_{endo}), 2.07 (dm, *J*=12.0 Hz, 1H, 13-H_{anti}), 2.53 (br dd, *J*=18.0 Hz, *J'*=5.0 Hz, 1H, 10-H_{exo}), 2.75 (m, 1H, 7''-H), 2.79 (br d, *J*≈18.0 Hz, 1H, 6''-H_{endo}), 3.14 (dd, *J*≈18.0 Hz, *J'*=5.5 Hz, 1H, 6''-H_{exo}), 3.37–3.48 (complex signal, 3H, 1'-H₂, 11''-H), 3.92 (dt, *J*=7.5 Hz, *J'*=4.0 Hz, 2H, 7'-H₂), 4.84 (s, NH, ⁺NH and OH), 5.58 (br d, *J*=5.0 Hz, 1H, 8''-H), 7.36 (dd, *J*=8.5 Hz, *J'*=1.0 Hz, 1H, 6-H), 7.44 (dd, *J*≈9.0 Hz, *J'*=2.0 Hz, 1H, 2''-H), 7.59 (d, *J*=2.0 Hz, 1H, 4''-H),

7.66 (d, $J \approx 2.0$ Hz, 1H, 3-H), 7.73 (dd, $J = 7.5$ Hz, $J' \approx 1.0$ Hz, 1H, 8-H), 7.78 (dd, $J \approx J' \approx 7.5$ Hz, 1H, 7-H), 8.09 (d, $J = 2.0$ Hz, 1H, 1-H), 8.26 (d, $J = 9.0$ Hz, 1H, 1''-H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 23.4 (CH_3 , 9''- CH_3), 27.3 (CH , C11''), 27.6 (CH_2), 27.7 (CH_2) (C4', C5'), 27.9 (CH , C7''), 29.3 (CH_2 , C13''), 29.7 (CH_2), 29.9 (CH_2) (C2', C3'), 31.1 (CH_2 , C6'), 36.0 (CH_2), 36.1 (CH_2) (C6'', C10''), 41.0 (CH_2 , C1'), 49.7 (CH_2 , C7'), 115.6 (C, C12a''), 117.1 (C, C10a), 117.6 (C, C11a''), 118.6 (C, C4a), 118.7 (CH , C1), 119.2 (CH , C4''), 120.9 (CH , C8), 123.7 (CH , C3), 125.1 (CH , C8''), 125.8 (CH , C6), 126.6 (CH , C2''), 129.3 (CH , C1''), 134.6 (C, C9''), 134.8 (C, C8a), 135.3 (C, C9a), 138.8 (CH , C7), 140.2 (C, C3''), 141.0 (C, C4a''), 143.7 (C, C2), 151.3 (C, C5a''), 156.6 (C, C12''), 163.5 (C, C4), 163.8 (C, C5), 167.4 (C, CONH), 182.2 (C, C9), 193.6 (C, C10); HRMS (ESI) calcd for $(\text{C}_{39}\text{H}_{38}^{35}\text{ClN}_3\text{O}_5 + \text{H}^+)$ 664.2573 found 664.2569. Anal. ($\text{C}_{39}\text{H}_{38}\text{ClN}_3\text{O}_5 \cdot \text{HCl} \cdot 0.5\text{H}_2\text{O}$) C, H, N, Cl.

($\pm$)-N-{8-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]octyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide [($\pm$)-7d].

It was prepared as described for ($\pm$)-7a. From reagent **3** (213 mg, 0.75 mmol), and amine ($\pm$)-6d (307 mg, 0.75 mmol), a red solid (740 mg) was obtained and purified by column chromatography (40–60 μm silica gel, $\text{CH}_2\text{Cl}_2/\text{MeOH}/50\%$ aq. NH_4OH mixtures, gradient elution). On elution with $\text{CH}_2\text{Cl}_2/\text{MeOH}/50\%$ aq. NH_4OH 98:2:0.2 to 80:20:0.2, hybrid ($\pm$)-7d (80 mg, 16% yield) was isolated as a red solid; R_f 0.30 ($\text{CH}_2\text{Cl}_2/\text{MeOH}/50\%$ aq. NH_4OH 9:1:0.05).

($\pm$)-7d·HCl: mp 180–182 °C ($\text{EtOAc}/\text{CH}_2\text{Cl}_2/\text{MeOH}$ 66:17:17); IR (KBr) ν 3500–2500 (max at 3222, 3047, 3007, 2924, 2852, 2640, O-H, N-H, $\text{N}^+\text{-H}$ and C-H st), 1766, 1674, 1628, 1607, 1582, 1566, 1522 (C=O, ar-C-C and ar-C-N st) cm^{-1} ; ^1H NMR (500 MHz, CD_3OD) δ 1.44 (complex signal, 8H, 3'- H_2 , 4'- H_2 , 5'- H_2 , 6'- H_2), 1.59 (s, 3H, 9''- CH_3), 1.67 (tt, $J \approx J' \approx 7.0$ Hz, 2H,

2'-H₂), superimposed in part 1.86 (tt, $J \approx J' \approx 7.0$ Hz, 2H, 7'-H₂), superimposed 1.85–1.94 (m, 1H, 13''-H_{syn}), superimposed in part 1.93 (br d, $J \approx 18.0$ Hz, 1H, 10''-H_{endo}), 2.06 (dm, $J = 12.0$ Hz, 1H, 13''-H_{anti}), 2.53 (br dd, $J \approx 18.0$ Hz, $J' = 4.5$ Hz, 1H, 10''-H_{exo}), 2.75 (m, 1H, 7''-H), 2.82 (br d, $J = 18.0$ Hz, 1H, 6''-H_{endo}), 3.13 (dd, $J = 18.0$ Hz, $J' = 5.0$ Hz, 1H, 6''-H_{exo}), 3.39–3.47 (complex signal, 3H, 1'-H₂, 11''-H), 3.87 (dt, $J \approx J' \approx 6.0$ Hz, 2H, 8'-H₂), 4.86 (s, NH, ⁺NH and OH), 5.58 (br d, $J = 5.0$ Hz, 1H, 8''-H), 7.26 (d, $J = 8.5$ Hz, 1H, 6-H), 7.35 (d, $J \approx 9.0$ Hz, 1H, 2''-H), 7.54 (s, 1H, 4''-H), 7.58 (s, 1H, 3-H), superimposed in part 7.57–7.61 (m, 1H, 8-H), 7.69 (dd, $J \approx J' \approx 8.0$ Hz, 1H, 7-H), 7.97 (s, 1H, 1-H), 8.19 (d, $J = 9.0$ Hz, 1H, 1''-H); ¹³C NMR (125.7 MHz, CD₃OD) δ 23.5 (CH₃, 9''-CH₃), 27.2 (CH, C11''), 27.5 (CH₂), 27.6 (CH₂), (C5', C6'), 27.8 (CH, C7''), 29.3 (CH₂, C13''), 29.86 (CH₂), 29.92 (CH₂), 30.0 (CH₂) (C2', C3', C4'), 31.1 (CH₂, C7'), 36.0 (2CH₂, C6'', C10''), 41.0 (CH₂, C1'), 49.7 (CH₂, C8'), 115.4 (C, C12a''), 116.7 (C, C10a), 117.5 (C, C11a''), 118.3 (C, C4a), 118.8 (CH, C1), 119.0 (CH, C4''), 120.8 (CH, C8), 123.7 (CH, C3), 125.1 (CH, C8''), 125.8 (CH, C6), 126.6 (CH, C2''), 129.2 (CH, C1''), 134.4 (C, C9''), 134.6 (C, C8a), 134.9 (C, C9a), 138.8 (CH, C7), 140.1 (C, C3''), 140.8 (C, C4a''), 143.6 (C, C2), 151.1 (C, C5a''), 156.5 (C, C12''), 163.3 (C, C4), 163.5 (C, C5), 167.1 (C, CONH), 181.8 (C, C9), 193.4 (C, C10); HRMS (ESI) calcd for (C₄₀H₄₀³⁵ClN₃O₅ + H⁺) 678.2729, found 678.2734. Anal. (C₄₀H₄₀ClN₃O₅·HCl·H₂O) C, H, N, Cl.

(±)-N-{9-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]nonyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide [(±)-7e].

It was prepared as described for (±)-7a. From rein, **3** (213 mg, 0.75 mmol), and amine (±)-6e (321 mg, 0.75 mmol), a red solid (550 mg) was obtained and purified by column chromatography (40–60 μ m silica gel, hexane/EtOAc/MeOH/Et₃N mixtures, gradient elution).

On elution with hexane/EtOAc/MeOH/Et₃N 20:80:0:0.2 to 0:80:20:0.2, hybrid (±)-**7e** (118 mg, 23% yield) was isolated as a red solid; *R_f* 0.33 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

(±)-**7e**·HCl: mp 192–193 °C (EtOAc/CH₂Cl₂/MeOH 70:15:15); IR (KBr) ν 3500–2500 (max at 3401, 2925, 2853, O-H, N-H, N⁺-H and C-H st), 1628, 1584, 1566, 1524 (C=O, ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 1.41 (complex signal, 10H, 3'-H₂, 4'-H₂, 5'-H₂, 6'-H₂, 7'-H₂), 1.59 (s, 3H, 9''-CH₃), superimposed in part 1.64 (m, 2H, 2'-H₂), superimposed in part 1.66 (tt, *J*≈*J'*≈6.0 Hz, 2H, 8'-H₂), superimposed 1.84–1.94 (m, 1H, 13''-H_{syn}), superimposed in part 1.92 (br d, *J*=17.0 Hz, 1H, 10''-H_{endo}), 2.06 (dm, *J*≈10.5 Hz, 1H, 13''-H_{anti}), 2.53 (dm, *J*≈17.0 Hz, 1H, 10''-H_{exo}), 2.76 (m, 1H, 7''-H), superimposed in part 2.79 (br d, *J*≈18.0 Hz, 1H, 6''-H_{endo}), 3.14 (dd, *J*=18.0 Hz, *J'*=3.5 Hz, 1H, 6''-H_{exo}), 3.38–3.42 (complex signal, 3H, 1'-H₂, 11''-H), 3.86–3.96 (m, 2H, 9'-H₂), 4.85 (s, NH, ⁺NH and OH), 5.58 (br d, *J*=4.0 Hz, 1H, 8''-H), 7.31 (d, *J*=8.5 Hz, 1H, 6-H), 7.38 (d, *J*=8.5 Hz, 1H, 2''-H), 7.57 (s, 1H, 4''-H), 7.63 (s, 1H, 3-H), superimposed in part 7.64 (d, *J*=7.0 Hz, 1H, 8-H), 7.72 (dd, *J*≈*J'*≈8.0 Hz, 1H, 7-H), 8.04 (s, 1H, 1-H), 8.20 (d, *J*=8.5 Hz, 1H, 1''-H), 8.82 (br s, 1H, CONH); ¹³C NMR (125.7 MHz, CD₃OD) δ 23.5 (CH₃, 9''-CH₃), 27.2 (CH, C11''), 27.5 (CH₂), 27.6 (CH₂) (C6', C7'), 27.8 (CH, C7''), 29.3 (CH₂, C13''), 29.7 (2CH₂), 30.0 (CH₂), 30.1 (CH₂) (C2', C3', C4', C5'), 31.2 (CH₂, C8'), 36.0 (2CH₂, C6'', C10''), 41.0 (CH₂, C1'), 49.6 (CH₂, C9'), 115.4 (C, C12a''), 116.8 (C, C10a), 117.5 (C, C11a''), 118.4 (C, C4a), 118.8 (CH, C1), 119.1 (CH, C4''), 120.8 (CH, C8), 123.7 (CH, C3), 125.1 (CH, C8''), 125.8 (CH, C6), 126.6 (CH, C2''), 129.2 (CH, C1''), 134.6 (2C, C8a, C9''), 135.1 (C, C9a), 138.7 (CH, C7), 140.2 (C, C3''), 140.8 (C, C4a''), 143.7 (C, C2), 151.0 (C, C5a''), 156.6 (C, C12''), 163.4 (C, C4), 163.6 (C, C5), 167.3 (C, CONH), 182.0 (C, C9), 193.5 (C, C10); HRMS (ESI) calcd for (C₄₁H₄₂³⁵ClN₃O₅ + H⁺) 692.2886, found 692.2873. Anal. (C₄₁H₄₂ClN₃O₅·1.3HCl·1.25H₂O) C, H, N, Cl.

