From Dual Binding Site Acetylcholinesterase Inhibitors to Multi-Target-Directed Ligands (MTDLs): A Step Forward in the Treatment of Alzheimer's Disease

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Abstract: Alzheimer's disease is a complex neurodegenerative disorder with a multifaceted pathogenesis. This fact has long halted the development of effective anti-Alzheimer drugs. Recently, however, basis for a therapeutic strategy based on multi-target-directed ligands has been formed. In this context, dual binding site acetylcholinesterase inhibitors represent a suitable starting point. The rational modification of their structures to provide them with additional biological properties has emerged as a successful approach.

Key Words: Dimeric compounds, bivalent ligands, multimodal therapeutics, multifunctional compounds, multifactorial diseases.

INTRODUCTION

Since its initial discovery, Alzheimer's disease (AD) has been recognized as one of the most important diseases amongst the elderly, currently afflicting 37 million people worldwide [1]. It remains for scientists an enigmatic disorder, for which no treatment to stop or reverse the relentless neurodegenerative process is presently available. One of the biggest obstacles in developing effective drug therapies has been lack of a comprehensive hypothesis able to explain all the mechanisms behind the different histopathological changes observed in AD patients. Clinical, experimental, microanatomic, and biochemical evidence indicate that AD is a complex disorder, characterized by widespread neurodegeneration of the CNS, with a major involvement of the cholinergic system, causing a progressive cognitive decline and dementia. Moreover, amyloid-β (Aβ) deposits in senile plaques and neurofibrillary tangles (NFT), mainly constituted of paired helical filaments of abnormally phosphorylated tau protein, have been identified as pathological hallmarks [2, 3]. In addition, several lines of evidence support a concomitant role for oxidative stress, metal ion dysregulation, inflammation and cell cycle regulatory failure in AD pathogenesis [4-7]. Trying to gather the threads of this debate, scientists have reached a consensus that AD is a multifactorial disease with a polyetiology, where different factors set in motion a self-sustaining, amplifying cycle which culminates in cell death processes [8]. To date, the most widely accepted and unifying hypothesis proposes that Aβ aggregates with metals are the main triggers of tau hyperphosphorylation and free-radical activity, and of the subsequent degeneration of affected neurons [9-11].

This integrated understanding of the mechanisms underlying the interrelationship between cholinergic dysfunction, Aβ formation and deposition, and tau pathology brings into question the validity of monotherapy in AD and clearly recommends for pharmacological interventions based on multimodal therapeutics. The identification of several other potential targets critically involved in this neurotoxic cycle strongly indicates that the polypharmacy regimen is the only worthwhile future direction for drug research [12-16].

On the clinical front, the approach that has so far produced the majority of marketed drugs is the cholinergic hypothesis [17], which proposed the cholinergic enhancement as an approach to improve cognitive function in AD. Acetylcholinesterase (AChE) inhibitors (AChEIs), which increase acetylcholine (ACh) levels at cholinergic synapses within the brain, are the primary medications used to treat AD. They have long been viewed as exclusively symptomatic medicines, whereas recent preclinical studies demonstrate that they exhibit a number of biological effects in addition to cholinesterase inhibition [18, 19]. Moreover, they are largely used in combination therapy with agents that have non overlapping or even synergistic mechanisms of action [20].

The multifactorial nature of AD and the routine use of combination therapy in clinical practice, prompted medicinal chemists to invest research efforts into the discovery of multifunctional pharmaceuticals. A growing number of compounds have been specifically developed to exhibit a multi-target-directed ligand (MTDL) profile against AD [21]. In designing novel MTDLs, dual binding site AChEIs have been viewed as a suitable starting point. These ligands, interacting simultaneously with AChE catalytic and peripheral sites, show the potential of alleviating the cognitive deficit in AD by restoring cholinergic activity. More importantly, they show the potential of addressing disease mechanisms by inhibiting Aβ aggregation [22-24]. In fact, different studies have highlighted that AChE binds through its peripheral anionic site (PAS) to the non amyloidogenic Aβ form and acts as a pathological chaperone inducing a conformational shift to the amyloidogenic form [25-27]. In this respect, AChEIs

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binding to both catalytic and peripheral sites of the enzyme do inhibit peptide aggregation. From a survey of recent literature, the rational modification of their molecular structures, to provide them with additional biological properties, all concurring in modulating AD relevant targets, has transpired to be a fruitful strategy to MTDLs [28-30].

