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Recent Advances in the Multitarget-Directed Ligands Approach for the Treatment of Alzheimer's Disease

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Published online 26 July 2011 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/med.20248

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Abstract: With 27 million cases worldwide documented in 2006, Alzheimer's disease (AD) constitutes an overwhelming health, social, economic, and political problem to nations. Unless a new medicine capable to delay disease progression is found, the number of cases will reach 107 million in 2050. So far, the therapeutic paradigm one-compound-one-target has failed. This could be due to the multiple pathogenic mechanisms involved in AD including amyloid β (Aβ) aggregation to form plaques, τ hyperphosphorylation to disrupt microtubule to form neurofibrillary tangles, calcium imbalance, enhanced oxidative stress, impaired mitochondrial function, apoptotic neuronal death, and deterioration of synaptic transmission, particularly at cholinergic neurons. Approximately 100 compounds are presently been investigated directed to single targets, namely inhibitors of β and γ secretase, vaccines or antibodies that clear Aβ, metal chelators to inhibit Aβ aggregation, blockers of glycogen synthase kinase 3β, enhancers of mitochondrial function, antioxidants, modulators of calcium-permeable channels such as voltage-dependent calcium channels, *N*-methyl-D-aspartate receptors for glutamate, or enhancers of cholinergic neurotransmission such as inhibitors of acetylcholinesterase or butyrylcholinesterase. In view of this complex pathogenic mechanisms, and the successful treatment of chronic diseases such as HIV or cancer, with multiple drugs having complementary mechanisms of action, the concern is growing that AD could better be treated with a single compound targeting two or more of the pathogenic mechanisms leading to neuronal death. This review summarizes the current therapeutic

Contract grant sponsor: Fundación CIEN; *Contract grant number:* PI 016/09; *Contract grant sponsor*: Agencia Laín Entralgo; *Contract grant number:* NDG07/9; *Contract grant sponsor*: MICINN; *Contract grant numbers:* SAF2006-08764-C02-01; SAF2009-07271; *Contract grant sponsor*: CSIC-GRICES; *Contract grant number:* 2007PT-13; *Contract grant sponsors*: ISCIII; MICINN; *Contract grant number:* RD06/0026/1002 Retic "RENEVAS"; *Contract grant sponsor*: Comunidad de Madrid; *Contract grant number:* S/SAL-0275-2006.

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strategies based on the paradigm one-compound-various targets to treat AD. A treatment that delays disease onset and/or progression by 5 years could halve the number of people requiring institutionalization and/or dying from AD. Med Res Rev., 33, No. 1, 139–189, 2013

Key words: Alzheimer's disease; multitarget drugs; dual AChE inhibitors; AChE peripheral anionic site; multiactive compounds; Ca^{2+} dyshomeostasis; 1,4-dihydropyridines; voltage-dependent calcium channels; amyloid–β antiaggregating agents; antioxidant drugs; anti-inflammatory drugs; NSAIDs; neuroprotection; Ca^{2+} overload; oxidative stress; metal chelators; BACE-1 inhibitors; GSK-3β inhibitors; ERK2-inhibitors; CDK5 inhibitors; CK-1 inhibitors

1. INTRODUCTION

When Alois Alzheimer treated his patient Auguste D. more than 100 years ago, he did not realize that he was about to describe one of the most complex and challenging diseases in the history of humanity.[1] Since then, a great research effort has been devoted to increase the knowledge and understanding of this disease. Many scientists are currently investigating the pathogenic mechanism of Alzheimer's disease (AD) and millions are invested every year; however, the molecular basis of the disease is yet to be determined. The estimated number of patients needing treatment is 7–8 million in Europe, 4–5 million in the USA contributing to a total of 24 million worldwide. The number is expected to be doubled to 42 million in 2020 due to aging population.[2] Owing to the sheer number of patients, AD is considered the most common neurodegenerative disorder and a major health concern to societies worldwide.

AD is characterized by progressive memory loss, language skills decline, and other cognitive impairments.[3] The etiology of AD is not completely known; however, there are diverse factors such as amyloid-β (Aβ) deposits,[4] τ-protein (τ) aggregation,[5] oxidative stress,[6] and decreased levels of acetylcholine (ACh)[7], which are thought to play significant roles in the pathophysiology of the disease.[8] Because of its complexity, AD has been described as a multifactorial disease.[9] AD develops as a complex network of events, which are all involved and interconnected with the symptoms to induce the subsequent evolution of the disease.

Amyloid plaques are formed, mainly, of Aβ peptides that aggregate after structural modifications. The interaction of Aβ peptides with acetylcholinesterase (AChE) increases the rate of Aβ aggregation and also its toxicity.[10] The τ-protein acts as a stabilizer of the neuron's cytoskeleton. After being hyperphosphorylated, it becomes detached of the microtubules to form intracellular aggregates called neurofibrilary tangles (NFTs). NFTs are aberrant structures linked to the Aβ-induced neurotoxicity.[11] Among the molecular factors linked to AD, the apolipoprotein E (APOE) genotype is implicated in AD pathogenesis. Hence, APOE4 mutations are a high risk factor that can lead to early onset AD.[12] Although a number of mechanisms have been proposed to link APOE4 to Aβ,[13,14] the general consensus is that APOE4 isoform activity increases Aβ peptide concentration by decreasing its clearance.[15–17] Simultaneously, APOE increases the activity of glycogen synthase kinase 3β (GSK3β),[18] (kinase that specifically hyperphosphorylates τ protein). On the other hand, it has been established that GSK3β is essential for Aβ-induced neurotoxicity.[19] Aβ not only activates GSK3β but also cyclin-dependent kinase 5 (CDK5) and extracellular signal-regulated kinase 2 (ERK2), leading to τ hyperphosphorylation and, ultimately, to apoptosis.[20–22]

The Ca^{2+} ion is one of the most important second messengers in the brain. Tight control of Ca^{2+} homeostasis is crucial for cell survival. In AD, Ca^{2+} imbalance is likely to be one of the main causes of neurodegeneration.[23,24] Since Khachaturian proposed the pathological role of Ca^{2+} in AD,[25] much evidence has substantiated this hypothesis.[26,27] Aβ is able to affect neuronal membranes by making them unable to regulate their internal concentration of ions, particularly

Ca^{2+}.[25,28,29] Aβ increases the Ca^{2+} influx that occurs when neurotransmitter glutamate activates the *N*-methyl D-aspartate receptors (NMDAr).[30] This finding is supported by the fact that memantine[31] (a NMDAr antagonist, the first non cholinergic drug approved for AD) blocks Aβ-induced Ca^{2+} influx, indicating that drugs restoring the Ca^{2+} balance in neurons might indeed be therapeutic options for the disease.[32] The fact that Ca^{2+} imbalance does cause neuron death during the disease evolution suggests that restoration of Ca^{2+} homeostasis may become a new therapeutic strategy.

Recent drug development projects were based on the emergence of new potential targets in different genomic and proteomic studies. Despite all efforts of drug development undertaken, the number of successful drugs and novel targets have been lower than expected during the past few decades.[33–35] The ''one-target one-compound'' paradigm was highly successful in the past, thanks to biochemical studies and discovery of the molecular mechanisms that underly common diseases. Biologists were able to define a key target for a particular disease, leading to medicinal chemists strategically designing a molecule that interacts with this target selectively, with a potential drug as the outcome. After 20 years, it is evident that this target-based approach does not always guarantee success. Drugs directed to a single target might not always modify complex systems, even if they act in the way they are expected to proceed. It is very common in the cell to have ''back-up'' systems yielding the same effect such as gene expression, protein synthesis, receptors response, and protein degradation. Proteins and intermediates involved in this back-up systems can be compleately different and therefore, drugs targeting primary pathways will have no effect over this back-up pathway, an effect known as redundancy.[36] Moreover, many cellular networks are buffered to prevent major changes in their outputs, despite dramatic changes in the constituents.[37–40] Complex disorders, such as cancer, cardiovascular disease, depression, and neurodegenerative diseases, tend to result from multiple molecular abnormalities and not from a single defect. By using target-directed drugs it is not always likely that the effect will modify the evolution of the illness.

In the last decade, new strategies are emerging to overcome the lack of efficiency of the ''one-target one-compound'' development based on the complexity of the disease and the relationship between different pathways in the pathological evolution of the illness. Many of these drug development programs aim to influence multiple targets in a parallel fashion. One of the most widespread multiple target approaches, the so-called combination therapy, is increasingly used to treat many types of diseases, including AIDS, atherosclerosis, cancer, and depression. As one of the newly developed combination therapies, ''multitarget lead discovery'' is a promising strategy for the identification of unexpectedly novel effects of drug combinations.[41–45]

A similar approach that might have inherent advantages is the development of ligands including multiple targets, or the so-called ''multitarget-directed ligands'' (MTDLs).[46] Multitarget therapeutics can be more efficacious making the biological system more sensitive to the action of a drug with two or more targets simultaneously, thereby, mitigating the redundancy effect. The idea that multitarget drugs might be more effective than those directed to a single target has emerged from efforts made in understanding the action of different treatments such as antipsychotic drugs. One example is Clorazil® (clozapine), which targets a large number of proteins and has moderate side effects. In an effort to reduce its side effects, a range of analogs were developed, attempting to bind fewer targets. Surprisingly, many of these analogs had reduced activity, but still showed similar side effects.[47] Another example is amitriptyline, a dual inhibitor of serotonin reuptake transporter (SERT) and norepinephrine transporter (NET). It appears to combine a faster onset of action with increased efficacy.[48,49] This effect results from the demonstration of antagonism of 5-hydroxytryptamine receptors (5-HT) subtypes namely 5-HT_{2A}, 5-HT_{2C}, 5-HT_6, 5-HT_7, α_1-adrenergic, histamine H_1, muscarinic ACh receptors, and σ_1 receptor agonist properties.[50–56]