(-)-N-{9-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]nonyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide [(-)-7e].

It was prepared as described for (±)-7a. From rhein, **3** (422 mg, 1.49 mmol), and amine (-)-6e (632 mg, 1.49 mmol), a red solid (1.10 g) was obtained and purified by column chromatography (40–60 μm silica gel, CH₂Cl₂/MeOH/50% aq. NH₄OH mixtures, gradient elution). On elution with CH₂Cl₂/MeOH/50% aq. NH₄OH 99:1:0.2, hybrid (-)-7e (101 mg, 10% yield) was isolated as a red solid; *R*_f 0.33 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

(-)-7e·HCl: [α]_D²⁰ = -154 (*c* = 0.10, MeOH); mp 168–170 °C (CH₂Cl₂/MeOH 83:17); IR (KBr) ν 3500–2500 (max at 3226, 3048, 3002, 2925, 2853, 2743, O-H, N-H, N⁺-H and C-H st), 1766, 1705, 1675, 1629, 1604, 1582, 1566, 1524, 1509 (C=O, ar-C-C and ar-C-N st) cm⁻¹; the ¹H NMR and ¹³C NMR spectra were identical to those of (±)-7e; HRMS (ESI) calcd for (C₄₁H₄₂³⁵ClN₃O₅ + H⁺) 692.2886; found 692.2891. Anal. (C₄₁H₄₂ClN₃O₅·HCl·H₂O) C, H, N, Cl.

(+)-N-{9-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]nonyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide [(+)-7e].

It was prepared as described for (±)-7a. From rhein, **3** (477 mg, 1.68 mmol), and amine (+)-6e (715 mg, 1.68 mmol), a red solid (1.50 g) was obtained and purified by column chromatography (40–60 μm silica gel, CH₂Cl₂/MeOH/50% aq. NH₄OH mixtures, gradient elution). On elution with CH₂Cl₂/MeOH/50% aq. NH₄OH 99:1:0.2, hybrid (+)-7e (191 mg, 16% yield) was isolated as a red solid; *R*_f 0.33 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

(+)-7e·HCl: [α]_D²⁰ = +154 (*c* = 0.10, MeOH); mp 164–165 °C (CH₂Cl₂/MeOH 88:12); IR (KBr) ν 3500–2500 (max at 3226, 3049, 2926, 2852, O-H, N-H, N⁺-H and C-H st), 1763, 1739, 1723, 1710, 1656, 1629, 1583, 1566, 1555, 1537, 1511, 1501 (C=O, ar-C-C and ar-C-N st) cm⁻¹; the ¹H NMR and ¹³C NMR spectra were identical to those of (±)-7e; HRMS (ESI) calcd for

(C₄₁H₄₂³⁵ClN₃O₅ + H⁺) 692.2886, found 692.2877. Anal. (C₄₁H₄₂ClN₃O₅·1.25HCl·0.75H₂O) C, H, N, Cl.

(±)-N-{10-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]decyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide [(±)-7f]. It was prepared as described for (±)-7a. From rhein, **3** (344 mg, 1.21 mmol), and amine (±)-**6f** (530 mg, 1.21 mmol), a red solid (900 mg) was obtained and purified by column chromatography (40–60 μm silica gel, CH₂Cl₂/MeOH/50% aq. NH₄OH mixtures, gradient elution). On elution with CH₂Cl₂/MeOH/50% aq. NH₄OH 99:1:0.2, hybrid (±)-**7f** (115 mg, 13% yield) was isolated as a red solid; *R_f* 0.53 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

(±)-7f·HCl: mp 171–173 °C (EtOAc/CH₂Cl₂/MeOH 72:14:14); IR (KBr) ν 3500–2500 (max at 3229, 3049, 3002, 2925, 2852, O-H, N-H, N⁺-H and C-H st), 1767, 1739, 1715, 1675, 1629, 1604, 1583, 1567, 1524 (C=O, ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 1.34–1.44 (complex signal, 12H, 3'-H₂, 4'-H₂, 5'-H₂, 6'-H₂, 7'-H₂, 8'-H₂), 1.58 (s, 3H, 9''-CH₃), 1.66 (tt, *J*≈*J'*≈7.0 Hz, 2H, 2'-H₂), 1.83 (tt, *J*≈*J'*≈7.0 Hz, 2H, 9'-H₂), superimposed 1.90–1.93 (m, 1H, 13''-H_{syn}), superimposed in part 1.92 (br d, *J*=17.0 Hz, 1H, 10''-H_{endo}), 2.06 (dm, *J*≈12.0 Hz, 1H, 13''-H_{anti}), 2.52 (br dd, *J*≈17.0 Hz, *J'*≈4.3 Hz, 1H, 10''-H_{exo}), 2.75 (m, 1H, 7''-H), 2.82 (br d, *J*=18.0 Hz, 1H, 6''-H_{endo}), 3.15 (dd, *J*≈18.0 Hz, *J'*=5.3 Hz, 1H, 6''-H_{exo}), 3.38–3.45 (complex signal, 3H, 1'-H₂, 11''-H), 3.87 (dt, *J*≈*J'*≈6.5 Hz, 2H, 10'-H₂), 4.85 (s, NH, ⁺NH and OH), 5.57 (br d, *J*=5.5 Hz, 1H, 8''-H), 7.27 (dd, *J*=8.5 Hz, *J'*=1.0 Hz, 1H, 6-H), 7.39 (dd, *J*=9.5 Hz, *J'*=1.5 Hz, 1H, 2''-H), superimposed in part 7.59 (s, 1H, 4''-H), 7.60 (d, *J*=1.5 Hz, 1H, 3-H), 7.63 (dd, *J*=7.5 Hz, *J'*=1.0 Hz, 1H, 8-H), 7.70 (dd, *J*≈*J'*≈8.0 Hz, 1H, 7-H), 8.01 (d, *J*=1.5 Hz, 1H, 1-H), 8.21 (d, *J*=9.5 Hz, 1H, 1''-H); ¹³C NMR (125.7 MHz, CD₃OD) δ 23.5 (CH₃, 9''-CH₃), 27.2 (CH, C11''), 27.7 (2CH₂), 27.8 (CH + CH₂) (C6', C7', C8', C7''), 29.3 (CH₂, C13''), 30.03

(CH₂), 30.05 (CH₂), 30.22 (CH₂), 30.25 (CH₂) (C2', C3', C4', C5'), 31.2 (CH₂, C9'), 36.0 (2CH₂, C6'', C10''), 41.1 (CH₂, C1'), 49.6 (CH₂, C10'), 115.5 (C, C12a''), 116.8 (C, C10a), 117.5 (C, C11a''), 118.4 (C, C4a), 118.9 (CH, C1), 119.1 (CH, C4''), 120.9 (CH, C8), 123.7 (CH, C3), 125.1 (CH, C8''), 125.8 (CH, C6), 126.7 (CH, C2''), 129.3 (CH, C1''), 134.6 (2C, C8a, C9''), 135.0 (C, C9a), 138.7 (CH, C7), 140.2 (C, C3''), 140.8 (C, C4a''), 143.7 (C, C2), 151.0 (C, C5a''), 156.6 (C, C12''), 163.3 (C, C4), 163.6 (C, C5), 167.2 (C, CONH), 181.9 (C, C9), 193.5 (C, C10); HRMS (ESI) calcd for (C₄₂H₄₄³⁵ClN₃O₅ + H⁺) 706.3042, found 706.3038. Anal. (C₄₂H₄₄ClN₃O₅·HCl·0.5H₂O) C, H, N, Cl.

(±)-N-{11-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]undecyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide [(±)-7g]. It was prepared as described for (±)-7a. From rhein, **3** (491 mg, 1.73 mmol), and amine (±)-6g (785 mg, 1.73 mmol), a red solid (1.60 g) was obtained and purified by column chromatography (40–60 μm silica gel, CH₂Cl₂/MeOH/50% aq. NH₄OH mixtures, gradient elution). On elution with CH₂Cl₂/MeOH/50% aq. NH₄OH 99:1:0.2, hybrid (±)-7g (338 mg, 27% yield) was isolated as a red solid; *R_f* 0.50 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