In this review we examine recent examples of new dual binding site AChEIs, which have been purposely designed to have a multifunctional profile against AD beyond AChE inhibition, and which were not included in the exhaustive report by Decker that appeared in this journal last year [31].

2. DUAL BINDING SITE ACHEIs BEARING A POLYAMINE SCAFFOLD AS MTDLs

The discovery of caproctamine (**1**) represents one of the first cases of a rationally designed AChEI endowed with a multimodal mechanism of action against AD (Fig. (**1**)). Following the cholinergic hypothesis, it was proposed that there might be additional therapeutic value in ligands with affinity for both AChE active and peripheral sites and for muscarinic M2 receptors. In fact, inhibition of AChE activity would potentiate the cholinergic transmission in functionally active neurons, while antagonism of muscarinic M2 autoreceptors would facilitate the release of ACh in the synapse in a very physiological way. At the same time, inhibition of PAS would prevent the aggregation of Aβ promoted by AChE. Exploiting the universal template approach [32], caproctamine was designed. It proved to be a promising hit compound against AD, due to a well-balanced affinity profile as an AChEI and an M2 receptor antagonist [33]. Moreover, docking studies investigating the putative binding mode of **1** at AChE gorge revealed that it was able to contact both sites.

A breakthrough in the field was the development of an *in vitro* test system for the evaluation of AChE-induced Aβ aggregation inhibitory activity by dual binding site AChEIs [34]. This basic evidence increased the confidence of medicinal chemists in these compounds and gave impetus to the discovery of a new generation of AChEIs, which could act as disease-modifying treatments since they are able to simultaneously modulate ACh levels and Aβ deposition in the brain [22, 28, 35].

To this end, a new series of **1** derivatives were designed by replacing the inner octamethylene spacer separating the two amide functions with less flexible dipiperidine and dianiline moieties. Compound **2** was the most potent AChEI of the series (Fig. (**1**)), with an increased inhibitory activity with respect to **1** (pIC_{50} value of 8.48 *vs* 6.77), while also displaying muscarinic M2 antagonistic activity (pK_b value of 6.18). Moreover, the existing availability of the test allowed to verify the AChE-induced Aβ aggregation inhibition by the newly synthesized compounds in comparison with **1**. Although all derivatives caused a mixed type of AChE inhibition (active site and PAS), only those bearing an inner con-

