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3. Development of hybrid compounds to tackle Alzheimer's disease

Francisco Javier Pérez-Areales and Diego Muñoz-Torrero
Laboratory of Pharmaceutical Chemistry (CSIC Associated Unit), Faculty of Pharmacy and Food Sciences, University of Barcelona, Av. Joan XXIII 27–31, E-08028 Barcelona, Spain
Institute of Biomedicine (IBUB), University of Barcelona, E-08028 Barcelona, Spain

Abstract. Alzheimer's disease (AD) is the main neurodegenerative disorder worldwide. Its pathogenesis involves a network where various mechanisms are interconnected. This complex pathological network makes it extremely challenging to find an efficacious treatment. Herein, we give an overview on the design of the so-called multi-target-directed ligands, i.e. compounds that concurrently hit several key pathogenic factors within the network, as a realistic option to tackle AD, with a particular emphasis on some structural classes of multitarget hybrids recently developed in our group.

Introduction

Alzheimer's disease (AD) is characterized by an inexorable progressive deterioration in cognitive ability and capacity for independent living [1]. AD is the most prevalent neurodegenerative disorder and one of the most important health-care problems in developed countries. Over 47 million people live with dementia worldwide, and this number is estimated to increase

Correspondence/Reprint request: Dr. Francisco Javier Pérez-Areales, Laboratory of Pharmaceutical Chemistry (CSIC Associated Unit), Faculty of Pharmacy and Food Sciences, and IBUB, University of Barcelona, Av. Joan XXIII 27–31, E-08028 Barcelona, Spain. E-mail: fjperezareales@gmail.com

to more than 131 million by 2050, as populations age. Dementia also has a huge economic impact, with the total estimated worldwide cost being US $818 billion [2]. To aggravate this situation, current treatments against AD afford only temporary relief of the cognitive and functional symptoms, but do not prevent, halt, or delay disease progression.

During the past 40 years, intensive research efforts have aimed to decipher the mechanisms of AD progression. However, the etiology of AD is not yet completely understood, and the unique neuropathological clearly defined hallmarks are the senile plaques and neurofibrillary tangles (NFTs), which are mainly composed of aggregated β-amyloid peptide (Aβ) and hyperphosphorylated tau protein, respectively, together with a degeneration of the neurons and synapses [3,4]. The lack of success in discovering novel pharmaceuticals to tackle AD is very likely caused by the multifactorial nature of the disease, which involves various complex mechanisms where several key proteins and pathological pathways are interconnected in a robust network. Thus, we must conceive AD as a pathological network instead of a continuous process [5].

Considering the mechanistic complexity involved in the pathological network of AD, it is easy to understand why the classic medicinal chemistry paradigm of developing drugs based on the reductionist approach of "one molecule-one target" has met with very limited success, which highlights the need for a more comprehensive pharmacological strategy to obtain effective outcomes.

In this context, some pharmacological approaches are available for the treatment of multifactorial diseases, such as AD. The most commonly used in general pharmacotherapy, referred to as multiple-medication therapy (MMT), consists of combining several drugs with different action mechanisms. However, this approach might imply patient compliance and pharmacokinetics issues [6,7]. An alternative approach relies on the use of a multiple-compound medication (MCM), which implies the incorporation of different drugs into the same formulation in order to simplify dosing regimens and improve patient compliance [6,7].

Finally, a third strategy is based on the assumption that a single molecule may be able to hit multiple targets. This approach, the so-called multi-target-directed ligand therapy (MTDL, Fig. 1), shows advantages over the aforementioned strategies, such as easier pharmacokinetics, improved efficacy due to synergistic effects, improved safety by preventing the risk of drug-drug interactions, and easier development, among others [6,8,9]. MTDLs can be rationally designed through the molecular assembly of distinct pharmacophore moieties of known bioactive molecules, where each drug entity has conserved the potential to interact with its specific site on the target [9].