Some examples of marketed multitarget drugs are based on cancer drug development.[57] Sutent® (sunitinib malate), marketed by Pfizer, is a multitargeted inhibitor of tyrosine kinase (RTC), which blocks epidermal growth factor receptor and two other similar kinases.[58] On the other hand, sorafenib (Nexavar®; Bayer, Leverkusen, North Rhine-Westphalia, Germany), used in the treatment of various types of cancer, is an inhibitor of several protein kinases, vascular endothelial growth factor (VEGF) receptor 2 and 3 kinases and c kit. Sorafenib was the first molecule targeting the Raf/Mek/Erk pathway (within the MAP kinase pathway).[59] Furthermore, Gleevec® (imatinib mesilate),[60] which represents one of the first developments approved in 2001 by the FDA, is a multikinase inhibitor.[61] Several efficient drugs such as salicylate, nonsteroidal anti-inflammatory drugs (NSAIDs), metformin, or antidepressants[44] also affect many targets simultaneously.[44,60,62–66]

The concept of the multitarget approach is particularly applicable to AD. Efficacious and durable responses in AD require multitarget drugs, as the pathogenic mechanisms leading to neurodegeneration are known to be multifactorial.[67] The molecular basis of AD can be considered as a complex network with multiple pathological crossroads in the system, which must be modified simultaneously in order to induce a positive effect. The above-mentioned complexity of AD expressed as a linked network of several pathological events lead to the final conclusion that the best approach to treat this illness will involve the multitarget approach.[68] Not surprisingly, some of the most important nodes in the pathological network of AD are the most relevant targets currently under development such as amyloid precursor protein[69] (APP), β-secretase-1[70] (BACE-1), GSK3β,[71] Ca^{2+},[72] NFTs, and oxidative stress.[73] The multitarget approach is the starting point for the development of ligands capable of modifying the pathological system at several nodes simultaneously, resulting in a major action over the network. This strategy is proposed to avoid redundant mechanisms that could escape to a single-target attack, facilitating the recovery of the cells to a healthy state. This review will focus on the recent efforts devoted to the development of multitarget modifying molecules (Fig. 1).

2. CURRENT TREATMENT OF AD

The most widely used concept for AD drug development is the cholinergic hypothesis,[74] used for the discovery of palliative drugs during the last 20 years. The cholinergic hypothesis is based on the observation of a deficiency of ACh in the central nervous system. Subsequently, Whitehouse et al.[75] discovered the degeneration of neurons from the nucleus basalis of Meynert, mainly constituted by cholinergic innervation, in patients with AD. The loss of cholinergic function is closely related to cognitive dysfunction.[76] Also, in early onset patients observation showed abnormalities such as diminished activity of choline acetyl transferase (ChAT),[77] enzyme responsible for the synthesis of ACh. Activities of AChE and butyryl-cholinesterase (BuChE) were increased and concentrations of nicotinic ACh receptors (nAChRs) were diminished.[77] As a result of these findings, the primary therapeutic approach to address cognitive loss associated with AD has been the cholinergic replacement strategy. This approach was attempted using muscarinic and nicotinic cholinergic ligands and AChE inhibitors (AChEIs).[78]

Current treatments for AD are mainly based on the inhibition of AChE; however, new hypotheses are emerging. In 1993, tacrine (**1**, Fig. 2), an AChEI, was approved by the FDA for clinical use against AD. After tacrine, three more AChEIs were approved for the treatment of AD namely, donepezil[79] (**2**, Fig. 2), galantamine[80] (**3**, Fig. 2), and rivastigmine[81] (**4**, Fig. 2). These three AChEIs are currently the most common treatments for mild to moderate stages of AD sufferers. However, their clinical usefulness is limited, largely because

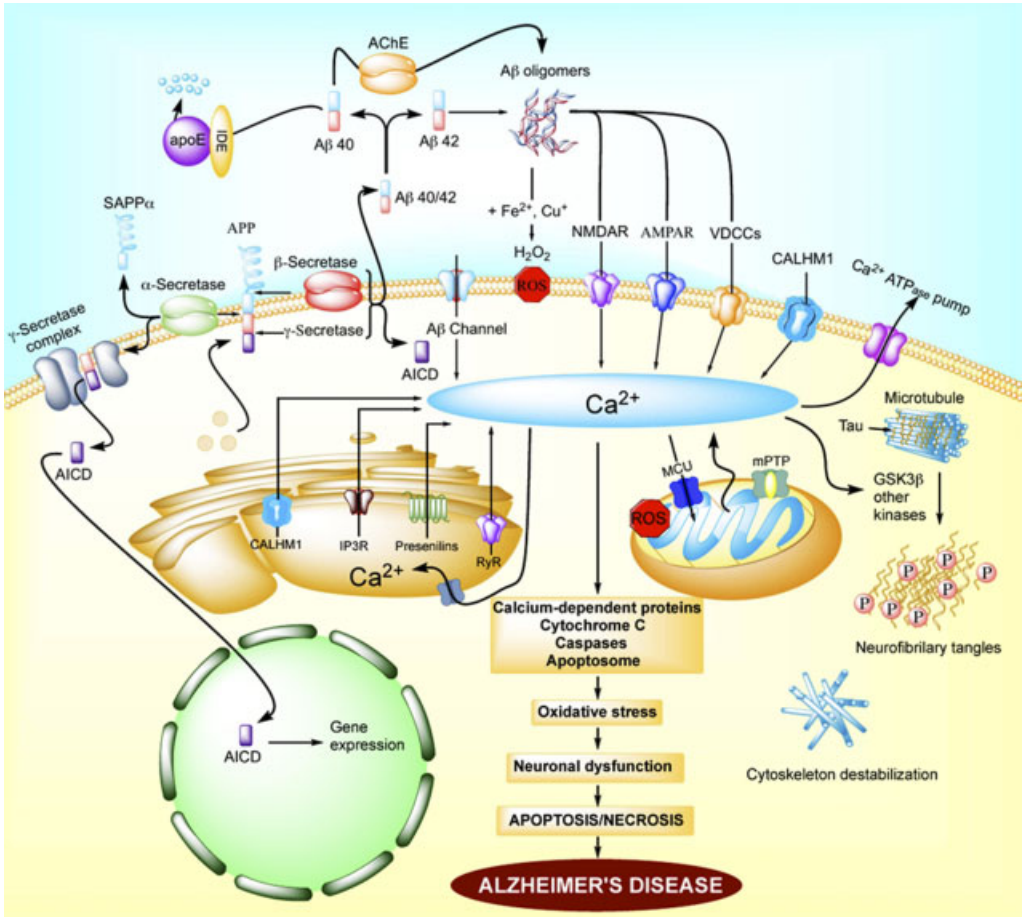


Figure 1. Interconnected pathways in Alzheimer's disease. The nonamyloidogenic pathway starts with the processing of the amyloid precursor protein (APP) by α-secretase to generate the soluble APP fragment α (sAPPα) and a second fragment that is further processed by the γ-secretase complex. In the amyloid pathway, APP is cleaved by β-secretase followed by γ-secretase complex to generate amyloid β peptide (Aβ 40/42), soluble APP β (sAPPβ), and amyloid intracellular domain (AICD). The Aβ monomers that are released outside neurons can be removed by microglia, which release insulin-degrading enzyme (IDE) that destroys them. This aberrant peptide increases the influx of Ca^{2+} by forming Ca^{2+} permeable channels in the plasma membrane. $Aβ_{42}$ has high potential to aggregate to form Aβ oligomers, which rate of aggregation is also increased by the interaction of Aβ with the peripheral anionic site of AChE. Aβ oligomers can interact with metal ions Fe^{2+} or Cu^{+} activating the generation of reactive oxygen species (ROS). These species damage the plasma membrane facilitating the influx of different ions resulting in membrane depolarization. Membrane depolarization and subproducts of lipid peroxidation affect the function of different receptor and channels such as *N*-methyl D–aspartate receptors (NAMDR), α–amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), voltage-dependent calcium channels (VDCCs). Aβ oligomers can affect also the activity of these receptors and channels directly. Presenilins (PS) function as Ca^{2+} leak channels at the endoplasmic reticulum (ER), mutated PS are highly related to familial AD. PS have been found at ER membranes. Mutations in PS modify their ability to regulate Ca^{2+} in the ER enhancing Ca^{2+} release through ryanodine receptors (RyR) and inositol triphosphate receptors (InsP3R) channels. There is also evidence that PS can interact directly with InsP3Rs, RyRs, and the SERCA pump to alter ER Ca^{2+} release and uptake. AICD migrates to the nucleus, interacts with transcription regulators, and modifies gene transcription to disrupt Ca^{2+} homeostasis. Elevated Ca^{2+} can also affect the attachment/detachment equilibrium of protein Tau (τ) to tubulin to form the cytoskeleton. Glycogen synthase 3 β (GSK3β) and other kinases are involved in this equilibrium; Ca^{2+} can modify their activity inducing τ hyperphosphorylation to generate, after its aggregation, the neurofibrillary tangles. Aβ also affect mitochondria inducing oxidative stress and Ca^{2+} dysregulation resulting in an increase in the production of radicals and decreased production of ATP. High concentrations of Ca^{2+} can be stored by mitochondria through mitochondrial Ca^{2+} uniporter (MCU), resulting in Ca^{2+} overload of mitochondria leading to opening of mitochondrial permeability-transition pore (mtPTP) and apoptosis.

of adverse peripheral effects arising from excessive activation of cholinergic systems, with side effects such as confusion, hallucinations, extreme or sudden changes in behavior, nausea, or stomach pain.[82] Additionally, the hepatotoxic effects[83] of tacrine led to its drop out from

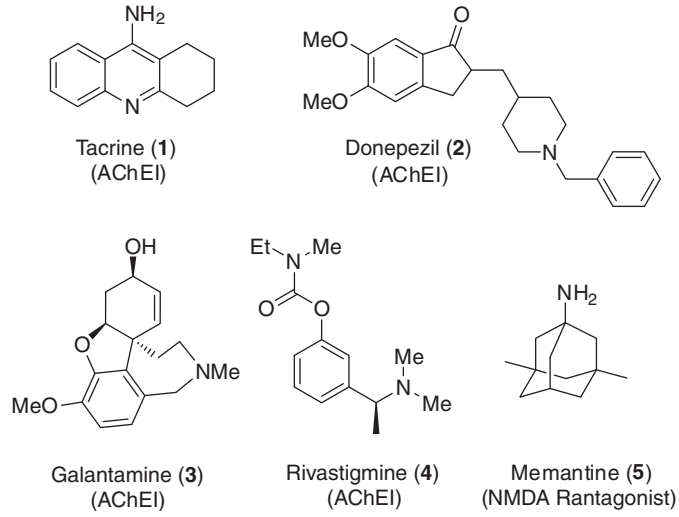


Figure 2. Tacrine and other drugs currently available to treat AD patients.