(±)-7g·HCl: mp 171–172 °C (EtOAc/CH₂Cl₂/MeOH 60:20:20); IR (KBr) ν 3500–2500 (max at 3226, 3048, 3007, 2924, 2852, 2645, O-H, N-H, N⁺-H and C-H st), 1765, 1675, 1628, 1607, 1583, 1567, 1522 (C=O, ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 1.33–1.43 (complex signal, 14H, 3'-H₂, 4'-H₂, 5'-H₂, 6'-H₂, 7'-H₂, 8'-H₂, 9'-H₂), 1.58 (s, 3H, 9''-CH₃), 1.66 (tt, *J*≈*J'*≈7.0 Hz, 2H, 2'-H₂), 1.82 (tt, *J*≈*J'*≈17.0 Hz, 2H, 10'-H₂), superimposed 1.89–1.93 (m, 1H, 13''-H_{syn}), superimposed in part 1.91 (br d, *J*=17.0 Hz, 1H, 10''-H_{endo}), 2.05 (dm, *J*≈10.5 Hz, 1H, 13''-H_{anti}), 2.52 (br dd, *J*≈17.0 Hz, *J'*≈4.5 Hz, 1H, 10''-H_{exo}), 2.75 (m, 1H, 7''-H), 2.82 (br d, *J*=18.0 Hz, 1H, 6''-H_{endo}), 3.14 (dd, *J*≈18.0 Hz, *J'*=5.5 Hz, 1H, 6''-H_{exo}), superimposed in

part 3.37 (m, 1H, 11''-H), superimposed in part 3.40 (tt, $J \approx J' \approx 7.0$ Hz, 2H, 1'-H₂), 3.86 (dt, $J \approx J' \approx 7.0$ Hz, 2H, 11'-H₂), 4.85 (s, NH, ⁺NH and OH), 5.57 (br d, $J = 5.5$ Hz, 1H, 8''-H), 7.23 (dd, $J = 8.5$ Hz, $J' = 1.0$ Hz, 1H, 6-H), 7.37 (dd, $J = 9.0$ Hz, $J' \approx 1.5$ Hz, 1H, 2''-H), superimposed in part 7.57 (d, $J = 1.5$ Hz, 1H, 4''-H), superimposed in part 7.58 (d, $J = 2.0$ Hz, 1H, 3-H), superimposed in part 7.58 (dd, $J = 8.0$ Hz, $J' = 1.0$ Hz, 1H, 8-H), 7.67 (dd, $J \approx J' \approx 8.0$ Hz, 1H, 7-H), 7.97 (d, $J = 1.5$ Hz, 1H, 1-H), 8.20 (d, $J = 9.0$ Hz, 1H, 1''-H); ¹³C NMR (125.7 MHz, CD₃OD) δ 23.5 (CH₃, 9''-CH₃), 27.2 (CH, C11''), 27.7 (CH₂), 27.82 (CH, C7''), 27.83 (2CH₂) (C7', C8', C9', C7''), 29.3 (CH₂, C13''), 30.1 (2CH₂), 30.2 (CH₂), 30.36 (CH₂), 30.40 (CH₂) (C2', C3', C4', C5', C6'), 31.2 (CH₂, C10'), 36.0 (2CH₂, C6'', C10''), 41.2 (CH₂, C1'), 49.6 (CH₂, C11'), 115.4 (C, C12a''), 116.7 (C, C10a), 117.5 (C, C11a''), 118.3 (C, C4a), 118.9 (CH, C1), 119.1 (CH, C4''), 120.8 (CH, C8), 123.7 (CH, C3), 125.1 (CH, C8''), 125.8 (CH, C6), 126.6 (CH, C2''), 129.3 (CH, C1''), 134.5 (C, C9''), 134.6 (C, C8a), 134.9 (C, C9a), 138.7 (CH, C7), 140.2 (C, C3''), 140.8 (C, C4a''), 143.7 (C, C2), 151.0 (C, C5a''), 156.6 (C, C12''), 163.3 (C, C4), 163.5 (C, C5), 167.1 (C, CONH), 181.8 (C, C9), 193.4 (C, C10); HRMS (ESI) calcd for (C₄₃H₄₆³⁵ClN₃O₅ + H⁺) 720.3199, found 720.3195. Anal. (C₄₃H₄₆ClN₃O₅·HCl·0.5H₂O) C, H, N, Cl.

(±)-N-{4-[(3-Chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinolin-12-yl)amino]methyl}benzyl}-9,10-dihydro-4,5-dihydroxy-9,10-dioxoanthracene-2-carboxamide [(±)-7h**].** It was prepared as described for (±)-**7a**. From rhein, **3** (548 mg, 1.93 mmol), and amine (±)-**6h** (780 mg, 1.93 mmol), a red solid (1.07 g) was obtained and purified by column chromatography (40–60 μ m silica gel, hexane/EtOAc/Et₃N mixtures, gradient elution). On elution with hexane/EtOAc/Et₃N 40:60:0.2, hybrid (±)-**7h** (113 mg, 9% yield) was isolated as a red solid; R_f 0.57 (CH₂Cl₂/MeOH/50% aq. NH₄OH 9:1:0.05).

(±)-7h·HCl: mp 129–130 °C (EtOAc/CH₂Cl₂/MeOH 66:17:17); IR (KBr) ν 3500–2500 (max at 3245, 3055, 2925, 2852, 2790, O-H, N-H, N⁺-H and C-H st), 1705, 1673, 1629, 1607, 1583, 1560, 1516 (C=O, ar-C-C and ar-C-N st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 1.59 (s, 3H, 9''-CH₃), superimposed 1.92–1.98 (m, 1H, 13''-H_{syn}), 1.97 (br d, J =16.8 Hz, 1H, 10''-H_{endo}), 2.08 (dm, J =12.4 Hz, 1H, 13''-H_{anti}), 2.53 (br dd, J ≈16.8 Hz, J' ≈5.0 Hz, 1H, 10''-H_{exo}), 2.78 (m, 1H, 7''-H), 2.86 (br d, J =18.0 Hz, 1H, 6''-H_{endo}), 3.20 (dd, J ≈18.0 Hz, J' =5.6 Hz, 1H, 6''-H_{exo}), 3.47 (m, 1H, 11''-H), 4.60 (s, 2H, CH₂NH), 4.85 (s, NH, ⁺NH and OH), 5.18 (s, 2H, CH₂NHCO), 5.59 (m, 1H, 8''-H), 7.28 (dd, J =9.2 Hz, J' =2.0 Hz, 1H, 2''-H), 7.34 (dd, J =8.0 Hz, J' =2.0 Hz, 1H, 6-H), 7.41 (d, J =8.4 Hz, 2H) and 7.47 (d, J =8.4 Hz, 2H) [2'(6')-H₂, 3'(5')-H₂], 7.67 (d, J =2.0 Hz, 1H) and 7.69 (d, J =2.0 Hz, 1H) (3-H, 4''-H), 7.76 (dd, J ≈ J' ≈7.2 Hz, 1H, 7-H), 7.79 (dd, J =7.2 Hz, J' =2.0 Hz, 1H, 8-H), 8.15 (d, J ≈2.0 Hz, 1H, 1-H), 8.21 (d, J =9.2 Hz, 1H, 1''-H); ¹³C NMR (125.7 MHz, CD₃OD) δ 23.5 (CH₃, 9''-CH₃), 27.5 (CH, C11''), 27.8 (CH, C7''), 29.3 (CH₂, C13''), 36.0 (CH₂, C6''), 36.2 (CH₂, C10''), 44.3 (CH₂, CH₂NH), 51.9 (CH₂, CH₂NHCO), 115.4 (C, C12a''), 116.9 (C, C10a), 118.2 (C, C11a''), 118.5 (C, C4a), 118.8 (CH, C1), 119.0 (CH, C4''), 120.9 (CH, C8), 123.7 (CH, C3), 125.0 (CH, C8''), 125.8 (CH, C6), 126.6 (CH, C2''), 128.1 (CH), 129.8 (CH) [C2'(6'), C3'(5')], 129.3 (CH, C1''), 134.6 (C, C9''), 134.7 (C, C8a), 135.0 (C, C9a), 137.8 (C) and 139.2 (C) (C1', C4'), 138.8 (CH, C7), 140.2 (C, C3''), 140.7 (C, C4a''), 143.1 (C, C2), 151.6 (C, C5a''), 157.1 (C, C12''), 163.3 (C, C4), 163.6 (C, C5), 167.1 (C, CONH), 182.0 (C, C9), 193.5 (C, C10); HRMS (ESI) calcd for (C₄₀H₃₂³⁵ClN₃O₅ + H⁺) 670.2103, found 670.2104. Anal. (C₄₀H₃₂ClN₃O₅·1.1HCl·1.4H₂O) C, H, N, Cl.

In Vitro Biological Studies. AChE and BChE Inhibition Assay. Human recombinant AChE (Sigma, Milan, Italy) inhibitory activity was evaluated spectrophotometrically by the method of Ellman *et al.*⁴⁶ Initial rate assays were performed at 37 °C with a Jasco V-530 double beam

spectrophotometer. Stock solutions of the tested compounds (1 mM) were prepared in MeOH and diluted in MeOH. The assay solution consisted of a 0.1 M potassium phosphate buffer, pH 8.0, with the addition of 340 μ M 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, used to produce the yellow anion of 5-thio-2-nitrobenzoic acid), 0.02 unit/mL hAChE and 550 μ M substrate (acetylthiocholine iodide). Assay solutions with and without inhibitor were preincubated at 37 °C for 20 min followed by the addition of substrate. Blank solutions containing all components except hAChE were prepared in parallel to account for the non-enzymatic hydrolysis of the substrate. Five increasing concentrations of the inhibitor were used, able to give an inhibition of the enzymatic activity in the range of 20–80%. The results were plotted by placing the percentage of inhibition in function of the decimal log of the final inhibitor concentration. Linear regression and IC₅₀ values were calculated using Microcal Origin 3.5 software (Microcal Software, Inc).

Human serum BChE inhibitory activities were also evaluated spectrophotometrically by the method of Ellman *et al.*⁴⁶ The reactions took place in a final volume of 300 μ L of 0.1 M phosphate-buffered solution pH 8.0, containing hBChE (0.02 u/mL) and 333 μ M DTNB solution. Inhibition curves were performed in duplicates using at least 10 increasing concentrations of inhibitor and preincubated for 20 min at 37 °C. One duplicate sample without inhibitor was always present to yield 100% of BChE activity. Then substrate butyrylthiocholine iodide (300 μ M, Sigma-Aldrich) was added and the reaction was developed for 5 min at 37 °C. The color production was measured at 414 nm using a labsystems Multiskan spectrophotometer. Data from concentration–inhibition experiments of the inhibitors were calculated by non-linear regression analysis, using the GraphPad Prism program package (GraphPad Software; San

Diego, USA), which gave estimates of the IC_{50} values. Results are expressed as mean $\pm$ S.E.M. of at least 4 experiments performed in duplicate.

Kinetic Analysis of AChE Inhibition. To assess the mechanism of action of hybrid (–)-**7e**, reciprocal plots of $1/\text{velocity}$ versus $1/[\text{Substrate}]$ were constructed at relatively low concentration of substrate (0.562–0.112 mM) by using Ellman's method⁴⁶ and human recombinant AChE (Sigma, Milan, Italy). Three concentrations of inhibitor were selected for this study: 0.84, 1.34, 3.65 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.).

Calculation of the inhibitor constant (K_i) value was carried out by re-plotting slopes of lines from the Lineweaver-Burk plot *versus* the inhibitor concentration and K_i was determined as the intersect on the negative x-axis. K'_i (dissociation constant for the enzyme–substrate–inhibitor complex) value was determined by plotting the apparent $1/v_{\text{max}}$ *versus* inhibitor concentration.⁸⁷

AChE-Induced A β 40 Aggregation Inhibition Assay.⁴⁹ Thioflavin T (Basic Yellow 1), human recombinant AChE lyophilized powder, and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma Chemicals. Buffers and other chemicals were of analytical grade. Absolute DMSO over molecular sieves was from Fluka. Water was deionised and doubly distilled. A β 40, supplied as trifluoroacetate salt, was purchased from Bachem AG (Germany). A β 40 (2 mg mL⁻¹) was dissolved in HFIP, lyophilized and then resolubilized in DMSO to get a 2.3 mM A β 40 solution. 1 mM solutions of tested inhibitors were prepared by dissolution in MeOH.