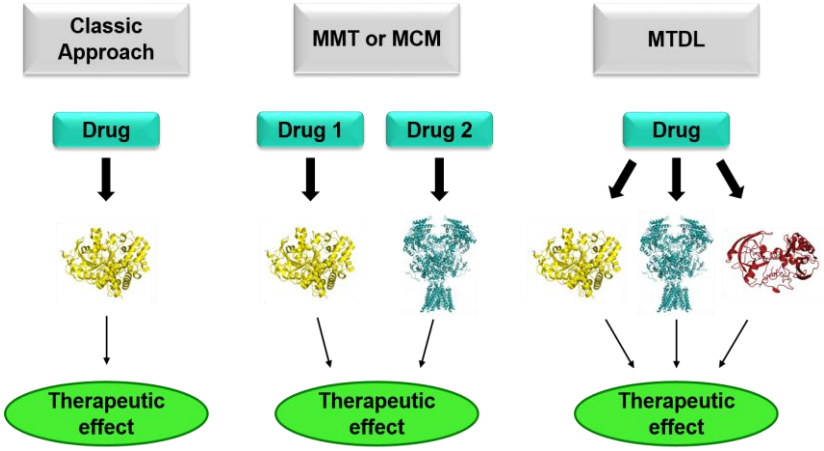


Figure 1. Different approaches to polypharmacological therapies against multifactorial diseases. Left: one-molecule-one-target strategy. Centre: multiple-medication therapy (MMT); in case of multiple-compound medication (MCM), both drugs are applied in the same pill. Right: multi-target-directed ligand (MTDL) approach.

In this chapter, we briefly review the design of hybrid molecules with the aim of combating AD, either by increasing the potency against a specific target, or by using a MDTL strategy in order to concurrently affect several targets within the AD network.

1. Increasing the potency against a key target, acetylcholinesterase

A common feature in AD patients is a cholinergic dysfunction, which is responsible for the clinical symptoms of the disease, which led to the postulation of the "cholinergic hypothesis of AD". This hypothesis proposed that degeneration of cholinergic neurons and the associated loss of cholinergic neurotransmission contributed significantly to the deterioration in cognitive function, perception, comprehension, reasoning, and short-term memory, observed in patients with AD [10,11]. This abnormal acetylcholine (ACh) neurotransmission is caused by dysregulation at different levels of synapses, such as a decreased availability of ACh because of high-affinity choline uptake, reduced ACh release or reduced ACh synthesis [11,12].

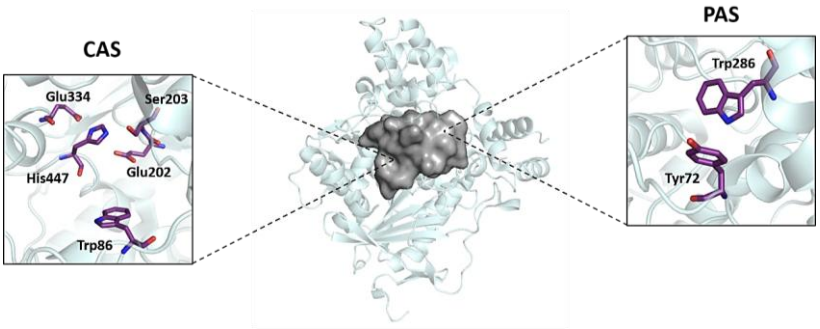


Figure 2. X-ray structure of *h*AChE (PDB ID: 3LII) with details of the CAS and the PAS.

At present, the most common therapeutic strategy aims at re-establishing the functional cholinergic neurotransmission by decreasing ACh metabolism through acetylcholinesterase inhibitors (AChEIs), which fit within the category of indirect cholinomimetic drugs [13]. Human AChE (*h*AChE) is the enzyme responsible for the hydrolysis of ACh, which takes place inside the catalytic anionic site (CAS) by means of the catalytic triad Ser203-His447-Glu334 (Fig. 2). A secondary binding site is the peripheral anionic site (PAS), which is located at the mouth of the narrow catalytic gorge and is responsible for the early binding and guiding of the substrate ACh towards the CAS [14,15].

The "cholinergic hypothesis" has led to four out of the five marketed anti-Alzheimer drugs, which act as AChEIs and are only symptomatic and effective for a limited time. The first approved drug of this group was tacrine (**1**, Fig. 3) [16,17], although it was withdrawn from the market due to hepatotoxicity issues [18].