clinical use. Later on, memantine (**5**, Fig. 2)[31] was approved for the treatment of AD. Memantine is an NMDAr antagonist developed considering the central role of Ca^{2+} in the pathogenesis of AD.[84]

Although the cholinergic hypothesis initiated palliative treatment, increasing evidence is accumulating suggesting that AChEIs have only minor, if any efficacy on AD.[85] Furthermore, these drugs do not address the underlying causes of the disease.[85–88] Recently, a new demonstrated effect of AChE, the so-called ''non-classical'' effect,[10] has arisen increased interest in developing dual interacting inhibitors of this enzyme. New developments under clinical trials include Aβ antiaggregating or plaque-dissolving agents like phenserine (an AChE inhibitor and Aβ synthesis inhibitor, phase III unsuccessful)[89] and ELND005 (anti-aggregating agent, phase II ongoing).[90,91]

Inflammation and glia activation observed in AD patients suggested that NSAIDs might be effective for the treatment and more importantly, for the prevention of AD.[92] Although phase III clinical trials with various NSAIDs were unsuccessful, new evidence has emerged supporting this theory, resulting in the development of new molecules.[93–98] Other targets such as GSK3β, BACE-1,[70] γ-secretase,[99] nAChRs,[100] Aβ-immunization, histamine-3 (H_3) receptors, α–amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), γ-aminobutyric acid (GABA) receptors, as well as neuroprotective and neurotrophic agents are now being addressed. In 2009, Sabbagh reviewed selected molecules and antibodies that are currently in clinical trials with diverse and interesting results.[101]

Many approaches and targets have been addressed in the treatment of AD.[23,46,102–107] However, important physiopathological aspects of AD remain unclear. Growing evidence suggests an underlying complex network that comprises genetics, enzyme activities, receptor expression, protein interactions, alteration of metal concentrations, cell cycle survival disruption, ion homeostasis dysregulation, protein misfolding, among others. Medicinal chemists were prompted by this evidence to accept the multifactorial hypothesis, and the idea of the ''MTDLs'' that Melchiorre and coworkers proposed.[46]

In this review, we will summarize the most recent approaches on basic developments that will bring the next generation of multitarget AD drugs during the next years. Our summary will reflect the recent understanding of new targets in the design process that leads to new multitarget ligand development.

3. DUAL BINDING SITE AChE INHIBITORS

The development of dual binding site AChEIs is based on the cholinergic hypothesis. As referred to above, the cholinergic system is the most affected in AD patients with almost 80% of cholinergic neuron loss.[74] Early observations have demonstrated a decrease in ACh concentration within the synaptic cleft. At the same time, a decrease in the activity of ChAT, and hyperactivity of AChE and BuChE is also observed.[74] These findings led to the rational development of the first generation of AD drugs, AChEIs. Inhibition of AChE increases the concentration of ACh at the synaptic cleft, thereby enhancing central cholinergic neurotransmission. The increased bioavailability of ACh was supposed to be the treatment of the cognitive and behavioral symptoms of the patients. More than a decade since the approval of the first AChEI, some concerns about their benefits are emerging.[108]

Nevertheless, in 1996,[109] the interest in this kind of drugs was renewed due to the first demonstration of the so-called "non cholinergic action of AChE."[110] Inestrosa et al. proved for the first time the relationship between AChE and the aggregation of Aβ.[109] Interaction of Aβ at the peripheral anionic site (PAS) of AChE greatly accelerates the aggregation of this toxic peptide.[111] Interactions at the PAS of AChE catalyze some conformational changes in Aβ fibrils to form the β-sheet with increased aggregating potential.[112] Thus, inhibitors targeting the PAS of the enzyme will decrease the aggregation rate of Aβ keeping it in solution, therefore facilitating its clearance. This strategy could represent a new area of research for the AD treatment based on both, the cholinergic and the amyloid hypothesis.

Early developments on dual inhibitors of AChE were based on the elucidation by Sussman et al. of the 3D structure of AChE[113] and the reported structure of the donepezil–AChE complex.[114] Donepezil (**2**, Fig. 2) is able to interact in both, catalytic and PAS binding sites of AChE via extended solvent organization.[115] Several studies demonstrated the ability of donepezil (22% inhibition)[112] and other AChEIs to decrease the AChE-induced Aβ aggregation. A large antiaggregating effect was observed for PAS ligands such as propidium (**9**, Fig. 3) (82% inhibition), giving further insight into the connection between PAS and amyloid plaques.[112] Subsequent developments of dual binding sites inhibitors were directed toward the modification of donepezil structure, in order to achieve a better pharmacological profile while keeping both interactions. A wide range of *N*-benzylpiperidine analogs were developed with different properties; a key review on those derivatives was reported by Muñoz-Torrero et al.[116] Following this hypothesis, new families of compounds were reported

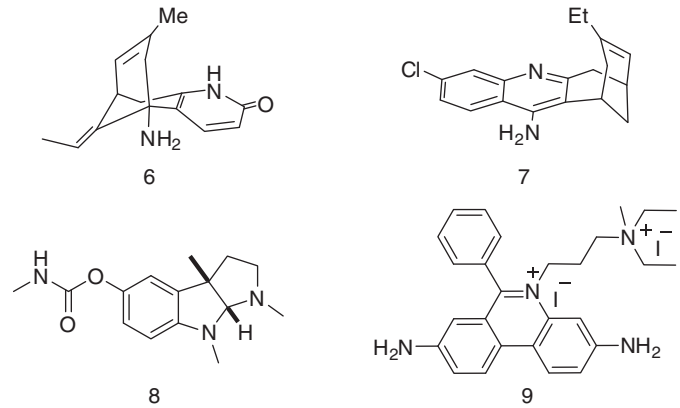
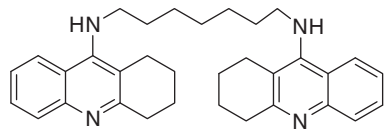


Figure 3. Chemical structure of known AChE inhibitors (−)-huperzine A (**6**), huprine X (**7**), physostigmine (**8**), and propidium iodide (**9**).

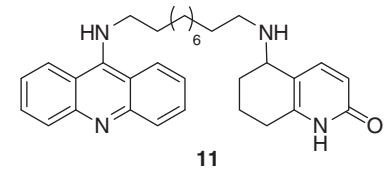
as homodimers or heterodimers of known inhibitors such as tacrine (**1**, Fig. 2), donepezil (**2**, Fig. 2),[117] galantamine (**3**, Fig. 2),[80] (−)-huperzine A[118] (**6**, Fig. 3), huprine X (**7**, Fig. 3),[119] physostigmine (**8**, Fig. 3),[120] and propidium (**9**, Fig. 3)[121] among others.

Bis(7)-tacrine (**10**, Fig. 4) was one of the first homodimers reported in the literature with increased potency as AChEI ($IC_{50} = 0.4$ nM), and showing good selectivity toward BuChE.[122] Bis(7)-tacrine also improved memory-enhancement capabilities.[123] Compound **10** increased the spontaneous quantal ACh release from peripheral cholinergic terminals in the electric organ of *Torpedo marmorata*, at lower concentrations than tacrine. It also had some effect on nAChRs.[124] Neuroprotective properties were tested in different models where



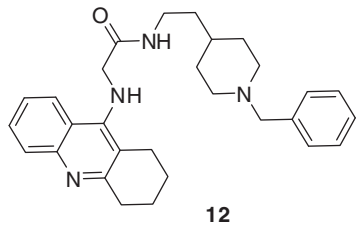
Bis(7)-tacrine: 10

AChE	IC_{50} = 0.40 nM
BuChE	IC_{50} = 390 nM
$A\beta_{(AChE)}$	IC_{50} = 41.7 μM
NMDA blockade	IC_{50} = 0.76 μM
$GABA_A$ blockade	IC_{50} = 5.6 μM
BACE-1	IC_{50} = 7.5 μM



11

AChE IC_{50} = 8.8 nM
BuChE IC_{50} = 81.5 nM



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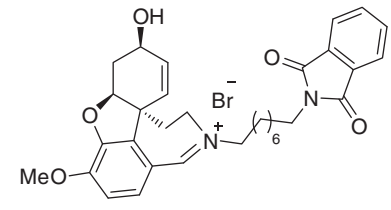
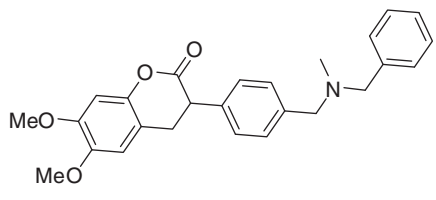
AChE IC_{50} = 6.0 nM
BuChE IC_{50} = 76 nM



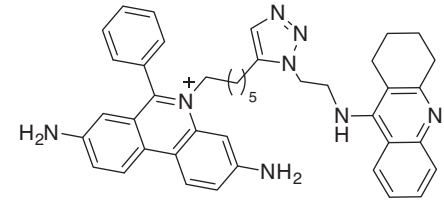
13

AChE IC_{50} = 10 nM



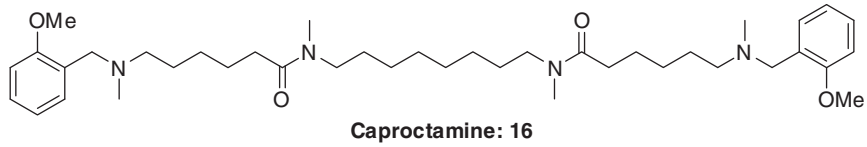
AP2238: 14

AChE	IC_{50} = 44.5 nM
BuChE	IC_{50} = 48.9 μM
$A\beta_{(AChE)}$	35% at 100 μM



15

AChE Kd = 77 fM



Caproctamine: 16

AChE IC_{50} = 0.17 μM
M_2 K_b 660 nM

Figure 4. Multitarget drug developments as dual binding site AChE inhibitors.