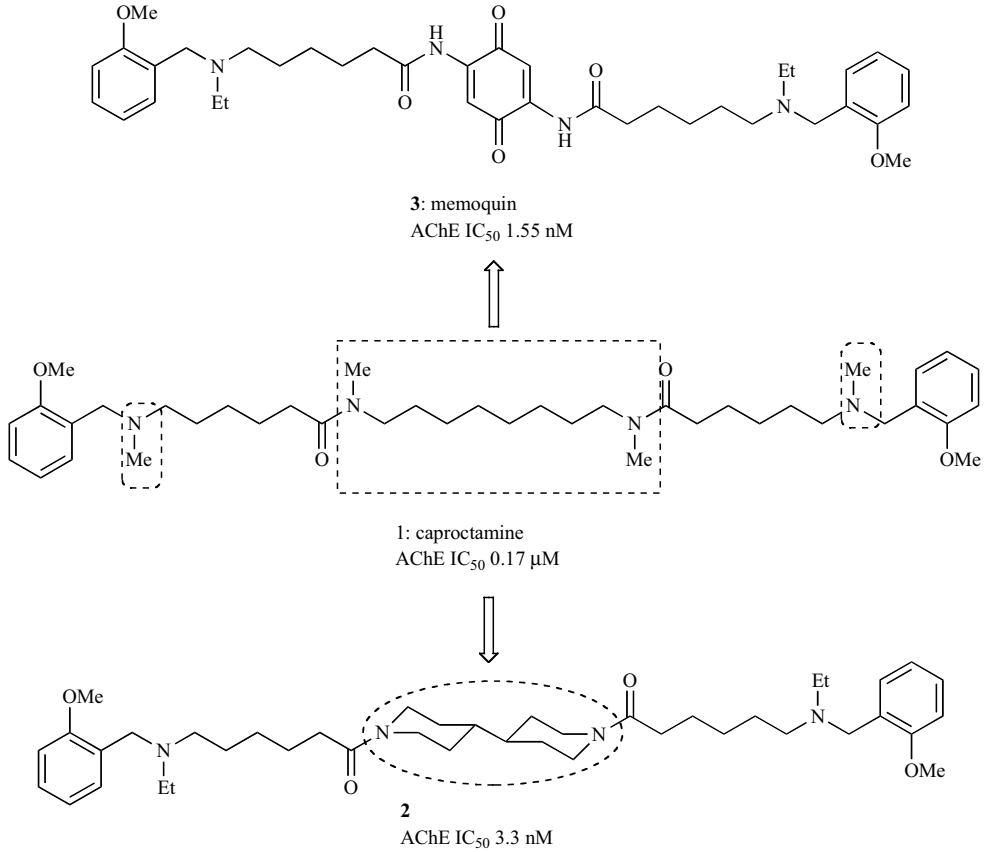

Fig. (1). Chemical structures of MTDLs **1**-**3** based on a polyamine backbone.

strained spacer, such as **2,** were able to inhibit AChE-induced Aβ aggregation. Notably, the ability of an AChEI, based on a linear polyamine backbone, to bind both AChE sites may not be a sufficient condition for the inhibition of AChE-induced Aβ aggregation [36].

To further expand the research emphasis on MTDLs, a program was begun to identify new ligands that possessed extra-properties, such as antioxidant properties, in addition to modulating the cholinergic system. Indeed, there was substantial evidence to place oxidative stress at centre stage in AD, and since it occurs early in the pathogenesis [37], it represented an ideal target for intervention. At the time the most pursued strategy for creating MTDLs was based on joining functionally distinct pharmacophores in a single molecule. In this regard, a starting point was a polyamine backbone. This is because the lack of tight molecular specificity for only one given target, typical of polyamines [32], would represent a prerequisite for the choice of a better lead compound. **1** was modified by incorporating into its structure the benzoquinone function of coenzyme Q10, as this natural antioxidant offered promise against AD both *in vitro* [38, 39] and *in vivo* [40]. Having learned from previous investigations that the biophoric space around the octamethylene chain of **1** could tolerate a variety of cyclic bulky moieties [36], the benzoquinone nucleus was introduced in place of the inner polymethylene spacer (see Fig. (**1**) for the design strategy). In the resulting 2,5-bis-diaminobenzoquinone derivative **3** (memoquin), a hydrophobic and planar π system was generated, which was suitable, in principle, for intercalating Aβ and perturbing protein-protein interactions in the fibrillogenesis process. As expected, **3** showed a relevant inhibitory activity toward AChE. Unexpectedly, it turned out to be an inhibitor of the amyloid precursor protein (APP) processing enzyme β-secretase (BACE-1). It was also able to considerably reduce self and AChE-mediated Aβ aggregation, together with oxidative processes. In particular, the ability of **3** to counteract the oxidative stress was strictly mediated by NADP(H): quinone oxidoreductase 1, an enzyme able to generate and maintain the reduced hydroquinone form, responsible of the antioxidant activity. *In vivo*, **3** showed the ability to rescue scopolamine-induced amnesia in mice. Moreover, it caused an effective recovery from the cholinergic deficit, tau hyperphosphorylation, Aβ deposition, and behavioral abnormalities in anti-NGF AD 11 neurodegeneration model [41], confirming that it is able to impinge on different points of the neurodegenerative cascade [42]. The significant effectiveness of **3** in reverting neurodegeneration *in vivo* strongly supported the feasibility of the MTDL approach to the treatment of AD. It also formed the basis for the development of a new class of MTDLs obtained through systematic modifications of **3**, as depicted in Fig. (**2**) [43]. The generated compound library behaved as a powerful tool both in understanding the role and function of the multiple