1.1. Huprines as a new class of highly potent AChEIs

An example of how the inhibitory activity against AChE can be greatly increased by achieving a larger number of interactions within the CAS of the enzyme was reported by the group of Camps and Muñoz-Torrero with the development of huprines, a new class of compounds that turned out to be among the most potent reversible AChEIs described so far [19-21]. Huprines were designed by a conjunctive approach, using as templates two well-known CAS inhibitors, namely (–)-huperzine A (**2**, Fig. 3), an alkaloid isolated from *Huperzia serrata* with potent AChE inhibitory activity that is commercialized

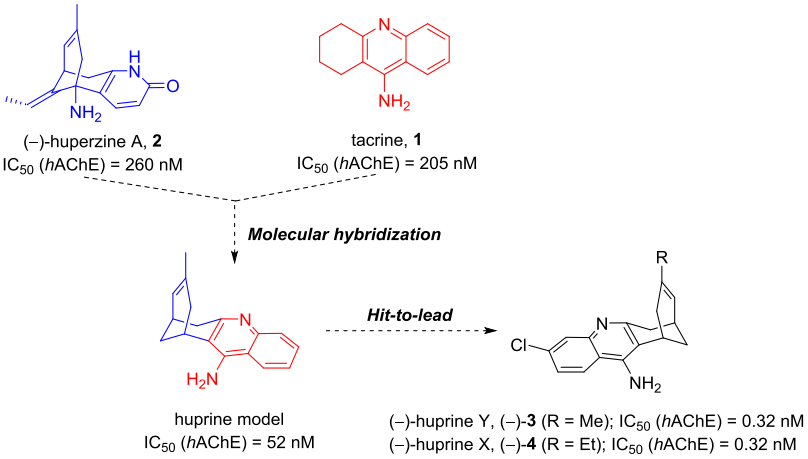


Figure 3. Design of huprines.

as a nutraceutical in the USA [21], and tacrine (**1**). More than thirty different huprines were designed, synthesized and pharmacologically tested. The most active huprines prepared to date are the so-called (–)-huprine Y, (–)-**3**, and (–)-huprine X, (–)-**4**, which are, in racemic form, up to 640- and 810-fold more potent *h*AChE inhibitors than the parent compounds tacrine and (–)-huperzine A, respectively [21]. X-Ray diffraction studies confirmed the extended binding of huprines within the CAS of AChE as compared with the binding mode of their parent compounds, which accounts in a great part for the higher AChE inhibitory potency of huprines, thereby confirming the success of the hybridization strategy [22].

1.2 Benzonaphthyridine−tacrine hybrids as novel AChEIs

As a further step to increase AChE inhibitory activity by enlarging the number of interactions with the enzyme, the so-called dual site binding consists of the simultaneous interaction of a compound with the two terminal binding sites within the catalytic gorge of AChE, i.e. with the CAS and the PAS. An attractive example of rational design of a dual binding site AChEI with a dramatic improvement of inhibitory potency is the development of the benzonaphthyridine−tacrine hybrid **9** [23]. This hybrid compound features a tacrine-based CAS interacting unit linked, by means of a tether of suitable length, to a previously developed PAS interacting unit.

Firstly, we carried out the design and synthesis of a PAS binding unit, structurally related to propidium (**5**, Fig. 4), a well-known PAS binding AChE inhibitor, which led to a pyrano[3,2-*c*]quinoline scaffold (**6**) [24]. Even though previous molecular dynamics (MD) simulations predicted that this structure would bind the PAS of AChE by means of π–π stacking interactions with residues Trp286 and Tyr72, compound **6** was found to be poorly active as AChEI (IC_{50} > 10 μM) [25]. Subsequent optimization of this PAS binding unit mainly involved the replacement of the oxygen atom at position 1 by a nitrogen. This structural modification should be accompanied by an increase in the basicity of the quinoline nitrogen atom, which, hence, should be protonated at physiological pH, thereby enabling additional cation–π interactions of the novel benzo[*h*][1,6]naphthyridine system (**7**, Fig. 4) at the PAS of AChE. MD simulations predicted an additional hydrogen bonding between the protonated pyridine nitrogen atom and the hydroxyl group of the PAS residue Tyr72 [26]. Compound **7** turned out to be a potent PAS AChEI (IC_{50} = 65 nM), being 500-fold more potent than propidium and more than 150-fold more potent than the hit **6**.