Aliquots of 2 μ L A β 40 peptide were incubated for 24 h at room temperature in 0.215 M sodium phosphate buffer (pH 8.0) at a final concentration of 230 μ M. For co-incubation

experiments aliquots (16 μ L) of hAChE (final concentration 2.30 μ M, A β /AChE molar ratio 100:1) and AChE in the presence of 2 μ L of the tested inhibitor (final inhibitor concentration 100 μ M) in 0.215 M sodium phosphate buffer pH 8.0 solution were added. Blanks containing A β 40 alone, human recombinant AChE alone, and A β 40 plus tested inhibitors in 0.215 M sodium phosphate buffer (pH 8.0) were prepared. The final volume of each vial was 20 μ L. 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_{em} =490 nm (λ_{exc} =446 nm). The percent inhibition of the AChE-induced aggregation due to the presence of the tested compound was calculated by the following expression: $100 - (IF_i/IF_o \times 100)$ where IF_i and IF_o are the fluorescence intensities obtained for A β plus AChE in the presence and in the absence of inhibitor, respectively, minus the fluorescence intensities due to the respective blanks.

A β 42 Self-Aggregation Inhibition Assay. As reported in a previously published protocol,⁵⁸ HFIP pretreated A β 42 samples (Bachem AG, Switzerland) were first solubilized with a CH₃CN/0.3 mM Na₂CO₃/250 mM NaOH (48.4:48.4:3.2) mixture to obtain a 500 μ M solution. 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 A β concentration 50 μ M) with and without inhibitor (10 μ M, A β /inhibitor = 5/1). Blanks containing the tested inhibitors were also prepared and tested. To quantify amyloid fibrils formation, the thioflavin T fluorescence method was used.⁸⁸ 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 μ M thioflavin T. A 300-second-time scan of fluorescence intensity was carried out (λ_{exc} =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 μ M 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: $100 - (IF_i/IF_o \times 100)$ where IF_i and IF_o are the fluorescence intensities obtained for A β 42 in the presence and in the absence of inhibitor, respectively.

BACE-1 Inhibition Assay. β -Secretase (BACE-1, Sigma) inhibition studies were performed by employing a peptide mimicking APP sequence as substrate (Methoxycoumarin-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys-dinitrophenyl, M-2420, Bachem, Germany). The following procedure was employed: 5 μ L of test compounds (or DMSO, if preparing a control well) were pre-incubated with 175 μ L of enzyme (in 20 mM sodium acetate pH 4.5 containing CHAPS 0.1% w/v) for 1 h at room temperature. The substrate (3 μ M, final concentration) was then added and left to react for 15 min. The fluorescence signal was read at λ_{em} =405 nm (λ_{exc} =320 nm). The DMSO concentration in the final mixture was maintained below 5% (v/v) to guarantee no significant loss of enzyme activity. The fluorescence intensities with and without inhibitor were compared and the percent inhibition due to the presence of test compounds was calculated. The background signal was measured in control wells containing all the reagents, except BACE-1 and subtracted. The % inhibition due to the presence of increasing test compound concentration was calculated by the following expression: $100 - (IF_i/IF_o \times 100)$ where IF_i and IF_o are the fluorescence intensities obtained for BACE-1 in the presence and in the absence of inhibitor, respectively. Inhibition curves were obtained by plotting the % inhibition versus the logarithm of inhibitor concentration in the assay sample, when possible. The linear regression parameters were determined and the IC_{50} extrapolated (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 ($IC_{50} = 13.0 \pm 0.1$ nM).

BBB Permeation Assay. The brain penetration of the synthesized hybrids was assessed using the parallel artificial membrane permeation assay for blood-brain barrier described by Di *et al.*⁷¹ The *in vitro* permeability (P_e) of fourteen commercial drugs through lipid extract of porcine brain membrane together with that of the test compounds was determined. Commercial drugs and synthesized hybrids were tested using a mixture of PBS/EtOH 70:30. Assay validation was made by comparing the experimental permeability of the different compounds with the literature values of the commercial drugs, which showed a good correlation: $P_e(\text{exp}) = 1.4525 P_e(\text{lit}) - 0.4926$ ($R^2 = 0.9199$). From this equation and taking into account the limits established by Di *et al.* for BBB permeation, we established the following ranges of permeability: compounds of high BBB permeation (CNS+): $P_e(10^{-6} \text{ cm s}^{-1}) > 5.3$; compounds of low BBB permeation (CNS-): $P_e(10^{-6} \text{ cm s}^{-1}) < 2.4$, and compounds of uncertain BBB permeation (CNS+/-): $5.3 > P_e(10^{-6} \text{ cm s}^{-1}) > 2.4$.

A β 42 Aggregation Inhibition Assay in Intact *Escherichia coli* Cells Overexpressing A β 42 and Tau. Cloning and overexpression of A β 42 peptide: *Escherichia coli* competent cells BL21 (DE3) were transformed with the pET28a vector (Novagen, Inc., Madison, WI, USA) carrying the DNA sequence of A β 42. Because of the addition of the initiation codon ATG in front of both genes, the overexpressed peptide contains an additional methionine residue at its N terminus. For overnight culture preparation, 10 mL of lysogeny broth (LB) medium containing $50 \mu\text{g}\cdot\text{mL}^{-1}$ of kanamycin were inoculated with a colony of BL21 (DE3) bearing the plasmid to be expressed at 37 °C. After overnight growth, the OD600 was usually 2–2.5. For expression of A β 42 peptide, 20 μL of overnight culture were transferred into eppendorf tubes of 1.5 mL containing 980 μL of LB medium with $50 \mu\text{g}\cdot\text{mL}^{-1}$ of kanamycin, 1 mM of isopropyl 1-thio- β -D-galactopyranoside (IPTG) and 10 μM of each hybrid 7 or reference compound to be tested in DMSO. The samples

were grown for 24 h at 37 °C and 1400 rpm using a Thermomixer (Eppendorf, Hamburg, Germany). In the negative control (without drug) the same amount of DMSO was added in the sample.

Cloning and overexpression of tau protein: *E. coli* BL21 (DE3) competent cells were transformed with pTARA containing the RNA-polymerase gen of T7 phage (T7RP) under the control of the promoter PBAD. *E. coli* BL21 (DE3) with pTARA competent cells were transformed with pRKT42 vector encoding four repeats of tau protein in two inserts. For overnight culture preparation, 10 mL of M9 medium containing 0.5% of glucose, 50 µg·mL⁻¹ of ampicillin and 12.5 µg·mL⁻¹ of chloramphenicol were inoculated with a colony of BL21 (DE3) bearing the plasmids to be expressed at 37 °C. After overnight growth, the OD600 was usually 2–2.5. For expression of tau protein, 20 µL of overnight culture were transferred into eppendorf tubes of 1.5 mL containing 980 µL of M9 medium containing 0.25% of arabinose, 0.5% of glucose, 50 µg·mL⁻¹ of ampicillin and 12.5 µg·mL⁻¹ of chloramphenicol and 10 µM of each hybrid **7** or reference compound to be tested in DMSO. The samples were grown for 24 h at 37 °C and 1400 rpm using a Thermomixer (Eppendorf, Hamburg, Germany). In the negative control (without drug) the same amount of DMSO was added in the sample.

Thioflavin-S (Th-S) steady-state fluorescence: Th-S (T1892) and other chemical reagents were purchased from Sigma (St. Louis, MO). Th-S stock solution (250 mM) was prepared in double-distilled water purified through a Milli-Q system (Millipore, USA). Fluorescent spectral scans of Th-S were analyzed using an Aminco Bowman Series 2 luminescence spectrophotometer (Aminco-Bowman AB2, SLM Aminco, Rochester, NY, USA). For the fluorescence assay, 25 µM of Th-S (20 µL of Th-S in 180 µL of sample) were added to samples and spectra were recorded after 15 min equilibration at 37 °C. Excitation and emission slit widths of 5 nm were

used. Finally, the fluorescence emission at 520 nm, when exciting at 440 nm, was recorded. In order to normalize the Th-S fluorescence as a function of the bacterial concentration, OD600 was obtained using a Shimadzu UV-2401 PC UV–Vis spectrophotometer (Shimadzu, Japan). Note that the fluorescence normalization has been carried out considering as 100% the Th-S fluorescence of the bacterial cells expressing the peptide or protein in the absence of drug and 0% the Th-S fluorescence of the bacterial cells non-expressing the peptide or protein.

***Ex Vivo* Studies in C57bl6 Mice. Electrophysiological Assays in Hippocampal Slices Incubated with A β Oligomers.** Hippocampal slices were prepared according to standard procedures previously described.^{89,90} Briefly, transverse slices (350 μ m) from the dorsal hippocampus were cut under cold artificial cerebrospinal fluid (ACSF, in mM: 124 NaCl, 2.6 NaHCO₃, 10 D-glucose, 2.69 KCl, 1.25 KH₂PO₄, 2.5 CaCl₂, 1.3 MgSO₄, and 2.60 NaHPO₄) using a Vibratome (Leica VT 1000s, Germany) and incubated in ACSF for 1 h at room temperature. In all experiments, 10 μ M picrotoxin (PTX) was added to suppress inhibitory GABAA transmission. Slices were transferred to an experimental chamber (2 mL), superfused (3 mL/min, at 20–22 °C) with gassed ACSF and visualized by trans-illumination with a binocular microscope (MSZ-10, Nikon, Melville, NY). To evoke field excitatory postsynaptic potentials (fEPSPs), we stimulated with bipolar concentric electrodes (Platinum/Iridium, 125 μ m OD diameter, FHC Inc., Bowdoin, ME) generated by a stimulator (Axon 700b, Molecular Devices, Sunnyvale, CA) and connected to an isolation unit (Isoflex, AMPI, Jerusalem, Israel). The stimulation was in the Stratum Radiatum within 100-200 μ m from the recording site. The paired pulse facilitation index was calculated by $((R2-R1)/R1)$, where R1 and R2 were the peak amplitudes of the first and second fEPSP respectively. To generate LTP we used Theta Burst Stimulation (TBS) consisting of 5 trains of stimulus with an inter-train interval of 20 s. Each

train consisted of 10 bursts at 5 Hz and each burst having 4 pulses at 100 Hz. To generate LTD we used Low Frequency Stimulation (LFS) consisting in 900 paired pulses at 1 Hz. Recordings were filtered at 2.0-3.0 kHz, sampled at 4.0 kHz using an A/D converter, and stored with pClamp 10 (Molecular Devices). Evoked postsynaptic responses were analyzed off-line, using an analysis software (pClampfit, Molecular Devices), which allowed visual detection of events, computing only those events that exceeded an arbitrary threshold.