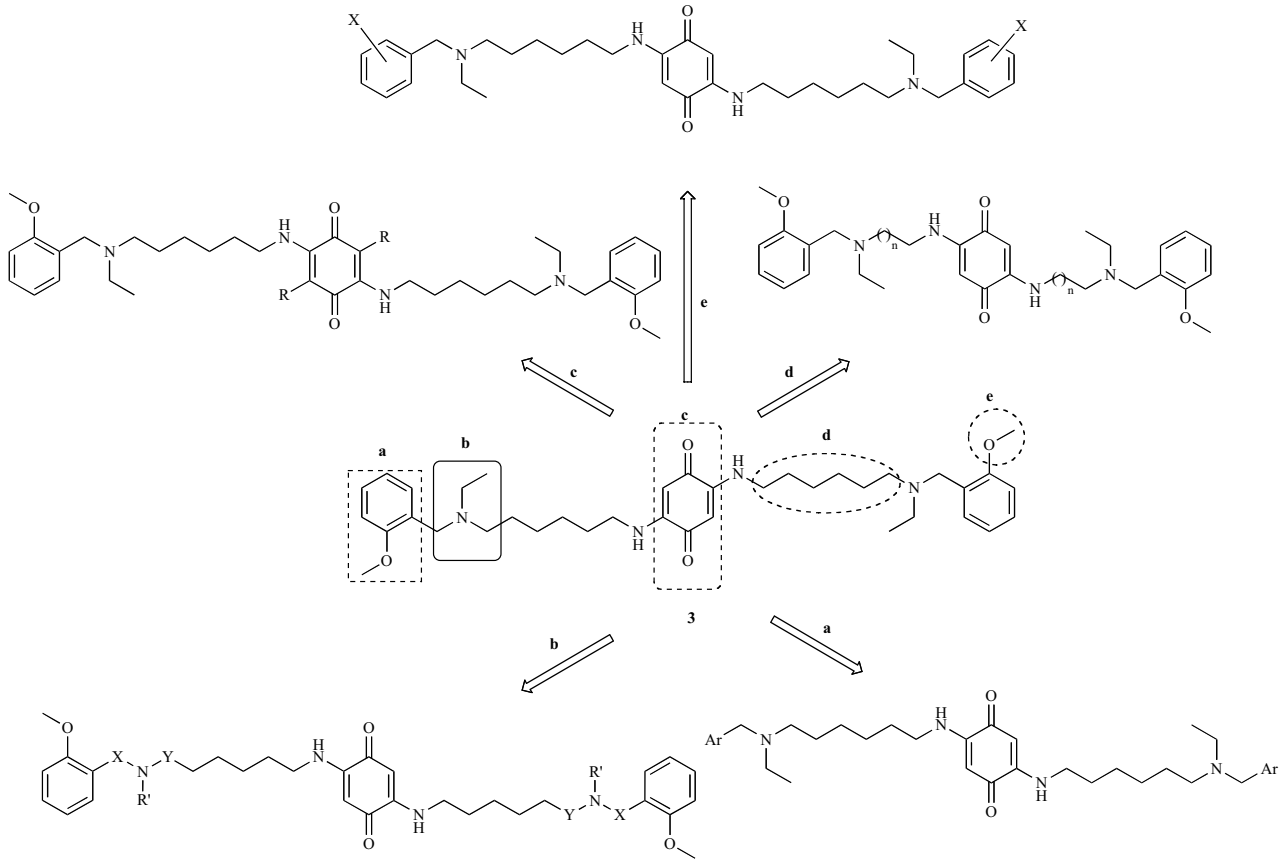

Fig. (2). Design strategy leading to a new library of **3**-derivatives. The structural modifications (**a**, **b**, **c**, **d**, **e**) performed are highlighted.

emerging biological targets and in further validating the design rationale. The wide range of pharmacological data on memoquin and derivatives suggests that this class of quinone-bearing polyamines are innovative and promising candidates for the treatment of AD [43].

3. BIVALENT DUAL BINDING SITE ACHEIs AS MTDLs

In designing dual binding site AChEIs, the bivalent ligand strategy, extensively exploited in the field of opioids [44], has received particular attention. This is because of the peculiar topology of the enzyme, which has two target sites at the top and the bottom of the gorge that share common molecular features [45, 46]. In the first documented application, two tacrine (**4**) units were linked at the amino group by a polymethylene spacer of optimized length to reach both the catalytic and peripheral sites. A computer aided drug design strategy resulted in the bivalent ligand **5**, with a marked superior affinity toward AChE over the monomeric **4** (Fig. (**3**)) [47]. After Pang's first report, very interesting and novel AChEIs were designed through increasing molecular complexity of known lead structures: (i) by duplication of the parent drug to obtain homodimeric structures, or (ii) by combination in the same chemical entity of scaffolds belonging to different lead compounds, giving rise to heterodimers. Thus, several examples of homo- or hetero-dimeric compounds, containing units of tacrine, donepezil, galantamine or huperzine linked by an oligomethylene chain have appeared in the literature, as has been recently reviewed [45, 46, 48].