Afterwards, we developed a hybrid (**9**) that featured the PAS binding pharmacophore of **7** and a unit of the well-known CAS binding ligand 6-chlorotacrine (**8**, an optimized derivative of tacrine, Fig. 5), a highly potent AChEI. Both moieties were connected through a 3-methylene linker, which was suggested by previous computational studies to be the most suitable to enable a dual site binding within AChE, thereby allowing the resulting hybrid to retain all the characteristic interactions of the parent compounds within the enzyme. Indeed, the 6-chlorotacrine fragment of the hybrid was predicted to be tightly bound at the CAS, with this moiety establishing cation–π interactions with Trp86 and Tyr337 and a hydrogen bond between

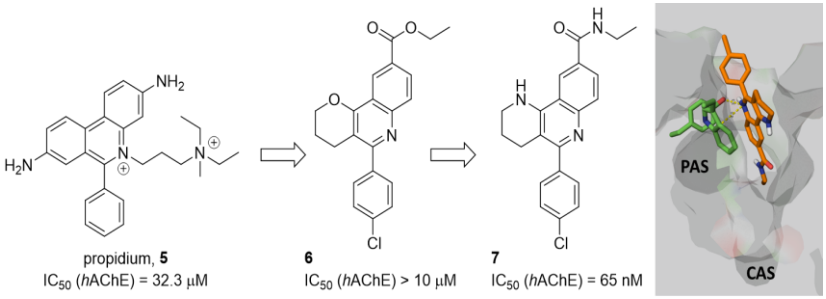


Figure 4. Left: optimization process of PAS AChEIs. Right: representation of the binding mode of compound **7** at the PAS of AChE [26].

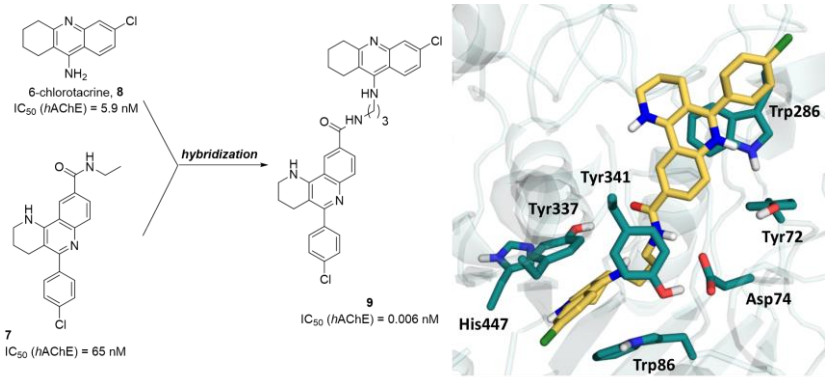


Figure 5. Left: design of hybrid **9**. Right: representation of the multi-site binding mode of hybrid **9** within AChE [23].

the protonated quinoline nitrogen with the carbonyl oxygen atom of His447. In turn, the benzo[*h*][1,6]naphthyridine moiety of the hybrid, whose quinoline nitrogen atom should be mostly protonated at physiological pH, was predicted to be firmly stacked against Trp286 at the PAS, establishing cation−π interactions. Remarkably, we found that an additional hydrogen bond could be formed between the amide group in the linker and Asp74. All this set of interactions along the catalytic gorge of AChE account for the extremely potent inhibitory activity of hybrid **9**, beyond our expectations, in the low picomolar range (IC_{50} = 6 pM), with this compound being 1000-fold more potent than the reference compound 6-chlorotacrine (IC_{50} = 5.9 nM) [23].

2. Huprine-based MTDLs against AD

Senile plaques and NFTs, mainly composed of aggregated Aβ and hyperphosphorylated tau protein, respectively, constitute two histopathological hallmarks clearly defined in AD patients. Consequently, both events have brought about the pertinent hypotheses about the origin of AD pathology. Firstly, the "amyloid hypothesis" postulates that AD is caused by an imbalance between Aβ production and clearance, resulting in increased amounts of Aβ, whose accumulation and aggregation into oligomers, and eventually fibrils and plaques, leads to neuronal damage and cell death [27]. The central event in the amyloid hypothesis is an alteration in the metabolism of the amyloid precursor protein (APP), which is directed

to an amyloidogenic pathway in AD patients, by which the sequential cleavage of APP through β-secretase (BACE1) and γ-secretase, affords a 39–43 amino acid polypeptide, Aβ, which is highly insoluble and shows strong tendency to aggregate [28]. In this regard, one of the most pursued targets in the search for new anti-Alzheimer drugs has been the modulation of Aβ production through BACE1 inhibitors [29]. BACE1 is an aspartic protease, whose active site contains two aspartate residues, Asp32 and Asp228, which are responsible for the initial cleavage of APP. The binding cleft is characterized for being partially covered by a highly flexible antiparallel hairpin-loop, referred to as the "flap", which guides the entrance of the substrate into the catalytic site (Fig. 6) [30].