Determination of Synaptic Protein Levels in Hippocampal Slices Incubated with A β Oligomers. Immunoblotting: The hippocampus of C57bl6 mice were dissected on ice and immediately frozen at -150°C or processed. Briefly, hippocampal tissue was homogenized in RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 1% SDS) supplemented with a protease inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitors (50 mM NaF, 1 mM Na_3VO_4 and 30 μM $\text{Na}_4\text{P}_2\text{O}_7$) using a Potter homogenizator and then passed sequentially through different caliber syringes. Protein samples were centrifuged at 14000 rpm at 4°C twice for 10 min. Protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). 20 and 40 μg of hippocampal samples were resolved by 10% SDS-PAGE, transferred to a PVDF membrane. The reactions were followed by incubation with primary antibodies, secondary antibodies anti-mouse, anti-goat or anti-rabbit IgG peroxidase conjugated (Pierce) and developed using an ECL kit (Western Lightning Plus ECL, PerkinElmer).

***In Vivo* Studies in APP-PS1 Mice.**

Animals and Treatment. APP^{swe}/PSEN1 ΔE9 (APP-PS1) mice, which express the Swedish mutation of APP (K595N/M596L) and PS1 with the deletion of exon 9 were obtained from The Jackson Laboratory (Bar Harbor, ME; mice stock #004462). All animals were housed in

temperature- and light-controlled rooms, with food and water *ad libitum* during the treatment. Six and ten month-old mice were treated and handled according to the National Institutes of Health guidelines (NIH, Baltimore, MD). Treatments were performed by intra-peritoneal (i.p.) injection of 2.0 mg/kg enantiopure rhein–huprine hybrids (+)-**7e** or (–)-**7e** with saline solution as vehicle, three times per week during 4 weeks. Transgenic control animals were injected only with the vehicle.

Immunoblotting. The hippocampus from control or treated APP-PS1 mice were dissected on ice and immediately frozen at –150 °C or processed as detailed previously.^{90,91} Briefly, hippocampus were homogenized in RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 1% SDS) supplemented with a protease inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitors (50 mM NaF, 1 mM Na₃VO₄ and 30 μM Na₄P₂O₇) using a Potter homogenizator and then passed sequentially through different caliber syringes. Protein samples were centrifuged at 14000 rpm at 4 °C twice for 20 min. Protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

Aβ detection. For the detection of soluble Aβ peptides, samples from hippocampus were centrifuged to 20.000g for 1 h and the protein concentration from supernatant was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). 80 μg of proteins were resolved in 17.5% SDS polyacrylamide Tris-tricine gels, followed by immunoblotting on PVDF membranes using mouse anti-Aβ protein 6E10 (Covance). To analyze the levels of APP the antibody MAB348 (Millipore) was utilized. Band intensities were visualized by ECL kit (Western Lightning Plus ECL, PerkinElmer), scanned and densitometrically quantified using ImageJ software.^{85,92}

Docking in AChE. AChE models were built up from the human recombinant AChE structure 3LII.⁹³ The structure was refined by addition of missing hydrogen atoms, removal of non-standard residues and generation of disulfide bonds Cys69–Cys96, Cys257–272 and Cys405–Cys524. Since the fragment between residues Gly264 and Pro258 is not observed in the X-ray structure, it was modeled from the equivalent fragment of *Torpedo californica* AChE (*TcAChE*). Finally, the enzyme was modeled in its physiological active form with neutral His447 and neutral Glu334, which together with Ser203 forms the catalytic triad. We considered the standard ionization state at physiological pH for the rest of ionizable residues with exception of Glu450, which was modeled in its neutral state.

The binding mode of (–)-**7h** and (+)-**7h** to AChE was explored by means of docking calculations performed with rDock, whose reliability in predicting binding mode for dual site inhibitors of AChE has been largely demonstrated in previous studies.⁹⁴ The orientation of Tyr337 residue was modified to mimic the conformation of the equivalent residue Phe330 in the complex between *TcAChE* and (–)-huprine X (PDB 1E66).³⁹ To explore the binding at the PAS, three different AChE models differing in the orientation of Trp286 were built. The side chain of Trp286 was oriented following the three orientations found in X-ray structures deposited in the Protein Data Bank, which can be characterized by dihedral angles χ_1 (N–C $_{\alpha}$ –C $_{\beta}$ –C $_{\gamma}$) and χ_2 (C $_{\alpha}$ –C $_{\beta}$ –C $_{\gamma}$ –C $_{\delta 2}$) close to i) –60 and –80, ii) –120 and +50, and iii) –160 and –120 degrees. PDB structures 1N5R (AChE–propidium complex), 2CKM (AChE–*bis*(7)-tacrine complex) and 1Q83 (AChE–*syn*-TZ2PA6 complex), respectively, are representative cases of these orientations and were used to generate the corresponding models. The docking volume was defined as the space covered by the catalytic, midgorge and peripheral sites and each compound was subjected to 100 docking runs and solutions clustered according to the RMSD among its heavy atoms using a

threshold of 2.0 Å, selecting the best ranked pose to be representative of the cluster. The resulting binding modes were analyzed by visual inspection in conjunction with the docking scores.

Docking and molecular dynamics in BACE-1. In order to explore the binding mode of rhein-huprine hybrids, druggable pockets were identified using the MDpocket program.⁹⁵ Calculations were performed for X-ray structures 1M4H, 1SGZ, 2OHL and 3CIB chosen to account for structural variability in the flexible ‘flap’ loop located over the catalytic site and of certain regions (loops 9–12 and 263–268) and side chains in the peptide binding groove. The structures were refined by addition of missing hydrogen atoms, removal of non-standard residues and generation of disulfide bonds Cys155–Cys359, Cys217–Cys382 and Cys269–Cys319. Only druggable pockets that shared α -spheres in at least three different structures were retained and ranked according to druggability criteria.⁹⁶

GLIDE⁹⁷ was used to explore the preferred binding sites of the huprine and hydroxyanthraquinone moieties (obtained by replacing the methylene chain by a methyl group) to the druggable sites and to explore the binding mode of compound **7h**. The docking cavity was defined as a 13824Å³ cube whose center was located among residues Gly13, Leu30, Tyr71, Ile110, Trp115 and Gly230. Three different protonation states were considered for the catalytic diad (Asp32, Asp228), including the doubly deprotonated form and the two single deprotonated forms. Default settings were used and the best poses were clustered according to the RMSD between its heavy atoms, with a threshold of 2.0 Å and subsequently evaluated using the Glide-XP scoring function.

The binding modes proposed for (–)-**7h** and (+)-**7h** were assessed by means of Molecular Dynamics (MD) using GPU-accelerated PMEMD⁹⁸ module from the AMBER12 software

package.⁹⁹ The Parm99SB¹⁰⁰ force field was used for the protein and the gaff force field¹⁰¹ was used to assign parameters to both ligands. Na⁺ cations added to neutralize the system were treated according with Joung and Cheatham parameters.¹⁰² The ligands charge distribution were determined from a fit to the HF/6-31G(d) electrostatic potential obtained with Gaussian09¹⁰³ using the RESP¹⁰⁴ protocol as implemented in the Antechamber module of AmberTools12 software package.

Each system comprised around 46000 atoms, including the protein–ligand complex and 8 Na⁺ cations solvated on a truncated octahedral box of 13000 TIP3P water molecules.¹⁰⁵ The geometry of the system was minimized in four steps. First, water molecules were refined through 4500 steepest descent algorithm followed by 10500 steps of conjugate gradient. Then, protein and ligand hydrogen atoms positions were optimized using 500 steps of steepest descent and 4500 of conjugate gradient. Next the ligand, water molecules and counterions were further optimized with 2000 steps of steepest descent and 6000 of conjugate gradient and, at the last step, the whole system was optimized with 2500 steps of steepest descent and 4500 of conjugate gradient. At this point, 5 different replicas for each ligand were generated by randomly assigning different sets of velocities to the initial coordinates, all fitting a Maxwell distribution for a temperature of 50K. For each one of the replicas, thermalization was performed in the canonical ensemble during five 25 ps steps, using a time step of 1 fs and increasing the temperature from 50 to 298 K. Concomitantly, the inhibitor and the residues in the binding site were restrained during thermalization using a variable restraint force that was decreased sequentially from 5 kcal mol⁻¹ Å⁻² to 0 kcal mol⁻¹ Å⁻² using increments of 1 kcal mol⁻¹ Å⁻² within the different thermalization stages. Prior to the production runs, a short 1 ns simulation in the isothermal-isobaric ensemble was performed in order to reach a stable density value of 1003 kg m⁻³. Production runs consisted

of 50 ns trajectories (accounting for a global simulation time of 200 ns per ligand) using SHAKE for bonds involving hydrogen atoms, allowing for a timestep of 2 fs, in conjunction with periodic boundary conditions at constant volume and temperature (298 K; Langevin thermostat with a collision frequency of 3 ps^{-1}), particle mesh Ewald for long-range electrostatic interactions, and a cutoff of 10 Å for nonbonded interactions.

ASSOCIATED CONTENT

Supporting Information. Synthetic procedures and chemical characterization of intermediate nitriles **5a–h**, and amines **6a–h**, additional figures of the molecular modeling studies on AChE and BACE-1, additional data of PAMPA-BBB studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by Ministerio de Ciencia e Innovación (MICINN) (CTQ2011-22433, SAF2009-10553, SAF2011-27642, start-up grant of the Ramón y Cajal program for R.S.), Generalitat de Catalunya (GC) (2009SGR1396, 2009SGR249), University of Bologna, Italian Ministry of Education (MIUR, FFO - Fondo Finanziamento Ordinario) and Uni-Rimini S.p.A., and CONICYT-PFB (12/2007). Fellowships from GC to E.V. and I.S., from FIS to J.J.-J., from CONICYT to C.T.R., a contract from the Ramón y Cajal program of MICINN to R.S., and the ICREA support to F.J.L. are gratefully acknowledged. The Center for Scientific and Academic Services of Catalonia (CESCA) is acknowledged for providing access to computational facilities.

ABBREVIATIONS USED

A β , β -Amyloid peptide; A β -o, A β oligomers; AChE, acetylcholinesterase; AChEI, AChE inhibitor; ACSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; APP, amyloid precursor protein; BBB, blood–brain barrier; BACE-1, β -site APP cleaving enzyme 1; BChE, butyrylcholinesterase; CAS, catalytic anionic site; CNS, central nervous system; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); fEPSP, field excitatory postsynaptic potentials; hAChE, human acetylcholinesterase; hBChE, human butyrylcholinesterase; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; IBs, inclusion bodies; LTP, long-term potentiation; PAS, peripheral anionic site; PBS, phosphate-buffered saline; SYN, synapsin; SYP, synaptophysin; *TcAChE*, *Torpedo californica* acetylcholinesterase.