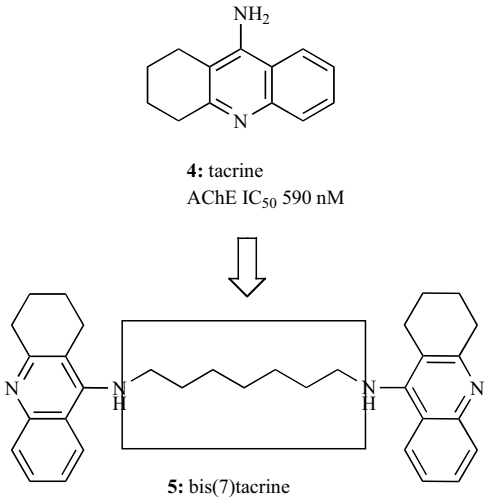

Fig. (3). Chemical structures of **4** and **5**. The latter compound represents the prototype of bivalent AChEIs.

3.1. Homodimeric Congeners

By testing **5** in the fluorometric assay, scientists recently obtained proof of concept that such a prototypic dual binding site inhibitor also decreased AChE-induced Aβ aggregation [49]. Indeed, **5** inhibited AChE-induced Aβ fibrillogenesis with an IC_{50} value of 41.7 ± 3.5 μM, only slightly higher than that of the PAS inhibitor propidium (12.6 ± 0.5 μM) [50]. In light of this new result and in the search for new MTDLs, it was argued to expand the role of the spacer of tacrine-derived bivalent inhibitors through the synthesis of different ligands, in which the distance spanning the two enzyme sites was featured by properly designed scaffolds. To rationally convert the dual inhibitor **5** into a triple MTDL against AD, the spacer was considered as the carrier of a third biological activity relevant to AD, such as metal chelation. The spacers of **6** (BW284c51) and ambenonium (**7**) were selected because these inhibitors carry carbonyl and oxalamide functions, likely endowed with the desired chelation property, and because their dual binding mode had been verified through X-ray diffraction [51] and molecular modeling studies [52], respectively (Fig. (**4**)). The new bis-tacrines **8** and **9** maintained a potent AChE-inhibiting activity (nanomolar range), were able to reverse AChE-induced amyloid fibrillogenesis, and at the same time had the additional property of acting as metal chelators. More importantly, the new MTDLs were not significantly larger or more complex than the parent compound **5**, which had already showed oral activity *in vivo* and therefore might have favourable pharmacokinetic properties [49].

Huperzine A (**10**) is a naturally occurring alkaloid that was isolated in the early 1980s from Chinese medicinal herb *Huperzia serrata* and approved as a drug for the treatment of AD in China. Huperzine B (**11**) is a natural analogue, showing a lower inhibition profile against AChE, but a higher therapeutic index in comparison to huperzine A (Fig. (**5**)). Therefore it was a good candidate for molecular duplication to afford new bis-derivatives with improved affinity towards the enzyme. In a recent report, tethers with two nitrogen atoms were again used to link two molecules of **11**. Derivatives with a 12–20 atom tether displayed significantly higher potency than the parental huperzine B, with inhibitory activity enhanced by about two to three orders of magnitude. The most potent derivative **12** exhibited a 1635-fold increase in AChE inhibition and a dual binding mode of interaction, as revealed by docking studies [53]. More interestingly, even if the authors did not test the AChE-induced Aβ aggregation activity, they disclosed a multifunctional profile for **12**, analogous with the multiple neuroprotective mechanism of action of huperzine A [54]. In cellular assays, **12** attenuated Aβ-induced cytotoxicity, ameliorated Aβ-induced redox disequilibrium, and was more potent than huperzine B in inducing neuroprotection and apoptosis. *In vivo* studies showed that **12** remarkably improved the spatial performance deficits provoked by scopolamine and by transient cerebral ischemia/reperfusion.

In addition to primary AChEIs, the structure of the AChE reactivator 2-PAM (**13**) has also been exploited as a monomeric unit in order to develop dimeric derivatives [55]. Recently, the anti-amyloid property of a series of this type of pyridinium derivatives [56] was evaluated to reveal potential supplementary pharmacological effects which may strengthen their therapeutic application. Aβ fibril formation studies were performed by means of the thioflavin T fluorescence assay. These revealed that at equimolar concentrations **14** and **15** (Fig. (**6**)) were able to inhibit Aβ fibril formation by 50%. Even though the AChE inhibition is in the submicromolar range, the fact that these compounds, in addition to