On the other hand, the "tau hypothesis" postulates that AD patients suffer from an increased kinase activity, which triggers tau hyperphosphorylation, and detachment of the resulting distorted protein from the microtubules, so that the axon disintegrates and the skeleton of the neuron is no longer maintained. Without the cytoskeleton, neurons degenerate, and connections between neurons are lost, what eventually leads to apoptosis due to the loss of function [31,32]. Moreover, defective tau protein has a strong tendency to aggregate, forming paired helical filaments (PHF) inside the neuron, whose abnormal accumulation results in NFTs formation. Tau aggregation occurs through a nucleation-dependent elongation mechanism [33]. In fact, tau may adopt stable seed structures, displaying prion-like characteristics [34,35]. Therefore, prevention of tau aggregation has emerged as another promising therapeutic approach.

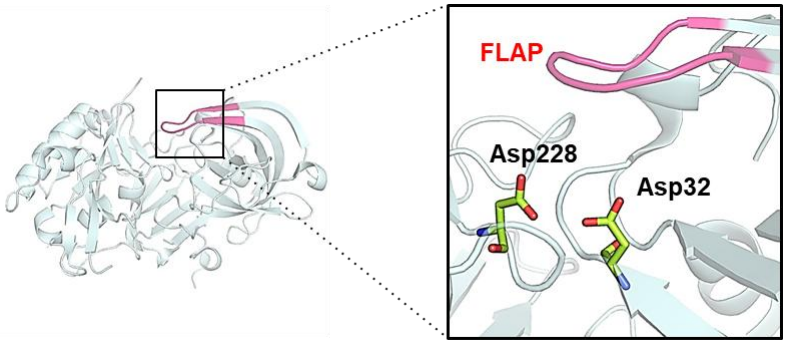
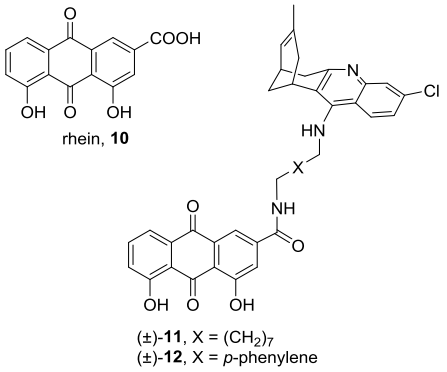


Figure 6. Structure of BACE1 (PDB ID: 1SGZ) with the details of the catalytic anionic dyad and the "flap".

2.1. Rhein−huprine hybrids as a new class of anti-Alzheimer MTDLs

The multifactorial nature of AD led to the establishment of the MTDL strategy as a promising, realistic therapeutic approach. In this context, rhein−huprine hybrids were designed as a novel structural family of MTDLs. This class of compounds had its origin in the finding that compounds sharing a core structure of hydroxyanthraquinone displayed tau anti-aggregating properties *in vitro* with IC_{50} values in the low micromolar range [36,37]. The structurally related compound rhein (**10**, Fig. 7, left) is a natural product found in the traditional Chinese herbal medicine rhubarb (*Rheum rhabarbarum*), which is well tolerated in humans [38]. We assumed that the hydroxyanthraquinone derivative rhein could also display tau anti-aggregating activity. Accordingly, the first generation of rhein–huprine hybrids was designed by connecting the hydroxyanthraquinone system of rhein and a moiety of the potent AChEI huprine Y (**3**) with a linker of suitable length. The lead compound of this family turned out to be the nonamethylene-linked hybrid (±)-**11** [39,40].

This family of hybrids was endowed with a very interesting *in vitro* and *in vivo* multi-target profile, especially the lead compound (±)-**11** (Fig. 7, right). Not unexpectedly, this compound displayed cholinergic activity through a potent inhibition of *human* AChE and butyrylcholinesterase (*h*BChE), and $A\beta_{42}$ and tau anti-aggregating activity. But more surprisingly,



In vitro
*h*AChE IC_{50} = 3.60 nM
*h*BChE IC_{50} = 620 nM
*h*BACE1 IC_{50} = 120 nM
***In vivo* (intact *E. coli* cells)**
48% inhibition of $A\beta_{42}$ aggregation at 10 μM
30% inhibition of tau aggregation at 10 μM
***Ex vivo* (mouse hippocampal slices)**
Prevention of Aß-induced loss of synaptic proteins
***In vivo* (APP/PS1 mice)**
Central Aß lowering effect

Figure 7. Left: rhein, **10**, the lead compound of the first generation of rhein–huprine hybrids, (±)-**11**, and the *p*-phenylene-linked analog (±)-**12**. Right: multi-target biological profile of the lead compound (±)-**11**.

the lead compound (±)-**11** was also found to be a potent inhibitor of *h*BACE1, which led to a significant Aβ lowering effect in a transgenic mouse model of AD (APP/PS1 mice) [39,40].