Bis(7)-tacrine revealed a NMDAR antagonist character, with an IC_{50} of 0.76 μM preventing glutamate-induced neuronal apoptosis.[125] Interestingly, a few years later a regulatory effect of L-type calcium channels was reported;[126] such an effect has several neuroprotective implications discussed in following paragraphs. Compound **10** also showed neuroprotection against hydrogen peroxide-induced oxidative stress.[127] Its large pharmacological profile did not include any Aβ implications until 2007 when Bolognesi et al.[128] reported for the first time such activity. Bis(7)-tacrine inhibited the AChE-induced Aβ aggregation with an IC_{50} of 41.7 μM. A year later, Fu et al. reported a decrease by **10** in the generation of both, secreted and intracellular $Aβ_{42}$ (48.5% reduction at 3 μM) and $Aβ_{40}$ (37.7% reduction at 3 μM).[129] An initial explanation might be the interaction of this drug at the PAS of AChE; however, surprisingly, it was able to increase the amount of soluble amyloid protein precursor α fragment (APPα) and decrease the aberrant insoluble β-fragment (APPβ). This indicates the activation of a different processing pathway. Fu et al.[129] demonstrated the possible activation of α-secretase, although it has a minor role (less than 10% activation). More importantly, Bis(7)-tacrine behaved as a BACE-1 inhibitor with an IC_{50} of 7.5 μM.[129]

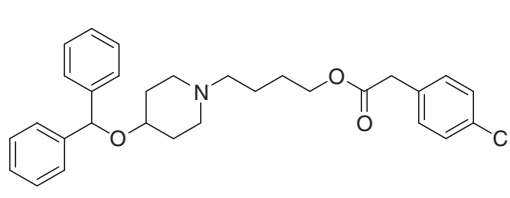
In addition to the homodimers of tacrine, other AChEIs have been used to form heterodimers; this is the case of compound **11**[130] (Fig. 4). Designed as heterodimer of tacrine and the 5-amino-5,6,7,8-tetrahydro-2(1*H*)-quinolone fragment from (−)-huperzine A (**6**, Fig. 3),[118] it is a selective and potent AChEI. Compound **11** (IC_{50} = 8.8 nM) is 13-fold more potent than (−)-huperzine A, 25-fold more potent than tacrine, and 10-fold more selective toward BuChE than tacrine. Several heterodimers were reported with improved activities and selectivity over the monomers.[130–132] The crystal structure was solved for this type of heterodimers.[132] The tacrine moiety binds to the PAS of AChE. To the best of our knowledge, no further studies were performed in order to elucidate their potential Aβ-antiaggregating effect. Donepezil was also dimerized with tacrine, resulting in a new family of inhibitors yielding compounds type **12** (Fig. 4), with similar potency as donepezil.[133] In the case of galantamine, homodimers led to better inhibitors than the parent compound.[134] Galantamine hetero–hybrid **13** (Fig. 4) was the lead compound with an IC_{50} of 10 nM.[135]

In 2003, Piazzi et al.[136] reported the synthesis and biological activity of AP2238 (**14**, Fig. 4), an interesting molecule based on the study of crystal structures of different AChE–ligand complexes and molecular modeling studies. This compound was designed as a merger of two optimal binders for both catalytic and PAS sites. A benzylamino group and a coumarin (2*H*-2-chromene) were fused with a linker able to interact with the residues at the gorge of the enzyme. AP2238 is a good inhibitor of AChE (IC_{50} = 44.5 nM), including good antiaggregating properties (35% inhibition at 100 μM, 1.6-fold more potent than donepezil).

An inspired approach for the design of dual binding site inhibitors of AChE was reported by Sharpless et al. in 2002.[137] Using click chemistry as a tool for lead optimization, they reported one of the most potent inhibitors known to date. Compound **15** (Fig. 4) inhibited AChE with a dissociation constant as low as 77 fM. Co-crystallization of **15** with AChE revealed a multivalent interaction, the expected interaction of the separated monomeric moieties and further hydrogen bonding of the triazole ring.[138] Another example of early developments of dual binding site AChEIs is caproctamine[139] (**16**, Fig. 4) a polyamine-based dual inhibitor of AChE designed from benextramine,[140] a muscarinic receptor (M) M_2 antagonist. Caproctamine was able to inhibit AChE (IC_{50} = 0.17 μM) and block the M2 muscarinic receptor (M_2 K_b = 0.66 μM).[141] Recently, new approaches to dual AChEIs with improved potencies and better Aβ anti-aggregating effects have been reported.[142–145] Some of these new structures are summarized in Figure 4.

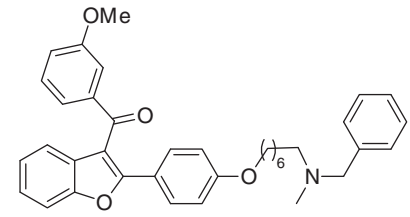
In 2007, Kwon et al.[142] reported a new family of compounds based on the structure of donepezil with the core structure of *N*-substituted piperidines. These have the ability to bind on both sites of the AChE, including effective inhibition of AChE-induced $Aβ_{1–42}$

aggregation and on self-aggregation. Compound **17** (Fig. 5), bearing a *p*-Cl substituent, was selected as the best inhibitor of the family. Compound **17** has an IC_{50} of 0.32 μM to inhibit AChE and a good selectivity (BuChE IC_{50} = 34.4 μM) being 120-fold more active toward AChE. Molecular modeling studies showed an interesting interaction with both catalytic and PAS sites. This suggests that the 4-chlorobenzene moiety in **17** might be placed at the bottom of the gorge, bound by π–π stacking to the aromatic indole-ring of Trp84. The aliphatic alkyl chain of **17** is placed in the middle of the gorge surrounded with phenyl rings of Tyr121, Phe330, and Tyr334. Compound **17** was approximately 37-fold less potent inhibiting AChE than donepezil; however, **17** was able to inhibit AChE-induced Aβ aggregation by 42.4% at 1 μM and by 55.5% at 100 μM. It is more effective than tacrine and donepezil, which were unable to inhibit the aggregation at these concentrations. Furthermore, compound **17** was able to inhibit Aβ self-aggregation with an IC_{50} of 28.2 μM, 3-fold more potent than donepezil.



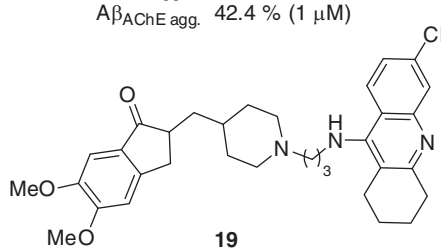
17

AChE	IC_{50} = 0.32 μM
BuChE	IC_{50} = 38.4 μM
$A\beta_{self\ agg.}$	IC_{50} = 28.2 μM
$A\beta_{AChE\ agg.}$	42.4 % (1 μM)



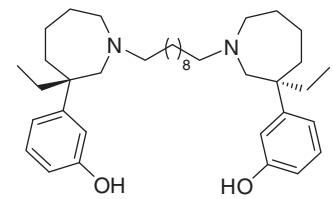
18

AChE	IC_{50} = 10.5 μM
BuChE	IC_{50} = 1.82 μM
$A\beta_{self\ agg.}$	IC_{50} = 13.0 μM



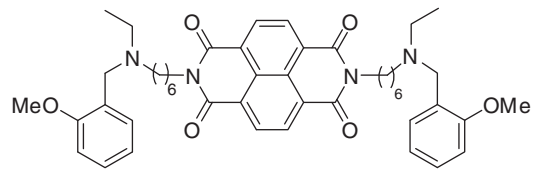
19

AChE	IC_{50} = 0.09 nM
BuChE	IC_{50} = 66.3 nM
$A\beta_{AChE\ agg.}$	46.1 % (100 μM)



20

AChE	IC_{50} = 9.5 nM
BuChE	IC_{50} = 17.0 nM
$A\beta_{AChE\ agg.}$	IC_{50} = 5.80 μM



21

AChE	IC_{50} = 0.37 nM
BuChE	IC_{50} = 1.91 μM
$A\beta_{self\ agg.}$	IC_{50} = 9.69 μM
$A\beta_{AChE\ agg.}$	IC_{50} = 8.13 μM

Figure 5. Dual binding site AChE inhibitors with improved anti-Aβ aggregating properties.

Evidence indicates that BuChE might be a co-regulator of the activity of the neurotransmiter ACh.[146] Remarkably, cortical levels of BuChE show a significant increase in AD patients. This observation prompted the development of selective inhibitors to determine the role of this enzyme and the therapeutic feasibility of its inhibition.[147,148] Compound **18** (Fig. 5)[143] was designed as a new selective BuChE inhibitor, maintaining the ability to bind to the PAS of AChE. Compound **18** was based on two molecules, *N*-Methyl-*N*-benzylamine,[149] an AChEI and compound SKF-64346, a benzofuran derivative with good properties as an inhibitor of Aβ-fibril formation.[150] *N*-methyl-*N*-benzylamine moiety was linked to the benzofuran residue with a heptyloxy chain. Compound **18** showed an IC_{50} of 1.82 μM to inhibit BuChE and was 5.7-fold more selective toward BuChE than AChE (IC_{50} = 10.5 μM). Some derivatives are even more selective, up to 100-fold difference in inhibition. Furthermore, compound **18** was able to inhibit the Aβ self-aggregation with an IC_{50} of 13 μM and showed a marked neuroprotective effect against $A\beta_{25-35}$ peptide-induced neurotoxicity with a maximum neuroprotective effect of 63% at 30 μM.