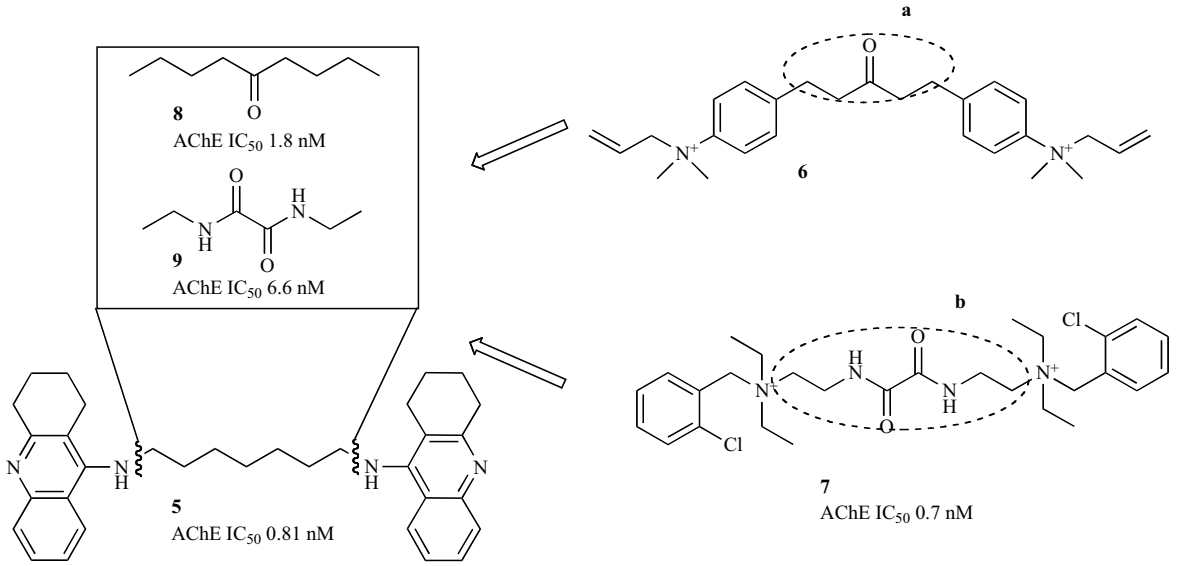

Fig. (4). Design strategy to novel bis-tacrines **8** and **9** with chelation properties (moieties **a** and **b**).

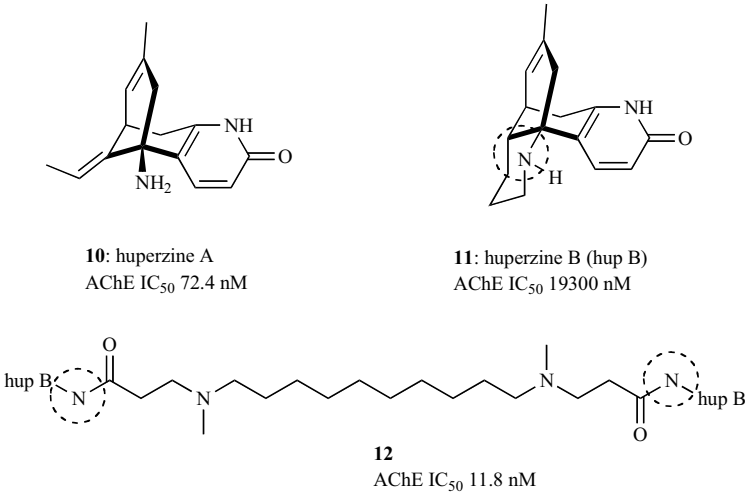

Fig. (5). Chemical structures of huperzine A (**10**), huperzine B (**11**) and the bivalent derivative **12**.

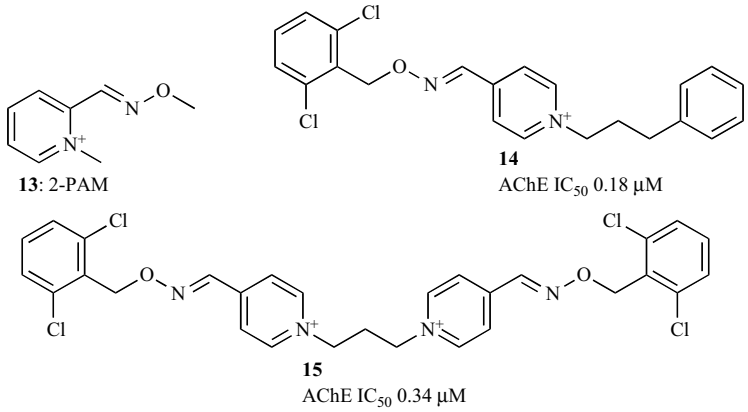

Fig. (6). Chemical structures of 2-PAM (**13**) and of the bivalent pyridinium derivatives **14** and **15**.

their antifibrillogenic activity, showed the ditopic binding mode at enzyme gorge, makes them interesting tools [57].