To shed light on the binding mode within *h*AChE, molecular modeling studies were carried out for the *p*-phenylene-linked rhein–huprine hybrid (±)-**12**, a less flexible analog of (±)-**11**, which was still a potent *h*AChEI, with an IC_{50} value of 18 nM. These studies suggested that the potent inhibitory activity of these hybrids against *h*AChE arises from a dual site binding within the enzyme [40]. Likewise, a dual site binding was also predicted with regard to *h*BACE1 inhibition, with the huprine moiety interacting with the catalytic dyad and the rhein fragment interacting with an unexplored secondary binding site [40].

Of note, the huprine moiety, protonated at physiological p*H*, remains tightly bound to the catalytic site in both *h*AChE and *h*BACE1 by means of hydrogen bonding interaction with His447 and cation–π interactions with Trp86 and Tyr337 at the CAS of AChE, and a salt bridge with the catalytic dyad of BACE1. The basicity of the huprine moiety of these hybrids is therefore crucial for AChE and BACE1 inhibition, due to the need of being protonated at physiological p*H* to enable these strong interactions [40].

2.2. Second generation rhein−huprine hybrids

In general, compounds with high basicity suffer from low brain exposure as a result of poor permeation through biological membranes, particularly the blood-brain barrier (BBB), and high P-glycoprotein (P-gp)-mediated efflux liability [41,42]. Hence, tuning of drugs pK_a has been an approach widely adopted to increase drug concentrations in brain [41,43]. In this light, a second generation of rhein−huprine hybrids was envisaged in order to explore how modulation of their basicity would affect their multiple biological activities, while trying to improve their pharmacokinetic properties. In the case of BACE1 inhibitors, the optimal balance between the relevant properties of enzymatic potency and pharmacokinetics has been reported for compounds with pK_a values between 7 and 7.5 [44].

For the design of the novel hybrids, the lead compound **11** was used as a template. Structural modification of its huprine moiety, i.e. the replacement of the chlorobenzene ring by other aromatic rings, should modify the basicity of the pyridine nitrogen. The selection of the novel huprines was made on the basis of their calculated pK_a values by means of high-level quantum mechanical (QM) computations. In this way, we selected the 1,4-difluorohuprine **13a** (Fig. 8, left) and the thienohuprine **13b**, with reduced basicity compared with huprine Y (pK_a = 8.2, for the *N*-methylated

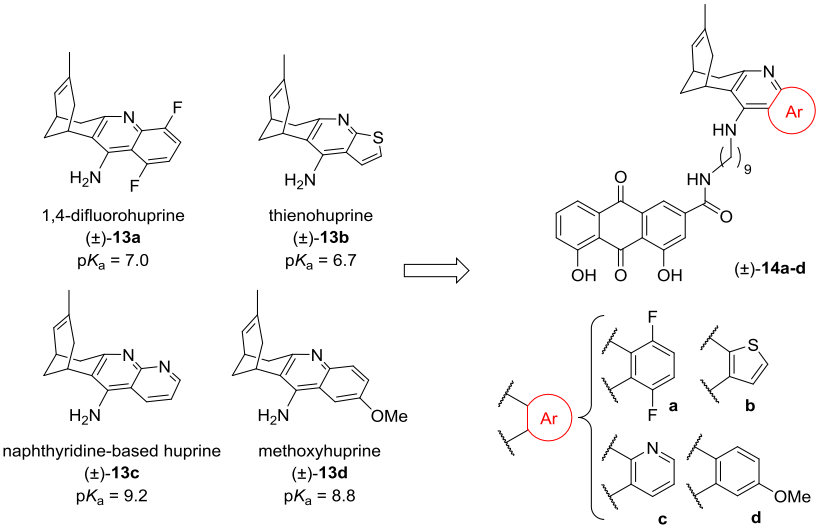


Figure 8. Left: selected modified huprines, (±)-**13a-d**, and their calculated pK_a values determined for the *N*-methylated derivatives by QM computations. Right: novel rhein–huprine hybrids, (±)-**14a-d**.