Compound **19** (Fig. 5) was developed as a dual binding site inhibitor based on donepezil and tacrine, reported by Camps et al. in 2008.[144] It was designed on the basis of the binding modes of donepezil[114] and tacrine[151] within TcAChE, by combining the 5,6-dimethoxy-2[(4-piperidinyl)-methyl-1-indanone] moiety of donepezil with tacrine. Most of the donepezil structure was maintained in order to allow favorable interactions of the indanone ring at the PAS, piperidine moiety at the centre of the gorge, and tacrine at the bottom of the cavity. This family were potent inhibitors of AChE, where compound **19** was a subnanomolar inhibitor with an IC_{50} of 0.09 nM, more potent than all parent compounds (tacrine, 6-chlorotacrine and donepezil). Derivative **19** was also a potent inhibitor of BuChE (IC_{50} = 66.3 nM). The higher inhibitory activity against AChE in comparison with BuChE of tacrine-based homo- and heterodimers is ascribed to the lack of PAS of BuChE;[122,152] however, recent studies have suggested that Phe278 would be responsible for π–π interactions with aromatic moieties of tacrine-based heterodimers, thus explaining the higher inhibitory potencies in this case.[153] Furthermore, compound **19** inhibits the AChE-induced Aβ aggregation up to 46.1% (at 100 μM) presumably by interaction at the PAS of the enzyme. In this line, **19** decreased thioflavin T (an Aβ-aggregate[154] and AChE-PAS[155] specific binder) fluorescence by 57% at 100 μM, an indirect proof of direct interaction of **19** with the PAS.

A new approach to the development of dual binding site AChE inhibitors was reported by Xie et al. in 2008.[145] Their approach was focused on meptazinol,[156] a racemic analgesic opioid with low addiction liability. Its minus enantiomer is a moderate AChE inhibitor.[157] Based on molecular modeling and pharmacological data, they synthesized a new series of homobivalent (−)-*N*-demethylmeptazinols; compound **20**[145] was found to be the family lead with high potency to inhibit AChE (IC_{50} = 9.5 nM). It was 1.8-fold more potent against AChE than BuChE. The crystal structure of compound **20**[158] demonstrates a dual interaction simultaneously with both the catalytic site and PAS of AChE. Key interactions in both sites are π-interaction, cation π-interaction and two hydrogen bonds at the catalytic site, explaining the activity on BuChE. Further testing with thioflavin T-based assay resulted in a high activity of compound **20,** preventing the AChE-induced Aβ-aggregation with an IC_{50} value of 5.8 μM (95% aggregation inhibition at 100 μM). Continuing an extensive program in the development of MTDLs performed by Melchiorre and co-workers, Tumiatti et al.[159] reported the synthesis and pharmacological profile of polyamine-based dual inhibitors with special emphasis on the investigation of the inner spacer. Caproctamine[160] (**16**, Fig. 4) and related derivatives were unable to inhibit the AChE-induced Aβ aggregation, although they made contact with both PAS and active AChE binding sites.[160] New molecules, such as compound **21,**[159] were designed aiming to test if this failure in the inhibition of Aβ aggregation was due to their high structural flexibility. Results revealed that the insertion of

constrained moieties strongly influenced the ability to inhibit AChE and BuChE. Compound **21** having a 1,4,5,8–naphthalenetetracarboxylic diimide moiety was the best inhibitor ($IC_{50} = 0.37$ nM), with a 9-fold improvement in potency in comparison with its parent analog (bis-piperidin derivative).[159] Compound **21** was also the most selective and potent of the series with an AChE/BuChE ratio greater than 5,000. Looking at its potential properties as Aβ aggregating inhibitor agent, kinetic studies reveal the dual ability of compound **21** to interact with both binding sites of AChE. Thioflavin-T-based assay determined an IC_{50} of 8.13 μM (90% inhibition at 100 μM) to inhibit AChE-induced Aβ aggregation. Finally, **21** was able to inhibit the Aβ-self-aggregation with an IC_{50} of 9.69 μM. In this case, the modification of the 1,4,5,8–naphthalenetetracarboxylic diimide structural motif results in a more potent activity and better pharmacological profile that deserves further in vivo development.

Proof of the interest of the dual binding site AChEIs type of compounds is the fact that NP-61 a member of the dual AChEIs type of compounds and is the first one to be in phase II clinical trials for AD.[161]

4. DUAL BINDING AChE AND BACE-1 INHIBITORS

Aβ is proposed to play a key role in the pathogenesis of AD.[69,162] Aβ is formed through the amyloidogenic pathway, in which APP is sequentially cleaved by BACE-1 and γ-secretase, rather than through nonamyloidogenic processing by α-secretase.[163] BACE-1, an aspartyl protease, cleaves APP in a first instance at the extracellular domain of APP.[99,164,165] Subsequent cleavage by γ-secretase produces Aβ fibrils in their soluble form.[99] Further structural modifications of soluble Aβ fibrils form high β-sheet content peptides, inducing their aggregation to form senile plaques. As before, AChE can catalyze these conformational changes accelerating the aggregation process. Activation of γ-secretase and the inhibition of BACE-1 and/or γ-secretase reduce the production of Aβ. Targeting these three secretase activities is currently an objective in the development of new treatments for AD.[166,167] According to the amyloid hypothesis, a drug able to reduce brain Aβ levels should act as an effective drug against the disease. Both enzymes, AChE and BACE-1 are connected by the production-aggregation process; therefore, development of agents targeting at both enzymes should be a good approach to design effective pharmaceutical agents.

Bis(7)-tacrine (**10**, Fig. 4) has been recently reported as a selective BACE-1 inhibitor.[129] In experiments performed in N2a APPswe cells, bis(7)-tacrine reduced the generation of both secreted and intracellular $A\beta_{42}$ and $A\beta_{40}$ in a concentration-dependent manner up to 48.5% ($A\beta_{42}$, 3 μM) and 37.7% ($A\beta_{40}$, 3 μM) without any toxic effect for cell viability at those concentrations. In order to rationalize this result, the authors measured the amounts of APP cleavage products in presence and in absence of bis(7)–tacrine. A reduction in the amount of C-terminal fragments β, and increased amount of C-terminal fragments α, was observed without affecting the overall expression of APP. This could indicate a significant inhibition of BACE-1 or an activation of α-secretase. Bis(7)–tacrine was found to be a moderate activator of α-secretase and a selective potent inhibitor of BACE-1 with an IC_{50} of 7.5 μM. Furthermore, bis(7)–tacrine mitigates Aβ-induced neuronal apoptosis,[126] corroborating the multiple-target activities in the amyloid pathological cascade of AD.

Dual inhibition of AChE and BACE-1 is not an easy task and only a few privileged structures are able to effectively inhibit both enzymes. In 2008, Piazzi et al. reported the design and evaluation of the first dual inhibitors, exemplified by compound **22**.[168] Its design was based on compound **14** (Fig. 4) (AP2238,[169] AChE dual binding inhibitor) and BACE-1 inhibitors bearing a dihalophenyl acid motif was reported in the literature.[170,171] Compound **22** contains the core structure of AP2243; the methoxy groups of the coumarin were

alternatively substituted by the amidic chain to extend the activity to BACE-1. Amidic-coumarin derivatives were found to be among the poorest inhibitors of AChE compared with the dimethoxy-derivatives; however, they were successful inhibitors of BACE-1. Compound **22** was the best balanced derivative able to inhibit AChE with an IC_{50} of 181 nM and BACE-1 with an IC_{50} of 150 nM. Compound **22** was designed to be a dual inhibitor of AChE and BACE-1. To the best of our knowledge, no data on its ability to modify Aβ aggregation or production have been reported.

Similar design methodology of hybridizing different molecules with known activity in one single entity was used to obtain derivative **23**.[172] This was reported in 2009 as dual AChE/BACE-1 inhibitor aiming to include both pharmacological profiles. Development was based on the reported isophthalamide, a widely used pharmacophore for BACE-1 inhibitors[173–177] and donepezil. It was envisaged that interactions may occur between the *N*-benzylpiperidine group and the catalytic site of AChE, and the isophthalamide moiety at the PAS. The best balanced inhibitor of the series was **23**. Moderate potencies against AChE and BACE-1 (IC_{50} = 1.83 and 0.567 μM respectively) were observed. In order to elucidate the activity of this compound on the Aβ production/aggregation by intracellular inhibition of endogenous BACE-1, compound **23** was tested in a cell-based assay using HEK293 cells transfected with human βAPP695wt. Compound **23** displayed an excellent inhibitory effect in the cell-based assay on Aβ production (IC_{50} = 98.7 nM). With this encouraging result, its potential neuroprotective profile of **23** against free radicals (relevancy of oxidative stress in AD will be discussed in following sections) was investigated. Measuring the ability of derivative **23** to protect against H_2O_2 showed a mild protective effect in PC12 cells. Owing to its favorable overall profile, in vivo efficacy was also investigated. Intracerebroventricular administration of compound **23** to APP transgenic mice induced a 29% decrease in endogenous $A\beta_{1-40}$ production compared with the vehicle-treated control mice.

Propidium (**9**, Fig. 3) binds to the PAS of AChE via π–π stacking interactions with the indole moiety of Trp286.[178] This is reinforced by concomitant cation–π interactions between a quaternary aromatic nitrogen atom and the same residue.[179] After development of a synthetic route to obtain pyrano[3,2-*c*]quinoline scaffold,[180] Camps et al. reported a new series of compounds, structurally similar to **24** (Fig. 6).[181] These were designed to interact at the PAS, without being protonated. This property can implement a better profile to cross the blood–brain barrier (BBB). Derivative **24** was a potent inhibitor of AChE (IC_{50} = 14.0 nM) and a mild inhibitor of BuChE (IC_{50} = 1.07 μM). Molecular modeling predicted interactions between **24** and both catalytic and PAS sites of AChE. With this dual binding site character, **24** was tested as potential Aβ antiaggregating agent, either AChE-induced or self-induced aggregation, showing good results in both assays (45.7% inhibition at 100 μM and 47.3% at 50 μM respectively). Based on the assumption that some dual AChEIs are able to inhibit BACE-1.[129,168,182–185] Compound **24** was also a potent BACE-1 inhibitor, exhibiting a 78% inhibition at 2.5 μM and emerging as a promising compound to treat AD. Finally, compound **24** proved to be able to cross the BBB and reach its pharmacological targets located in the central nervous system (CNS).