REFERENCES

- (1) Batsch, N. L.; Mittelman, M. S. World Alzheimer Report 2012. Overcoming the stigma of dementia, London: Alzheimer's Disease International, 2012 (<http://www.alz.co.uk>).
- (2) Hardy, J.; Selkoe, D. J. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **2002**, *297*, 353–356.
- (3) Kung, H. F. The β -amyloid hypothesis in Alzheimer's disease: seeing is believing. *ACS Med. Chem. Lett.* **2012**, *3*, 265–267.
- (4) Citron, M. Alzheimer's disease: strategies for disease modification. *Nat. Rev. Drug Discovery* **2010**, *9*, 387–398.
- (5) Pimplikar, S. W. Reassessing the amyloid cascade hypothesis of Alzheimer's disease. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 1261–1268.
- (6) Cavalli, A.; Bolognesi, M. L. Minarini, A. ; Rosini, M.; Tumiatti, V.; Recanatini, M.; Melchiorre, C. Multi-target-directed ligands to combat neurodegenerative diseases. *J. Med. Chem.* **2008**, *51*, 347–372.
- (7) Bajda, M.; Guzior, N.; Ignasik, M.; Malawska, B. Multi-target-directed ligands in Alzheimer's disease treatment. *Curr. Med. Chem.* **2011**, *18*, 4949–4975.
- (8) Geldenhuys, W. J.; Van der Schyf, C. J. Rationally designed multi-targeted agents against neurodegenerative diseases. *Curr. Med. Chem.* **2013**, *20*, 1662–1672.
- (9) Chen, X.; Decker, M. Multi-target compounds acting in the central nervous system designed from natural products. *Curr. Med. Chem.* **2013**, *20*, 1673–1685.

- (10) Russo, P.; Frustaci, A.; Del Bufalo, A.; Fini, M.; Cesario, A. Multitarget drugs of plants origin acting on Alzheimer's disease. *Curr. Med. Chem.*, **2013**, *20*, 1686–1693.
- (11) For recent examples see references 11–15. Butini, S.; Brindisi, M.; Brogi, S.; Maramai, S.; Guarino, E.; Panico, A.; Saxena, A.; Chauhan, V.; Colombo, R.; Verga, L.; De Lorenzi, E.; Bartolini, M.; Andrisano, V.; Novellino, E.; Campiani, G.; Gemma, S. Multifunctional cholinesterase and amyloid beta fibrillization modulators. Synthesis and biological investigation. *ACS Med. Chem. Lett.* **2013**, *4*, in press (DOI: 10.1021/ml4002908).
- (12) Capurro, V.; Busquet, P.; Lopes, J. P.; Bertorelli, R.; Tarozzo, G.; Bolognesi, M. L.; Piomelli, D.; Reggiani, A.; Cavalli, A. Pharmacological characterization of memoquin, a multi-target compound for the treatment of Alzheimer's disease. *PLoS One* **2013**, *8*, e56870.
- (13) Kochi, A.; Eckroat, T. J.; Green, K. D.; Mayhoub, A. S.; Lim, M. H.; Garneau-Tsodikova, S. A novel hybrid of 6-chlorotacrine and metal-amyloid- β modulator for inhibition of acetylcholinesterase and metal-induced amyloid- β aggregation. *Chem. Sci.* **2013**, *4*, 4137–4145.
- (14) Xie, S.-S.; Wang, X.-B.; Li, J.-Y.; Yang, L.; Kong, L.-Y. Design, synthesis and evaluation of novel tacrine-coumarin hybrids as multifunctional cholinesterase inhibitors against Alzheimer's disease. *Eur. J. Med. Chem.* **2013**, *64*, 540–553.
- (15) Prinz, M.; Parlar, S.; Bayraktar, G.; Alptüzün, V.; Erciyas, E.; Fallarero, A.; Karlsson, D.; Vuorela, P.; Burek, M.; Förster, C.; Turunc, E.; Armagan, G.; Yalcin, A.; Schiller, C.; Leuner, K.; Krug, M.; Sotriffer, C. A.; Holzgrabe, U. 1,4-Substituted 4-(1H)-pyridylene-

- hydrazone-type inhibitors of AChE, BChE, and amyloid- β aggregation crossing the blood–brain barrier. *Eur. J. Pharm. Sci.* **2013**, *49*, 603–613.
- (16) Viayna, E.; Sabate, R.; Muñoz-Torrero, D. Dual inhibitors of β -amyloid aggregation and acetylcholinesterase as multi-target anti-Alzheimer drug candidates. *Curr. Top. Med. Chem.* **2013**, *13*, 1820–1842.
- (17) Inestrosa, N. C.; Alvarez, A.; Pérez, C. A.; Moreno, R. D.; Vicente, M.; Linker, C.; Casanueva, O. I.; Soto, C.; Garrido, J. Acetylcholinesterase accelerates assembly of amyloid- β -peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. *Neuron* **1996**, *16*, 881–891.
- (18) Alvarez, A.; Alarcón, R.; Opazo, C.; Campos, E. O.; Muñoz, F. J.; Calderón, F. H.; Dajas, F.; Gentry, M. K.; Doctor, B. P.; De Mello, F. G.; Inestrosa, N. C. Stable complexes involving acetylcholinesterase and amyloid- β -peptide change the biochemical properties of the enzyme and increase the neurotoxicity of Alzheimer's fibrils. *J. Neurosci.* **1998**, *18*, 3213–3223.
- (19) De Ferrari, G. V.; Canales, M. A.; Shin, I.; Weiner, L. M.; Silman, I.; Inestrosa, N. C. A structural motif of acetylcholinesterase that promotes amyloid beta-peptide fibril formation. *Biochemistry* **2001**, *40*, 10447–10457.
- (20) Sussman, J. L.; Harel, M.; Frolov, F.; Oefner, C.; Goldman, A.; Toker, L.; Silman, I. Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. *Science* **1991**, *253*, 872–879.

- (21) Du, D.-M.; Carlier, P. R. Development of bivalent acetylcholinesterase inhibitors as potential therapeutic drugs for Alzheimer's disease. *Curr. Pharm. Des.* **2004**, *10*, 3141–3156.
- (22) Recanatini, M.; Valenti, P. Acetylcholinesterase inhibitors as a starting point towards improved Alzheimer's disease therapeutics. *Curr. Pharm. Des.* **2004**, *10*, 3157–3166.
- (23) Castro, A.; Martinez, A. Targeting beta-amyloid pathogenesis through acetylcholinesterase inhibitors. *Curr. Pharm. Des.* **2006**, *12*, 4377–4387.
- (24) Li, W. M.; Kan, K. K. W.; Carlier, P. R.; Pang, Y. P.; Han, Y. F. East meets West in the search for Alzheimer's therapeutics - Novel dimeric inhibitors from tacrine and huperzine A. *Curr. Alzheimer Res.* **2007**, *4*, 386–396.
- (25) Holzgrabe, U.; Kapková, P.; Alptüzün, V.; Scheiber, J.; Kugelmann, E. Targeting acetylcholinesterase to treat neurodegeneration. *Expert Opin. Ther. Targets* **2007**, *11*, 161–179.
- (26) Musial, A.; Bajda, M.; Malawska, B. Recent developments in cholinesterases inhibitors for Alzheimer's disease treatment. *Curr. Med. Chem.* **2007**, *14*, 2654–2679.
- (27) Haviv, H.; Wong, D. M.; Silman, I.; Sussman, J. L. Bivalent ligands derived from huperzine A as acetylcholinesterase inhibitors. *Curr. Top. Med. Chem.* **2007**, *7*, 375–387.
- (28) Muñoz-Torrero, D. Acetylcholinesterase inhibitors as disease-modifying therapies for Alzheimer's disease. *Curr. Med. Chem.* **2008**, *15*, 2433–2455.
- (29) Rampa, A.; Belluti, F.; Gobbi, S.; Bisi, A. Hybrid-based multi-target ligands for the treatment of Alzheimer's disease. *Curr. Top. Med. Chem.* **2011**, *11*, 2716–2730.

- (30) Pickhardt, M.; Gazova, Z.; von Bergen, M.; Khlistunova, I.; Wang, Y.; Hascher, A.; Mandelkow, E.-M.; Biernat, J.; Mandelkow, E. Anthraquinones inhibit tau aggregation and dissolve Alzheimer's paired helical filaments *in vitro* and in cells. *J. Biol. Chem.* **2005**, *280*, 3628–3635.
- (31) Bulic, B.; Pickhardt, M.; Schmidt, B.; Mandelkow, E.-M.; Waldmann, H.; Mandelkow, E. Development of tau aggregation inhibitors for Alzheimer's disease. *Angew. Chem. Int. Ed.* **2009**, *48*, 1740–1752.
- (32) Yang, X.; Sun, G.; Yang, C.; Wang, B. Novel rhein analogues as potential anticancer agents. *ChemMedChem* **2011**, *6*, 2294–2301.
- (33) Shi, D.-H.; Huang, W.; Li, C.; Wang, L.-T.; Wang, S.-F. Synthesis, biological evaluation and molecular modeling of aloe-emodin derivatives as new acetylcholinesterase inhibitors. *Bioorg. Med. Chem.* **2013**, *21*, 1064–1073.
- (34) Orhan, I.; Tosun, F.; Şener, B. Coumarin, anthroquinone and stilbene derivatives with anticholinesterase activity. *Z. Naturforsch.* **2008**, *63c*, 366–370.
- (35) Guo, J.-P.; Yu, S.; McGeer, P. L. Simple in vitro assay to identify amyloid- β aggregation blockers for Alzheimer's disease therapy. *J. Alzheimer's Dis.* **2010**, *19*, 1359–1370.
- (36) Camps, P.; Contreras, J.; Font-Bardia, M.; Morral, J.; Muñoz-Torrero, D.; Solans, X. Enantioselective synthesis of tacrine–huperzine A hybrids. Preparative chiral MPLC separation of their racemic mixtures and absolute configuration assignments by X-ray diffraction analysis. *Tetrahedron: Asymmetry* **1998**, *9*, 835–849.