3.2. Heterodimers Congeners

A successful example of heterodimeric dual binding site AChEIs with anti-amyloid properties is represented by AP2238 (**16**) (Fig. (**7**)), a rationally designed bivalent ligand composed of two different moieties optimal for binding at each enzyme site, connected by a phenyl spacer. The two selected moieties were a benzylamino group for interacting with the active site and a coumarin nucleus, for the peripheral one [58]. The same authors recently conducted extensive investigation into **16**-SAR leading to the discovery of AP2243 (**17**), which, by replacing a methyl group with an ethyl one, showed an improved inhibitory potency against both AChE catalityc (IC_{50} = 18.3 nM) and pro-aggregating activity [59]. In a more recent work aimed at identifying MTDL coumarin derivatives, the structure of **17** was modified to extend the activity to BACE-1, a very attractive target for lowering Aβ levels in the brain. Halophenylalkylamidic functions were introduced in positions 6 or 7 of the coumarin. This is because these moieties emerged as a leitmotif in different BACE-1 inhibitors which appeared in the literature [60]. Although the new structures showed decrease in activity toward AChE, they still maintained the dual binding mode. Of these, compound **18**, which turned out to inhibit both enzymes at very similar submicromolar concentration (0.18 and 0.15 μM), is expected to be a promising hit [61].

A similar design rationale underlies the development of dual binding site AChEIs endowed with a piperidine scaffold. In this case, an aromatic ester was chosen as a moiety for binding at the AChE catalytic site, another aromatic group for PAS, whereas an elongated lipophilic structure was considered optimal for intercalating between Aβ fibrils (Fig. (**8**)). Docking studies confirmed that the most potent compound of the series, **19** (IC_{50} = 320 nM)**,** was able to interact through a π-π stacking with the Trp of the active site *via* the 4-Cl-phenyl moiety, whereas the benzhydryl substituent covered the PAS, assuming a funnel-like shape. To further explore the dual action, **19** was examined in the thioflavin T fluorometric assay, where it showed inhibitory capabilities against Aβ oligomerization and AChE-induced aggregation [62].

Tacrine, as well as being a prototypical cholinesterase inhibitor, is also an allosteric modulator of muscarinic receptors [63, 64]. Similarly, gallamine is a muscarinic allosteric agent, but at high concentrations it also inhibits cholinesterases [65]. Moreover, bis-tacrine **5** has revealed a higher allosteric affinity than **4**. Motivated by these findings, hybrids of tacrine and gallamine connected by a previously established linker [66] were envisaged [67]. All synthesized hybrids, such as **20** (Fig. (**8**)), exhibited a profound inhibitory potential toward AChE (nanomolar range), consistent with the dual binding mode revealed by docking simulations. Moreover, the allosteric potency at M2 receptors was highly increased when compared to the building blocks gallamine and tacrine.

CONCLUDING REMARKS

While AChEIs remain a dynamic and evolving research field in the AD treatment, the rationale for MTDL design strategy clearly stems from the multifactorial etiological ba-