Finally, Cavalli et al.[185] reported the synthesis and pharmacological profile of the highly promising compound **25** (Fig. 6), called memoquin. Memoquin constitutes the natural evolution of the polyamine-core-based derivatives previously studied in the same group.[136,141,186–188] Design was based on the existing polyamine core, an anticholinesterasic agent with other interesting properties including muscarinic antagonism and Aβ anti-aggregating effects. The authors aimed at adding these properties to the ability to counteract oxidative stress introducing a 1,4-benzoquinone fragment from coenzyme Q10 (CoQ10), a fragment with antioxidant properties.[189,190] Initial evaluation of compound **25** as an anti-cholinesterasic agent showed promising results with improved ability to inhibit AChE

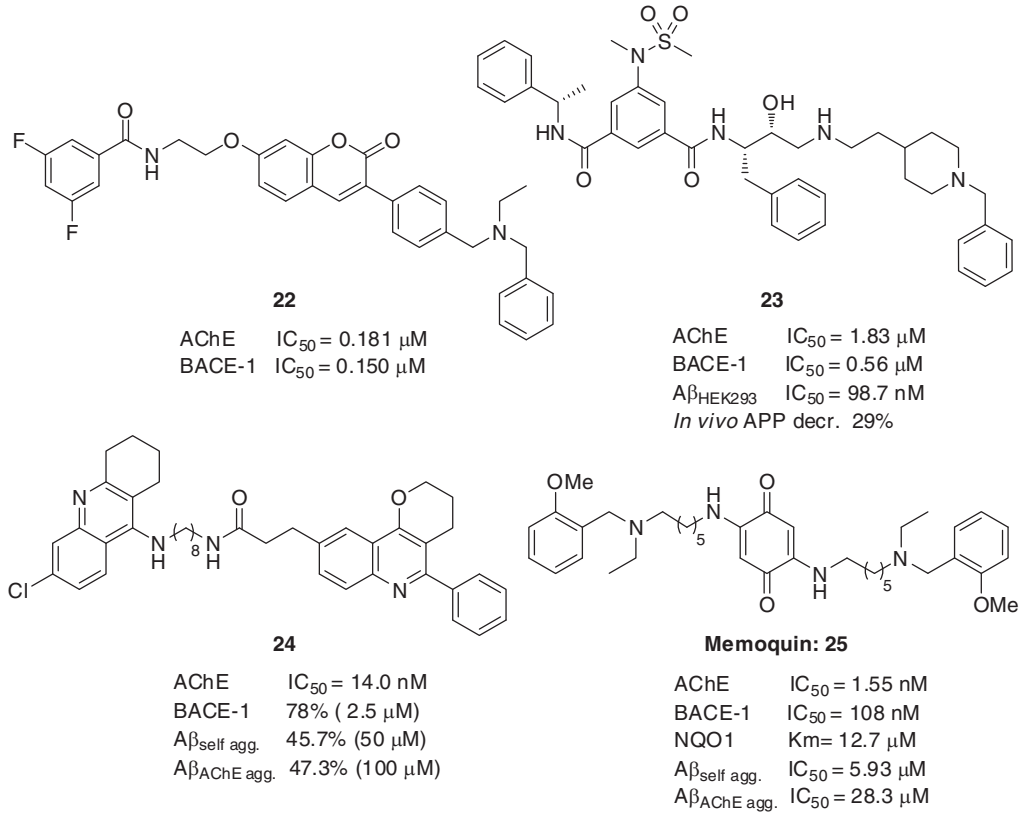


Figure 6. Multitarget-directed ligands as dual inhibitors of AChE and BACE-1.

($IC_{50} = 1.55$ nM), almost 15-fold more potent than the reference compound.[185] Related derivatives proved to be both AChE and self-induced Aβ antiaggregating agents. Memoquin was tested with encouraging results. Memoquin was able to inhibit AChE-induced $A\beta_{1-40}$ aggregation with an IC_{50} of 28.3 μM and the self-induced $A\beta_{1-42}$ aggregation with an IC_{50} of 5.93 μM. This revealed the ability of memoquin to interact with Aβ directly. Furthermore, memoquin was found to inhibit BACE-1 activity at submicromolar concentrations with an IC_{50} value of 108 nM, more potent than other molecules designed to have the same activity. Finally, memoquin's antioxidant properties were investigated. Memoquin was able to reduce the formation of free radicals by 44.1%, a value slightly lower to that for trolox;[191] however, this activity was tested only in the oxidized form. In vivo, the 1,4-benzoquinone moiety can be reduced to the 1,4-dihydroquinone form, increasing its antioxidant potential and scavenging properties. The enzyme NAD(P)H:quinine oxidoreductase 1 (NQO1) was shown to be responsible for the reduction of CoQ10 oxidized state increasing its scavenging properties. The capability of this enzyme to reduce memoquin to the 1,4-dihydroquinone form was tested showing that memoquin is a good substrate for NQO1 with K_m and V_{max} values of 12.7 μM and 3,480 (μmol/min)/mg, respectively. On in vitro cell-based experiments, these authors corroborated the expected increased antioxidant properties of the reduced form by improving its ability to reduce reactive oxygen species (ROS) formation in the presence or absence of sulforaphane (a potent inducer of NQO1).[192] The most potent antioxidant form of memoquin is directly generated in situ in the affected cells, avoiding possible secondary effects. Further in vivo characterization was recently reported,[191] demonstrating the

capabilities of memoquin to restore cholinergic deficit, by reverting the neuronal death observed in a mouse model. Memoquin was also able to successfully reduce Aβ expression and accumulation at the same time as reducing τ hyperphosphorylation. Finally, memoquin proved to ameliorate the behavioral deficits in scopolamine-based object recognition test. The wide pharmacological profile of memoquin gives evidence of the excellent potential of this derivative, endowed with a plethora of activities that could become a disease modifying drug.

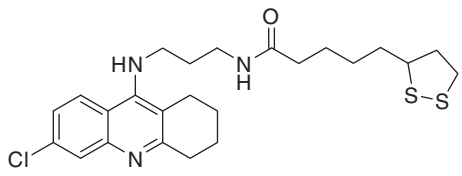
5. AChE INHIBITORS AND ANTIOXIDANTS

Oxidative stress is one of the main causes of neuronal death in AD. It refers to the production of high concentrations of ROS in AD patients.[193] Abnormal pathological mitochondria produce much more O_2^-, increasing the concentration of H_2O_2 in the cytoplasm. Interaction of this hydrogen peroxide with iron can induce the production of free radicals via Fenton chemistry.[194] This interaction is possible due to the increased concentrations of free iron arising from decreased concentrations of ferritin observed in AD.[195] ROS oxidize lipids and damage membranes in the brains of AD patients.[196] Lipid peroxidation products, for example, aldehydes, arising from polyunsaturated fatty acids oxidation, have much longer half-lives than the radicals. Thus, aldehydes can diffuse to other sites within the cell and react there.[197,198] Free radicals can oxidize proteins to form protein carbonyl species, which are observed in increased levels in several brain regions in AD patients. Build up of these oxidized proteins results in the loss of their activity leading to the destabilization of several systems or equilibriums inside neurons.[196] Free radicals can also oxidize DNA and RNA and their oxidation products were found to be elevated in vulnerable neurons in the brains of AD patients. Oxidation would impact on ribosomal functioning and reduce protein synthesis.[199] In addition to iron homeostasis disturbance, modifications in the concentration of zinc and copper are also important in AD pathology.[200] Aβ binds copper ions with high affinity[201] due to three histidine residues and a tyrosine,[202] after some redox changes, Cu^{2+} is able to reduce oxygen to generate H_2O_2.[203]

By considering this evidence, lipocrine (**26**, Fig. 7)[187] was designed to have the capability to reduce free radical concentrations and their toxic effects. Rational design was based on the structure of lipoic acid, a potent antioxidant with multiple neuroprotective effects,[204] and tacrine. Several series of derivatives yield lipocrine as one of the most potent AChEIs ($IC_{50} = 0.25$ nM).[187] Owing to the linked lipoic acid moiety, a linear extension of the molecule was argued that lipocrine can interact with the PAS of AChE with the ability to decrease AChE-induced Aβ aggregation. Lipocrine reduced Aβ aggregation with an IC_{50} of 45 μM. Considering its antioxidant effect, lipocrine decreased by 51% the production of ROS species at 10 μM. Moreover, compound **26** demonstrated a good profile as a neuroprotectant against oxidative stress to a larger extent than its parent compound lipoic acid.[46]

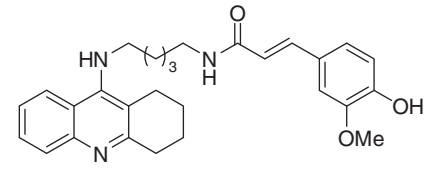
Compound **27**[205] (Fig. 7) was the lead compound of a series of hybrids of tacrine and feluric acid, a potent phenolic natural antioxidant, which concomitantly appears as a secondary metabolite with antioxidant properties.[206,207] It has been proven to reduce the toxicity in the brain of rats exposed to $A\beta_{1-42}$.[208] Compound **27** was able to inhibit AChE with high potency ($IC_{50} = 4.4$ nM), 10-fold more effective than tacrine. Interestingly, this family of derivatives kept antioxidant properties of the parent compound. In experiments performed by the oxygen radical absorbance capacity (ORAC) method,[209] compound **27** showed a value of 1.5 trolox equiv. in its ability to decrease ROS species.

Compound **28** (Fig. 7) was developed by Rodríguez-Franco et al. in 2006,[210] as multitargeted drug for AD. The design of **28** was based on the structure of tacrine and melatonin,



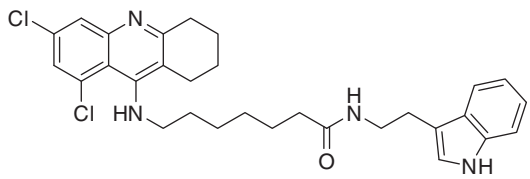
Lipocrine: 26

AChE	IC_{50} = 0.25 nM
BuChE	IC_{50} = 10.8 nM
% Inh. ROS	51 % at 10 μM
$A\beta_{AChE\ agg.}$	IC_{50} = 45 μM



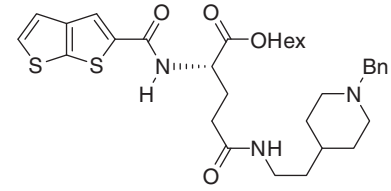
27

AChE	IC_{50} = 4.4 nM
BuChE	IC_{50} = 6.7 nM
ORAC	1.5 Trolox eq.