- (37) Camps, P.; El Achab, R.; Morral, J.; Muñoz-Torrero, D.; Badia, A.; Baños, J. E.; Vivas, N. M.; Barril, X.; Orozco, M.; Luque, F. J. New tacrine–huperzine A hybrids (huprines): Highly potent tight-binding acetylcholinesterase inhibitors of interest for the treatment of Alzheimer’s disease. *J. Med. Chem.* **2000**, *43*, 4657–4666.
- (38) Camps, P.; Cusack, B.; Mallender, W. D.; El Achab, R.; Morral, J.; Muñoz-Torrero, D.; Rosenberry, T. L. Huprine X is a novel high-affinity inhibitor of acetylcholinesterase that is of interest for the treatment of Alzheimer’s disease. *Mol. Pharmacol.* **2000**, *57*, 409–417.
- (39) Dvir, H.; Wong, D. M.; Harel, M.; Barril, X.; Orozco, M.; Luque, F. J.; Muñoz-Torrero, D.; Camps, P.; Rosenberry, T. L.; Silman, I.; Sussman, J. L. 3D Structure of *Torpedo californica* acetylcholinesterase complexed with huprine X at 2.1 Å resolution: Kinetic and molecular dynamics correlates. *Biochemistry* **2002**, *41*, 2970–2981.
- (40) Galdeano, C.; Viayna, E.; Sola, I.; Formosa, X.; Camps, P.; Badia, A.; Clos, M. V.; Relat, J.; Ratia, M.; Bartolini, M.; Mancini, F.; Andrisano, V.; Salmona, M.; Minguillón, C.; González-Muñoz, G. C.; Rodríguez-Franco, M. I.; Bidon-Chanal, A.; Luque, F. J.; Muñoz-Torrero, D. Huprine–tacrine heterodimers as anti-amyloidogenic compounds of potential interest against Alzheimer’s and prion diseases. *J. Med. Chem.* **2012**, *55*, 661–669.
- (41) Camps, P.; Formosa, X.; Galdeano, C.; Muñoz-Torrero, D.; Ramírez, L.; Gómez, E.; Isambert, N.; Lavilla, R.; Badia, A.; Clos, M. V.; Bartolini, M.; Mancini, F.; Andrisano, V.; Arce, M. P.; Rodríguez-Franco, M. I.; Huertas, O.; Dafni, T.; Luque, F. J. Pyrano[3,2-

- c]quinoline–6-chlorotacrine hybrids as a novel family of acetylcholinesterase- and β -amyloid-directed anti-Alzheimer compounds. *J. Med. Chem.* **2009**, 52, 5365–5379.
- (42) Best, M.; Gifford, A. N.; Kim, S. W.; Babst, B.; Piel, M.; Rösch, F.; Fowler, J. S. Rapid radiosynthesis of [^{11}C] and [^{14}C]azelaic, suberic, and sebacic acids for *in vivo* mechanistic studies of systemic acquired resistance in plants. *J. Labelled Compd. Radiopharm.* **2012**, 55, 39–43.
- (43) Taber, D. F.; Kong, S. Alkylation of acetonitrile. *J. Org. Chem.* **1997**, 62, 8575–8576.
- (44) Lethesh, K. C.; Van Hecke, K.; Van Meervelt, L.; Nockemann, P.; Kirchner, B.; Zahn, S.; Parac-Vogt, T. N.; Dehaen, W.; Binnemans, K. Nitrile-functionalized pyridinium, pyrrolidinium, and piperidinium ionic liquids. *J. Phys. Chem. B* **2011**, 115, 8424–8438.
- (45) Lane, R. M.; Potkin, S. G.; Enz, A. Targeting acetylcholinesterase and butyrylcholinesterase in dementia. *Int. J. Neuropsychopharmacol.* **2006**, 9, 101–124.
- (46) Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Featherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **1961**, 7, 88–95.
- (47) Rydberg, E. H.; Brumshtein, B.; Greenblatt, H. M.; Wong, D. M.; Shaya, D.; Williams, L. D.; Carlier, P. R.; Pang, Y.-P.; Silman, I.; Sussman, J. L. Complexes of alkylene-linked tacrine dimers with *Torpedo californica* acetylcholinesterase: Binding of bis(5)-tacrine produces a dramatic rearrangement in the active site gorge. *J. Med. Chem.* **2006**, 49, 5491–5500.

- (48) Bolognesi, M. L.; Cavalli, A.; Valgimigli, L.; Bartolini, M.; Rosini, M.; Andrisano, V.; Recanatini, M.; Melchiorre, C. Multi-target-directed drug design strategy: from a dual binding site acetylcholinesterase inhibitor to a trifunctional compound against Alzheimer's disease. *J. Med. Chem.* **2007**, *50*, 6446–6449.
- (49) Bartolini, M.; Bertucci, C.; Cavrini, V.; Andrisano, V. β -Amyloid aggregation induced by human acetylcholinesterase: Inhibition studies. *Biochem. Pharmacol.* **2003**, *65*, 407–416.
- (50) Rouleau, J.; Iorga, B. I.; Guillou, C. New potent human acetylcholinesterase inhibitors in the tetracyclic triperpene series with inhibitory potency on amyloid β aggregation. *Eur. J. Med. Chem.* **2011**, *46*, 2193–2205.
- (51) Mohamed, T.; Yeung, J. C. K.; Rao, P. P. N. Development of 2-substituted-*N*-(naphth-1-ylmethyl) and *N*-benzhydrylpyrimidin-4-amines as dual cholinesterase and $A\beta$ -aggregation inhibitors: synthesis and biological evaluation. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5881–5887.
- (52) Bolognesi, M. L.; Bartolini, M.; Mancini, F.; Chiriano, G.; Ceccarini, L.; Rosini, M.; Milelli, A.; Tumiatti, V.; Andrisano, V.; Melchiorre, C. Bis(7)-tacrine derivatives as multitarget-directed ligands: focus on anticholinesterase and antiamyloid activities. *ChemMedChem* **2010**, *5*, 1215–1220.
- (53) Minarini, A.; Milelli, A.; Tumiatti, V.; Rosini, M.; Simoni, E.; Bolognesi, M. L.; Andrisano, V.; Bartolini, M.; Motori, E.; Angeloni, C.; Hrelia, S. Cystamine-tacrine dimer: a new multi-target-directed ligand as potential therapeutic agent for Alzheimer's disease treatment. *Neuropharmacology* **2012**, *62*, 997–1003.

- (54) Rizzo, S.; Bisi, A.; Bartolini, M.; Mancini, F.; Belluti, F.; Gobbi, S.; Andrisano, V.; Rampa, A. Multi-target strategy to address Alzheimer's disease: design, synthesis and biological evaluation of new tacrine-based dimers. *Eur. J. Med. Chem.* **2011**, *46*, 4336–4343.
- (55) Belluti, F.; Bartolini, M.; Bottegoni, G.; Bisi, A.; Cavalli, A.; Andrisano, V.; Rampa, A. Benzophenone-based derivatives: a novel series of potent and selective dual inhibitors of acetylcholinesterase and acetylcholinesterase-induced beta-amyloid aggregation. *Eur. J. Med. Chem.* **2011**, *46*, 1682–1693.
- (56) Cavalli, A.; Bolognesi, M. L.; Capsoni, S.; Andrisano, V.; Bartolini, M.; Margotti, E.; Cattaneo, A.; Recanatini, M.; Melchiorre, C. A small molecule targeting the multifactorial nature of Alzheimer's disease. *Angew. Chem. Int. Ed.* **2007**, *46*, 3689–3692.
- (57) Muñoz-Ruiz, P.; Rubio, L.; García-Palomero, E.; Dorronsoro, I.; del Monte-Millán, M.; Valenzuela, R.; Usán, P.; de Austria, C.; Bartolini, M.; Andrisano, V.; Bidon-Chanal, A.; Orozco, M.; Luque, F. J.; Medina, M.; Martínez, A. Design, synthesis, and biological evaluation of dual binding site acetylcholinesterase inhibitors: New disease-modifying agents for Alzheimer's disease. *J. Med. Chem.* **2005**, *48*, 7223–7233.
- (58) Bartolini, M.; Bertucci, C.; Bolognesi, M. L.; Cavalli, A.; Melchiorre, C.; Andrisano, V. Insight into the kinetic of amyloid beta (1–42) peptide self-aggregation: Elucidation of inhibitors' mechanism of action. *ChemBioChem* **2007**, *8*, 2152–2161.

- (59) May, P. C.; Dean, R. A.; Lowe, S. L.; Martenyi, F.; Sheehan, S. M.; Boggs, L. N.; Monk, S. A.; Mathes, B. M.; Mergott, D. J.; Watson, B. M.; Stout, S. L.; Timm, D. E.; Smith LaBell, E.; Gonzales, C. R.; Nakano, M.; Jhee, S. S.; Yen, M.; Ereshefsky, L.; Lindstrom, T. D.; Calligaro, D. O.; Cocke, P. J.; Hall, D. G.; Friedrich, S.; Citron, M.; Audia, J. E. Robust central reduction of amyloid- β in humans with an orally available, non-peptidic β -secretase inhibitor. *J. Neurosci.* **2011**, *31*, 16507–16516.
- (60) Butini, S.; Brogi, S.; Novellino, E.; Campiani, G.; Ghosh, A. K.; Brindisi, M.; Gemma, S. The structural evolution of β -secretase inhibitors: a focus on the development of small-molecule inhibitors. *Curr. Top. Med. Chem.* **2013**, *13*, 1787–1807.
- (61) Galdeano, C.; Viayna, E.; Arroyo, P.; Bidon-Chanal, A.; Blas, J. R.; Muñoz-Torrero, D.; Luque, F. J. Structural determinants of the multifunctional profile of dual binding site acetylcholinesterase inhibitors as anti-Alzheimer agents. *Curr. Pharm. Design* **2010**, *16*, 2818–2836.
- (62) Fu, H.; Li, W.; Luo, J.; Lee, N. T. K.; Li, M.; Tsim, K. W. K.; Pang, Y.; Youdim, M. B. H.; Han, Y. Promising anti-Alzheimer's dimer bis(7)-tacrine reduces β -amyloid generation by directly inhibiting BACE-1 activity. *Biochem. Biophys. Res. Commun.* **2008**, *366*, 631–636.
- (63) Hanessian, S.; Yun, H.; Hou, Y.; Yang, G.; Bayrakdarian, M.; Therrien, E.; Moitessier, N.; Roggo, S.; Veenstra, S.; Tintelnnot-Blomley, M.; Rondeau, J.-M.; Ostermeier, C.; Strauss, A.; Ramage, P.; Paganetti, P.; Neumann, U.; Betschart, C. Structure-based design, synthesis, and memapsin 2 (BACE) inhibitory activity of carbocyclic and heterocyclic peptidomimetics. *J. Med. Chem.* **2005**, *48*, 5175–5190.