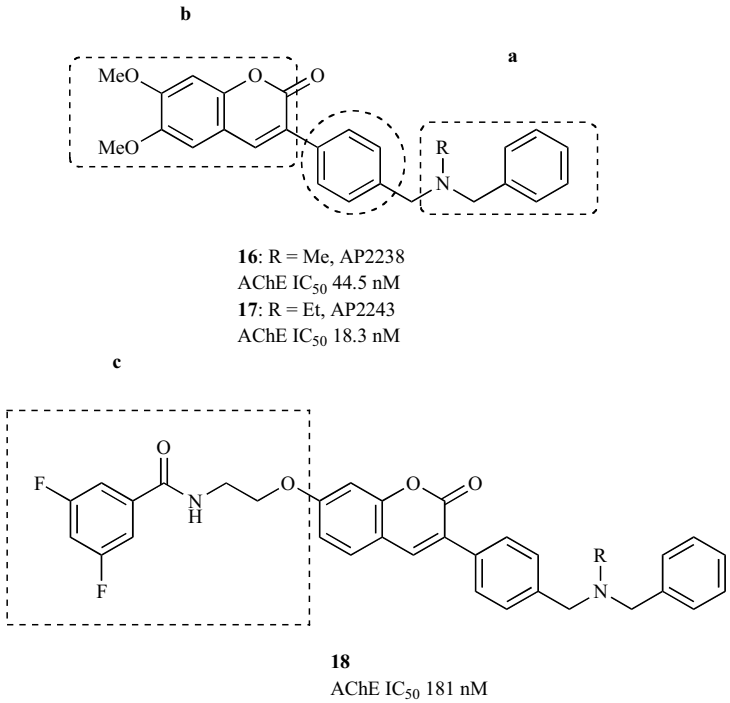

Fig. (7). Chemical structures of AP2238 (**16**) and derivatives **17** and **18**. The moieties responsible for the binding at the catalytic (**a**) and peripheral (**b**) sites, and for BACE-1 activity (**c**) are highlighted.

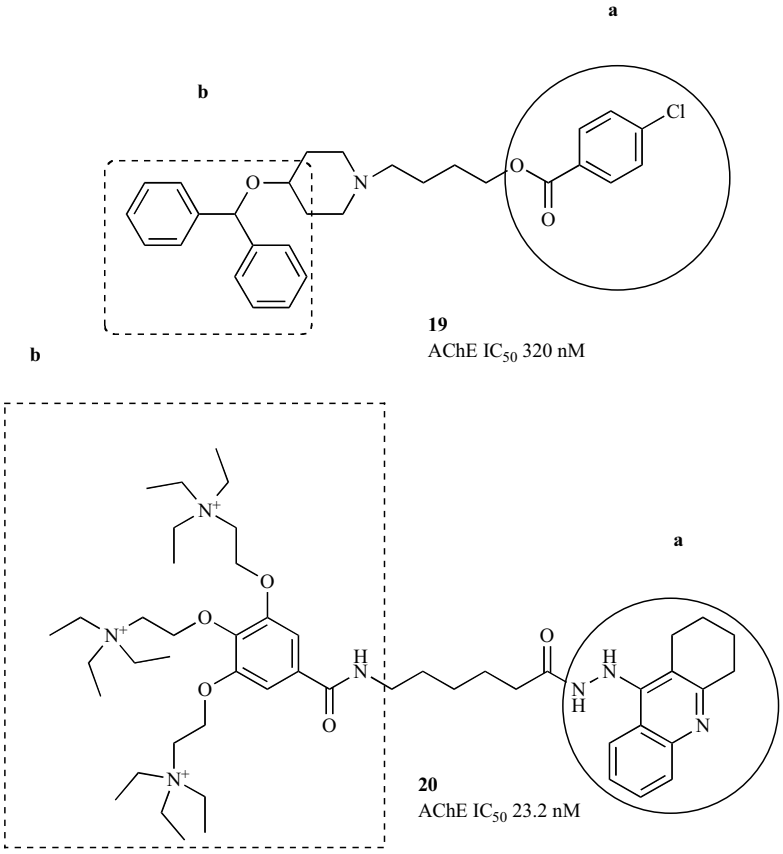

Fig. (8). Chemical structures of heterodimers **19** and **20**. The moieties responsible for the binding at the catalytic (**a**) and peripheral (**b**) sites are highlighted.

sis of AD. In the meantime, new therapeutic targets continue to emerge. This has led to the rapid development of new therapeutic agents investigated in clinical trials. Optimizing the therapeutic potential of dual binding site AChEIs by the addition of one of these activities in the arsenal for fighting neurodegeneration, is an on-going challenge for medicinal chemists.

Different approaches have been utilized to design MTDLs, but all take advantage of the combination in a single molecule of different smaller fragments of carriers of a given specific activity. All the reported examples seem to suggest that this medicinal-chemistry-driven fragonomics [68] approach is the best way to deliver new chemical entities, provided that the molecular enlargement is not too detrimental for the pharmacokinetic properties [69]. Further *in vivo* studies on the MTDLs under investigation or on further developments are urgently required and clearly needed to clarify these topics. Nevertheless, we would emphasize that these therapeutic tools, which simultaneously act at multiple pathways, may have great potential in the development of drugs against AD and other neurodegenerative diseases, and may become therapeutic entities in their own right in the future.

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