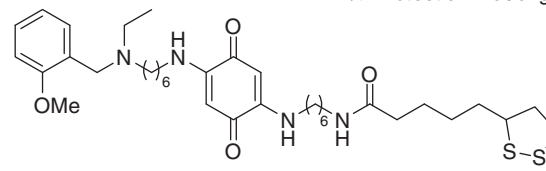
28

AChE	IC_{50} = 8.00 pM
BuChE	IC_{50} = 7.8 nM
ORAC	2.5 Trolox eq.



29

AChE	IC_{50} = 300 nM
BuChE	IC_{50} = 350 nM
Prop. Iodide displ.	42.2% at 0.3 μM
% Protection H_2O_2	54% at 1 μM
% Protection Rot/oligo	45% at 1 μM



30

AChE	IC_{50} = 0.1 μM
BuChE	IC_{50} = 4.99 μM
Self-Aβ aggreg. inh.	45.4% at 50 μM
Antimicin ROS prod. inh.	at 10 μM
^{t}BuOOH ROS prod. inh.	at 10 μM

Figure 7. Chemical structure of dual AChE inhibitors and antioxidants.

a neurohormone that possesses strong antioxidant properties, and also acts as scavenger for some ROS species.[211] Melatonin has also proved to exert neuroprotective effect against Aβ toxic insult in microglial cells.[212] Compound **28** was a high potent inhibitor of human AChE (IC_{50} = 8 pM). Interesting selectivity between AChE and BuChE was observed, compound **28** was a 1,000-fold more active toward AChE than toward BuChE, and 40,000 times more effective AChEI than tacrine. Interestingly, compound **28** was 2.5 times more potent than trolox in its antioxidant properties. Some member of this chemical family had even more potency, up to 4-fold higher. To complete its exciting profile, compound **28** was found to cross the BBB with a permeability of 7.610^{-6} cm s^{-1}.[213] Recently, derivatives of this family were evaluated as potential $A\beta_{1-40}$ self-aggregation inhibitors.[213] An analog compound of **28** reduced Aβ self-aggregation up to 63% at 100 μM. Members of this family were neuroprotectant agents against Ca^{2+} overload and oxidative stress models, showing a moderate neuroprotective profile. Further developments of this class of compounds have been recently

achieved with a tacrine–melatonin hybrid *N*–(2–(1*H*–indol–3–yl)ethyl–7–(1,2,3,4–tetrahydroacridin–9-ylamino)heptanamide.[214] This molecule successfully decreased the Aβ deposits in organotypic brain slices of APP/Ps1 mouse.[215] This compound induced a significant decrease in brain Aβ levels; moreover, it reduced the Aβ-cytotoxicity in primary neuronal cultures as well as in the APP/Ps1 mouse model. Evidence indicates that the observed neuroprotective activity may involve caspase-3 and -9 expression modifications. Finally, this derivative alleviated behavioral deficits in an APP/Ps1 mice model, with cognition deficits; the compound exerted an effective action only after 6 weeks of treatment.

In 2009, a new family of derivatives inspired in compound **29** was investigated.[216] Design was based in dicarboxylic amino acids as a biocompatible scaffold, allowing the attachment of different bioactive moieties. The authors reported L-glutamic acid as a linker for three groups: (a) A ω-situated *N*-benzylpiperidine moiety able to bind to the catalytic active site of AChE, based on the structure of donepezil (**2**, Fig. 2). (b) An *N*-protecting group able to interact with the PAS of AChE proposed to inhibit Aβ-aggregation. Finally, (c) a lipophilic α-hexyl ester that could favor the crossing of the BBB.[217] Overall properties displayed by exemplar compound **29** fits the hypothesis and therefore, it was selected as the lead compound of the family. Compound **29** showed high inhibitory activity of both cholinesterases in the nanomolar range. Furthermore, it decreased in a significant manner (42.2% at 0.3 μM) the fluorescence elicited by propidium, demonstrating the interaction at the PAS of AChE. This result is an indirect test of the potential Aβ anti-aggregating effect of these molecules. Neuroprotective potential was assessed against two different oxidative stress models. Generation of ROS from H_2O_2, and incubation with a combination of rotenone/ oligomicin-A, which blocks complexes I and V of the electron transport chain in the mitochondrion, leading to the generation of ROS. Compound **29** showed good properties as a neuroprotectant agent rescuing 54.5% of cell death originated by 60 μM H_2O_2 and 45.4% of cell death induced by rotenone/oligomicin-A cocktail. Finally, in vitro permeability experiments demonstrated the potential of **29** to cross the BBB.

Based on the structure of memoquin[191] (**25**, Fig. 6) and lipocrine[187] (**26**, Fig. 7), compound **30**[68] (Fig. 7) was designed trying to summarize the excellent multitarget profile of memoquin, and the inherent antioxidant activity of lipoic acid.[188] Bolognesi et al.[68] reported **30**, a new hybrid with good AChE inhibitory activity ($IC_{50} = 0.1$ μM) and selectivity (50-fold more potent against AChE). Furthermore, **30** successfully reduced Aβ self-aggregation up to 45.4% at 10 μM. Insertion of the benzoquinone (from CoQ10) and lipoyl moieties (from lipoic acid) into **30**, direct its antioxidant action to mitochondria. Thus, the ability of compound **30** to reduce the production of ROS was tested. Compound **30** reduced almost to 50% ROS production at 10 μM in the oxidized form. As observed with memoquin, antioxidant character of **30** enhance when quinone core is reduced by NQO1[185] (confirmed by experiments in cells treated with sulforaphane[68]). Reduced form of compound **30** exerted an increased antioxidant activity, decreasing ROS production to basal levels.

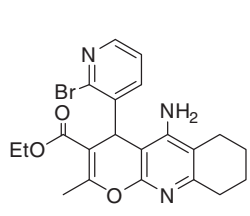
Considering oxidative damage as a key process in AD pathogenesis,[193] several targets have been defined with potential interest for further therapeutic developments. Monoamino oxidase (MAO) enzymatic family releases ROS during its catalytic deamination of neurotransmitters (noradrenaline, dopamine, and serotonin).[218] Inhibition of MAO will reduce ROS production, decreasing oxidative stress in AD patients. Several compounds have been designed and synthesized to include AChE and MAO inhibition in one molecule inserting propargylamine moieties.[219] Several families with dual activity over AChE and MAO have been reported; key compounds have been reported in recent reviews.[46] A prominent example of this type of compounds is ladostigil,[220] a novel multifunctional AChEI/MAO-AB inhibitor for the treatment of AD that currently is in phase II clinical trial.[221]

6. AChE INHIBITORS AND CALCIUM CHANNEL BLOCKERS

As stated before, Ca^{2+} overload is a major pathway initiating the processes leading to cell death.[222] Much evidence has shown that Ca^{2+} dysfunction is involved in the pathogenesis of AD,[223] increasing Aβ formation,[224] and τ hyperphosphorylation.[225] In pathological conditions, Ca^{2+} entry through L-channels causes calcium overload and mitochondrial disruption, leading to the activation of the apoptotic cascade and cell death.[226] Modulation of Ca^{2+} enty through this specific Ca^{2+} channel subtype could be an effective strategy to prevent cell death.

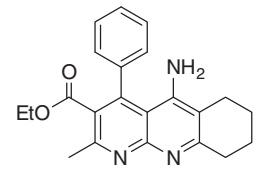
Compounds **31**,[227] **32,**[228] and **33**[229,230] (Fig. 8) were developed in an extensive program targeting Ca^{2+} dyshomeostasis produced in the pathological progression of AD.[227–240] Families were designed as hybrids of tacrine and 1,4-dihydropyridines, which are voltage-dependent Ca^{2+} channel (VDCC) modulators. 1,4-dihydropyridines demonstrated both antagonist (nimodipine)[241] or agonist (Bay K 8644)[242] activities due to their affinity for L channels. Combining both structures in one molecule, the new compounds were expected to have both activities. Derivative **31**, bearing the 4*H*-pyrano[2,3-*b*]quinoline core was reported as part of a new family of AChEIs with Ca^{2+} antagonism activities.[227] Compound **31** selectively inhibited AChE at the micromolar range (IC_{50} = 1.86 μM) with almost no activity over BuChE. More importantly, it blocked up to 43.4% the increase in cytosolic Ca^{2+} evoked by K^+ in SH–SY5Y neuroblastoma cells, thus providing evidence for the dual activity exerted by this hybrid. Furthermore, compound **31** showed neuroprotective effect in two AD in vitro models, Ca^{2+} overload induced by 70 mM K^+, and oxidative stress induced by 60 μM H_2O_2. Compound **31** rescued 42% of the cell death induced by K^+ and 52% of the toxicity induced in the oxidative stress model. Nevertheless, no evidence of the neuroprotection mechanism was given, and there was no correlation between neuroprotection effect and VDCC blockade. This lack of correlation implies a different effect by this family of compounds.

Neuroprotective effect may be explained by different studies performed with compound **32,** named as ITH4012.[228] ITH4012 was reported in 2004 as the best AChE inhibitor of the 1,8-naphtiridine family, with an IC_{50} of 820 nM.[236] This compound blocked the Ca^{2+} influx induced by 70 mM K^+ up to 20% at 3 μM. Even more interesting was its Ca^{2+} promoting activity, demonstrated in the chromaffin cell model. ITH4012 increased the cytosolic basal Ca^{2+} concentration of the cells from 47 to 250 nM at 10 nM. ITH4012 was found to have neuroprotective properties against thapsigargin (calcium overload model), H_2O_2-induced



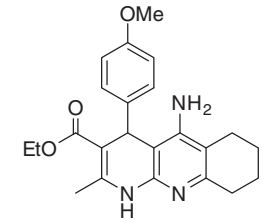
31

AChE IC_{50} = 1.86 μM
BuChE IC_{50} >100 μM
VDCC blockade 43.4% at 0.3 μM



ITH4012: 32

AChE IC_{50} = 0.82 μM
BuChE IC_{50} = 5.0 μM
VDCC blockade 20.4% at 3 μM
Ca^{2+} promoting act. at 10 nM



Tacripyrine: 33

AChE IC_{50} = 45 nM
BuChE IC_{50} >100 μM
VDCC blockade 32.7% at 0.3 μM
$Aβ_{AChE\ agg.}$ 30% at 100 μM
$Aβ_{self\ agg.}$ 35% at 50 μM

Figure 8. Multipotent AChE inhibitors with antagonist voltage-dependent Ca^{2+} channel activity.