derivative of huprine Y), and the naphthyridine-based huprine **13c** [45] and the methoxyhuprine **13d**, which were predicted to be slightly more basic than huprine Y [46]. BACE1 localizes and is fully active in acidic endosomal compartments (p*H* 4.5–6.5) [47,48,49], where all the novel rhein–huprine hybrids, **14a-d** (Fig. 8, right), should be mostly in protonated form and therefore able to form a salt bridge with the aspartate residues of the catalytic dyad. On the other hand, AChE is located at physiological p*H* in synapses, where the most basic hybrids **14c** and **14d** should be mostly protonated, thereby retaining their AChE inhibitory activity, while the least basic hybrids **14a** and **14b** should predominate in the neutral form, with the consequent loss of hydrogen bond and cation−π interactions at the CAS of AChE.

It has been previously reported that replacement of the chlorobenzene ring of huprines by other aromatic systems is detrimental for the AChE inhibitory activity [20,21,45]. In agreement with these previous findings, all novel hybrids were clearly less potent than the lead compound **11**, but they still exhibited IC_{50} values in the submicromolar to low micromolar range, in most cases. As anticipated, the most potent second-generation hybrids were those of increased basicity, especially the naphthyridine derivative **14c**

(IC_{50} = 180 nM), since they should retain their ability to bind at the CAS of AChE. The lower inhibitory potency of hybrid **14c** compared to the lead **11** was studied by means of QM computations and showed unfavorable secondary interactions due to the electrostatic repulsion between the lone pairs of the nitrogen atom at position 1 and of the His447 carbonyl oxygen [46]. Moreover, the decreased activity of **14c** might be ascribed to the absence of the chlorine atom present at position 3 of huprine Y, which fills a hydrophobic pocket near the CAS.

On the other hand, hybrids **14a** and **14b** displayed some *h*BACE1 inhibitory activity (22% inhibition at 1 μM, and 34% inhibition at 80 nM, respectively), whereas compounds **14c** and **14d** turned out to be essentially inactive. Again, this series of compounds was clearly less potent than the lead **11**, despite the fact that all novel second-generation rhein–huprine hybrids should be protonated at the acidic p*H* in endosomal compartments where BACE1 is located. According to QM calculations, unfavorable electrostatic interactions of the thiophene derivative **14b** with the carboxylate oxygens of the catalytic dyad of BACE1 might account for its lower potency compared with the lead compound **11** [46].

Furthermore, this second generation of rhein–huprine hybrids retained the $A\beta_{42}$ anti-aggregating activity, while displayed slightly increased tau anti-aggregating properties, compared with the lead compound **11**. A common feature of AD is the oxidative damage in cellular structures, which occurs after an overproduction of reactive oxygen species and a deficiency of the antioxidant systems. Thus, we also assessed the antioxidant capacity of this novel series of compounds because of the presence of phenolic groups in their structure, and since it had been previously reported that rhein as well as huprine Y and a class of huprine-based hybrids were endowed with antioxidant properties [50,51,52]. Very interestingly, all the novel hybrids turned out to be potent antioxidant agents, being 10–22-fold and 12–13-fold more potent than trolox in the $ABTS^{\cdot+}$ and DPPH assays, respectively, and slightly more potent than gallic acid [46]. Interestingly, using the PAMPA-BBB assay, all the hybrids were predicted to have good BBB permeability, a necessary requirement for all CNS drugs.

3. Conclusions

Novel approaches have to be explored to identify drugs that can efficiently treat AD. Focusing on the symptomatic treatment of AD by means of cholinomimetic agents, we have shown that molecular

hybridization is an effective strategy to derive extremely potent (subnanomolar or picomolar) AChEIs that display a wide array of interactions either at the CAS of the enzyme (e.g. huprines) or in a dual site manner, from the CAS to the PAS, all along the AChE catalytic gorge (e.g. benzonaphthyridine-chlorotacrine hybrids). More interestingly, molecular hybridization is an essential tool to design MTDLs, in a very promising approach to derive new drugs that are able to confront the complex pathological network of AD, and, hence, to modify the natural course of this devastating disease. Results from preclinical studies with animal models of AD support a disease-modifying effect for this kind of compounds (e.g. rhein-huprine hybrids).

Acknowledgements

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