- (64) Ballatore, C.; Brunden, K. R.; Piscitelli, F.; James, M. J.; Crowe, A.; Yao, Y.; Hyde, E.; Trojanowski, J. Q. ; Lee, V. M.-Y. ; Smith, III, A. B. Discovery of brain-penetrant, orally bioavailable aminothienopyridazine inhibitors of Tau aggregation. *J. Med. Chem.* **2010**, *53*, 3739–3747.
- (65) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *23*, 3–25.
- (66) Morphy, R.; Rankovic, Z. Designing multiple ligands – medicinal chemistry strategies and challenges. *Curr. Pharm. Des.* **2009**, *15*, 587–600.
- (67) García-Palomero, E.; Muñoz, P.; Usan, P.; Garcia, P.; De Austria, C.; Valenzuela, R.; Rubio, L.; Medina, M.; Martínez, A. Potent β -Amyloid Modulators. *Neurodegener. Dis.* **2008**, *5*, 153–156.
- (68) Spuch, C.; Antequera, D.; Fernandez-Bachiller, M. I.; Rodríguez-Franco, M. I.; Carro, E. A new tacrine–melatonin hybrid reduces amyloid burden and behavioral deficits in a mouse model of Alzheimer’s disease. *Neurotox. Res.* **2010**, *17*, 421–431.
- (69) Efremov, I. V.; Vajdos, F. F.; Borzilleri, K. A.; Capetta, S.; Chen, H.; Dorff, P. H.; Dutra, J. K.; Goldstein, S. W.; Mansour, M.; McColl, A.; Noell, S.; Oborski, C. E.; O’Connell, T. N.; O’Sullivan, T. J.; Pandit, J.; Wang, H.; Wei, B.; Withka, J. M. Discovery and optimization of a novel spiropyrrolidine inhibitor of β -secretase (BACE1) through fragment-based drug design. *J. Med. Chem.* **2012**, *55*, 9069–9088.

- (70) Lu, K.; Jiang, Y.; Chen, B.; Eldemenky, E. M.; Ma, G.; Packiarajan, M.; Chandrasena, G.; White, A. D.; Jones, K. A.; Li, B.; Hong, S.-P. Strategies to lower the Pgp efflux liability in a series of potent indole azetidine MCHR1 antagonists. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5310–5314.
- (71) Di, L.; Kerns, E. H.; Fan, K.; McConnell, O. J.; Carter, G. T. High throughput artificial membrane permeability assay for blood–brain barrier. *Eur. J. Med. Chem.* **2003**, *38*, 223–232.
- (72) Ami, D.; Natalello, A.; Lotti, M.; Doglia, S. M. Why and how protein aggregation has to be studied *in vivo*. *Microb. Cell Fact.* **2013**, *12*, 17.
- (73) Villar-Piqué, A.; Ventura, S. Modeling amyloids in bacteria. *Microb. Cell Fact.* **2012**, *11*, 166.
- (74) Pouplana, S.; Espargaró, A.; Galdeano, C.; Viayna, E.; Sola, I.; Ventura, S.; Muñoz-Torrero, D.; Sabate, R. Thioflavin-S staining of bacterial inclusion bodies for the fast, simple, and inexpensive screening of amyloid aggregation inhibitors. *Curr. Med. Chem.* **2014**, *21*, in press.
- (75) Porat, Y.; Abramowitz, A.; Gazit, E. Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism. *Chem. Biol. Drug Des.* **2006**, *67*, 27–37.
- (76) Zhang, H.-Y. Same causes, same cures. *Biochem. Biophys. Res. Commun.* **2006**, *351*, 578–581.

- (77) Narlawar, R.; Pickhardt, M.; Leuchtenberger, S.; Baumann, K.; Krause, S.; Dyrks, T.; Weggen, S.; Mandelkow, E.; Schmidt, B. Curcumin-derived pyrazoles and isoxazoles: Swiss army knives or blunt tools for Alzheimer's disease? *ChemMedChem* **2008**, *3*, 165–172.
- (78) LaFerla, F. M. Pathways linking A β and tau pathologies. *Biochem. Soc. Trans.* **2010**, *38*, 993–995.
- (79) Shankar, G. M.; Walsh, D. M. Alzheimer's disease: synaptic dysfunction and A β . *Mol Neurodegener.* **2009**, *4*, 48.
- (80) Lacor, P. N.; Buniel, M. C.; Chang, L.; Fernandez, S. J.; Gong, Y.; Viola, K. L.; Lambert, M. P.; Velasco, P. T.; Bigio, E. H.; Finch, C. E.; Krafft, G. A.; Klein, W. L. Synaptic targeting by Alzheimer's-related amyloid beta oligomers. *J. Neurosci.* **2004**, *24*, 10191–10200.
- (81) Walsh, D. M.; Klyubin, I.; Fadeeva, J. V.; Cullen, W. K.; Anwyl, R.; Wolfe, M. S.; Rowan, M. J.; Selkoe, D. J. Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation *in vivo*. *Nature* **2002**, *416*, 535–539.
- (82) Serrano-Pozo, A.; Frosch, M. P.; Masliah, E.; Hyman, B. T. Neuropathological alterations in Alzheimer disease. *Cold Spring Harb. Perspect. Med.* **2011**, *1*:a006189.
- (83) Alkondon, M.; Albuquerque, E. X.; Pereira, E. F. R. Acetylcholinesterase inhibition reveals endogenous nicotinic modulation of glutamate inputs to CA1 stratum radiatum interneurons in hippocampal slices. *Neurotoxicology* **2013**, *36*, 72–81.

- (84) Ye, L.; Qi, J. S.; Qiao, J. T. Long-term potentiation in hippocampus of rats is enhanced by endogenous acetylcholine in a way that is independent of N-methyl-D-aspartate receptors. *Neurosci. Lett.* **2001**, *300*, 145–148.
- (85) Ardiles, A. O.; Tapia-Rojas, C. C.; Mandal, M.; Alexandre, F.; Kirkwood, A.; Inestrosa, N. C.; Palacios, A. G. Postsynaptic dysfunction is associated with spatial and object recognition memory loss in a natural model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 13835–13840.
- (86) Garcia-Alloza, M.; Robbins, E. M.; Zhang-Nunes, S. X.; Purcell, S. M.; Betensky, R. A.; Raju, S.; Prada, C.; Greenberg, S. M.; Bacsikai, B. J.; Frosch, M. P. Characterization of amyloid deposition in the APP^{swe}/PS1^{dE9} mouse model of Alzheimer disease. *Neurobiol. Dis.* **2006**, *24*, 516–524.
- (87) Silverman R. B. The organic chemistry of enzyme-catalyzed reactions. Academic Press, San. Diego, 2000.
- (88) Naiki, H.; Higuchi, K.; Nakakuki, K.; Takeda, T. Kinetic analysis of amyloid fibril polymerization in vitro. *Lab Invest* **1991**, *65*, 104–110.
- (89) Varela-Nallar, L.; Alfaro, I. E.; Serrano, F. G.; Parodi, J.; Inestrosa, N. C. Wingless-type family member 5a (Wnt-5a) stimulates synaptic differentiation and function of glutamatergic synapses. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 21164–21169.
- (90) Inestrosa, N. C.; Carvajal, F. J.; Zolezzi, J. M.; Tapia-Rojas, C.; Serrano, F.; Karmelic, D.; Toledo, E. M.; Toro, A.; Toro, J.; Santos, M. J. Peroxisome proliferator reduce spatial

- (91) Carvajal, F. J.; Zolezzi, J. M.; Tapia-Rojas, C.; Godoy, J. A.; Inestrosa, N. C. Tetrahydrohyperforin decreases cholinergic markers associated with amyloid- β plaques, 4-hydroxynonenal formation, and caspase-3 activation in A β PP/PS1 mice. *J. Alzheimers Dis.* **2013**, *36*, 99–118.
- (92) Dinamarca, M. C.; Sagal, J. P.; Quintanilla, R. A.; Godoy, J. A.; Arrázola, M. S.; Inestrosa, N. C. Amyloid- β -acetylcholinesterase complexes potentiate neurodegenerative changes induced by the A β peptide. Implications for the pathogenesis of Alzheimer's disease. *Mol. Neurodegener.* **2010**, *5*, 4.
- (93) Dvir, H.; Silman, I.; Harel, M.; Rosenberry, T. L.; Sussman, J. L. Acetylcholinesterase: From 3D structure to function. *Chem.-Biol. Interact.* **2010**, *187*, 10–22.
- (94) Morley, S. D.; Afshar, M. Validation of an empirical RNA-ligand scoring function for fast flexible docking using RiboDock. *J. Comput.-Aided Mol. Des.* **2004**, *18*, 189–208.
- (95) Schmidtke, P.; Bidon-Chanal, A.; Luque, F. J.; Barril, X. MDpocket: Open source cavity detection and characterization on molecular dynamics trajectories. *Bioinformatics* **2011**, *27*, 3276–3285.
- (96) Schmidtke, P.; Barril, X. Understanding and predicting druggability. A high-throughput method for detection of drug binding sites. *J. Med. Chem.* **2010**, *53*, 5858–5867.
- (97) Friesner, R. A.; Murphy, R. B.; Repasky, M. P.; Frye, L. L.; Greenwood, J. R.; Halgren, T. A.; Sanschagrin, P. C.; Mainz, D. T. Extra precision Glide: Docking and

- (98) Salomon-Ferrer, R.; Goetz, A. W.; Poole, D.; Le Grand, S.; Walker, R. C. Routine microsecond molecular dynamics simulations with AMBER on GPUs. 2. Explicit solvent Particle Mesh Ewald, *J. Chem. Theory Comput.* **2013**, *9*, 3878–3888.
- (99) Case, D. A.; Darden, T. A.; Cheatham, III, T. E.; Simmerling, C. L.; Wang, J.; Duke, R. E.; Luo, R.; Walker, R. C.; Zhang, W.; Merz, K. M.; Roberts, B.; Hayik, S.; Roitberg, A.; Seabra, G.; Swails, J.; Goetz, A. W.; Kolossváry, I.; Wong, K. F.; Paesani, F.; Vanicek, J.; Wolf, R. M.; Liu, J.; Wu, X.; Brozell, S. R.; Steinbrecher, T.; Gohlke, H.; Cai, Q.; Ye, X.; Wang, J.; Hsieh, M.-J.; Cui, G.; Roe, D. R.; Mathews, D. H.; Seetin, M. G.; Salomon-Ferrer, R.; Sagui, C.; Babin, V.; Luchko, T.; Gusarov, S.; Kovalenko, A.; Kollman, P. A. (2012), *AMBER 12*, University of California, San Francisco.
- (100) Hornak, V.; Abel, R.; Okur, A.; Strockbine, B.; Roitberg, A.; Simmerling, C. Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins* **2006**, *65*, 712–725.
- (101) Wang, J. M.; Wolf, R. M.; Caldwell, J. W.; Kollman, P. A.; Case, D. A. Development and testing of a general amber force field, *J. Comput. Chem.* **2005**, *26*, 1157–1174.
- (102) Joung, I. S.; Cheatham, T. E. Molecular dynamics simulations of the dynamic and energetic properties of alkali and halide ions using water-model-specific ion parameters, *J. Phys. Chem. B* **2009**, *113*, 13279–13290.

- (103) Gaussian 09, Revision C.01; Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, J. M.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. Gaussian, Inc., Wallingford CT, 2009.
- (104) Bayly, C. I.; Cieplak, P.; Cornell, W.; Kollman, P. A. A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges: the RESP model. *J. Phys. Chem.* **1993**, *97*, 10269–10280.
- (105) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **1983**, *79*, 926–935.

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