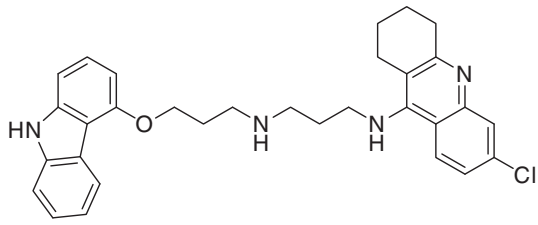
oxidative stress, Aβ-induced toxicity, and veratridine.[236] Further studies showed that protein synthesis inhibition by cycloheximide reverted ITH4012 cytoprotective effect. Investigations revealed overexpresion of Bcl-2 (antiapoptotic protein), which possibly mediates the ITH4012 antiapoptotic effect.[228]

Continuing this line of research, compound **33** (Fig. 8), named as tacripyrine, was developed as the third generation of AChEIs–VDCC antagonists. In this new family, the 1,4-dihydropyridine moiety was integrated into the tetrahydroacridine core of tacrine.[230] Compound **33** was the most potent AChEI with an IC_{50} of 45 nM (4-fold more potent than tacrine) and a highly selective, 2,000-fold more active against AChE compared with BuChE. Kinetic studies and molecular modeling data proposed that this compound may interact with the PAS of the AChE. Thus, compound **33** was tested in both AChE-induced and Aβ-self aggregation models, and was found to be moderately active in both models (30 and 35% inhibition respectively). Compound **33** also exhibits Ca^{2+} blocking activity, showing a 32% blockade of the Ca^{2+} signal in the same model. As previous generations, tacripyrine **33** included neuroprotective activity decreasing mortality induced by Ca^{2+} overload and oxidative stress. Finally, tacripyrine **33** successfully penetrated the BBB. These multiple activities and properties have made this family one of the most interesting candidates for further development in the search of future treatment for AD.

7. AChE AND OTHER SYSTEM MODIFYING TARGETS

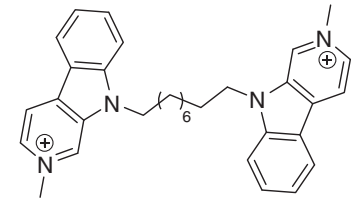
The links between cognitive impairment, neuronal death, Aβ production–aggregation, oxidative stress, metal accumulation, and Ca^{2+} dyshomeostasis are widely accepted.[9] All the above are mostly related with the cholinergic system. Nevertheless, in the last decade advances in the understanding of the molecular basis of the disease have led to the description of new important targets related with several stages of AD.[102] As an example, mitochondrial abnormalities are known markers for AD. Brains of AD patients show decreased activity of important enzymes such as cytochrome oxidase (COX, enzyme responsible for reducing molecular oxygen), pyruvate dehydrogenase complex, and the α-ketoglutarate dehydrogenase complex.[102] However, similar mitochondria abnormalities are found in neurons that lack pathological neurofibrils, indicating that these abnormalities occur at very early stages of the disease.[243] Compromised mitochondria activity may induce Ca^{2+} dyshomeostasis as mitochondria can buffer large quantities of this key ion. Mitochondria are involved in the tight control of intracellular Ca^{2+} homeostasis. Thus, mitochondrial damage leads to high basal cytosolic Ca^{2+} concentrations. In these pathological conditions, any stimulation can lead to Ca^{2+} overload toxicity for example, activation of NMDARs by glutamate cause greater neurotoxicity when mitochondria are damaged.[243] This effect has been proved in rats cholinergic neurons after compromising mitochondrial activity.[102] In fact, memantine (**5**, Fig. 2) (the only non AChEI treatment for AD) is an NMDAR antagonist, which is able to block the toxicity elicited by soluble Aβ oligomers. This observation suggests a direct link between soluble Aβ oligomers and NMDAR at early stages of the disease.

With these observations in mind, Melchiorre et al.[32] designed a new multitarget ligand based on carvedilol. Carvedilol[244] is a vasodilating β-blocker and antioxidant approved for the treatment of mild-to-moderate hypertension, which shows low-affinity NMDAR antagonism.[244] In this project, the tetrahydroacridine moiety from tacrine and the carbazole core from carvedilol were linked. The resulting dimeric molecules were expected to inhibit Aβ fibril formation, since carbazols are efficient inhibitors of Aβ aggregation.[245] Carbacrine (**34,** Fig. 9),[32] had effective anticholinesterase activity at the nanomolar range ($IC_{50} = 2.15$ nM),



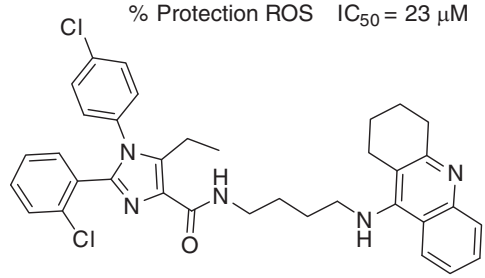
carbacrine: 34

AChE	IC_{50} = 2.15 nM
BuChE	IC_{50} = 296 nM
$\%A\beta_{AChE\ agg.}$	57.7% at 100 μM
$\%A\beta_{Self\ agg.}$	36.0% at 50 μM
NMDAR block.	IC_{50} = 0.74 μM
% Protection ROS	IC_{50} = 23 μM



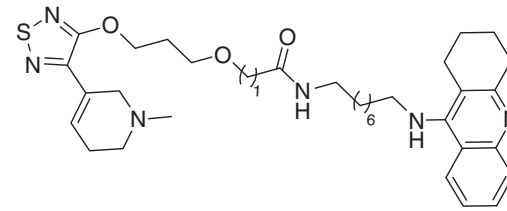
35

AChE	IC_{50} = 0.50 nM
BuChE	IC_{50} = 5.7 nM
NMDAr block. (NR1-1a/N2A)	IC_{50} = 1.4 μM
NMDAr block. (NR1-1a/N2B)	IC_{50} = 2.9 μM
Blockade NMDA med. tox.	IC_{50} = 1.4 μM



36

AChE	IC_{50} = 316 nM
Ki CB1	= 48 nM
Ki CB2	> 1000 nM



37

AChE	IC_{50} = 6.60 nM
BuChE	IC_{50} = 5.12 nM
M1 $logK_x$, diss.	6.35
M1 $logK_x$,	6.88

Figure 9. Chemical structure of AChE inhibitors including activity over NMDA, cannabinoid, or muscarinic receptors as potential targets for AD treatment.

more potent than tacrine and with good selectivity against AChE. Docking of carbacrine predicted interactions in both catalytic and PAS sites of AChE. Potential antiaggregating effect was therefore investigated. A good profile was observed, inhibiting both AChE and self-induced Aβ aggregation. As this family was designed as NMDAR modulators, their potential activity was tested. Interestingly, **34** NMDAR antagonistic effect (IC_{50} = 0.74 μM) was more potent than the reference compound carvedilol. Further experiments with carbacrine suggested that it can be a noncompetitive open-channel blocker. This ability may imply a well-tolerated antagonism that preferentially blocks excessive NMDAR activity without affecting normal neuronal function. Finally, and as an effect of all the intrinsic activity included in this molecule, neuroprotective potential was investigated. This potential effect was tested in ROS-generating models where carbacrine showed neuroprotective effects against ROS formation in a significant manner (IC_{50} = 23 μM). In the ORAC text, compound **34** was a good antioxidant (IC_{50} = 0.07 μM), more potent than trolox.

Based on the structure of monovalent β-carbolines (potent AChEI),[246,247] a new family for bivalent β-carbolines (pyrido[3,4-*b*]indoles) has recently been developed. Rook et al.[248] found that several bivalent β-carboline compounds were also potent NMDAR blockers, providing a possible multitarget approach to the treatment of AD. Compound **35** was the most potent anticholinesterasic agent of the series with IC_{50} to inhibit AChE as low as 0.5 nM. Also, compound **35** proved to be a potent inhibitor of the Ca^{2+} transient induced by glutamate, with an IC_{50} of 1.4 μM, 4-fold more potent than memantine (IC_{50} = 5.6 μM in the

same assay conditions).[248] No experiments were performed in order to elucidate its possible ability to inhibit Aβ-aggregation and to determine its potential antioxidant properties. However, the presence of a quaternary pyridine moiety may imply these activities and further exploration will be interesting in this area.

Cannabinoid receptors (CBR) are also interesting targets for AD treatment.[249,250] Cognitive disorders constitute a potential therapeutic area for cannabinoid CB_1 receptor antagonists.[250,251] CB_1 receptor antagonists increased ACh release in certain brain areas including cortical regions and the hippocampus.[249] Co-application of subthreshold doses of rimonabant (a CB_1 receptor antagonist) and donepezil (**2**, Fig. 2), in noneffective doses, induce memory enhancement.[252] Lange et al.[253] have recently targeted this type of receptors including two activities, AChE inhibition and CB_1 receptor antagonism. Design was based on tacrine and two known CB_1 antagonists, 3,4-diarylpyrazoline moiety[254] and 1,2-diarylimidazol.[255] Thus, imidazol **36** (Fig. 9) was a potent inhibitor of AChE ($IC_{50} = 316\,nM$), while it was able to block CB_1 receptors with high affinity ($K_i = 48.0\,nM$). Furthermore, a high CB_1/CB_2 receptor subtype selectivity (>20-fold) was observed. Molecular modeling with both (AChE and CB1 receptor) pharmacophores explained both activities; the *p*-Cl-phenyl moiety is known to be important in the interaction with CB_1R, while the pyrazoline core is surprisingly well accommodated at the PAS of AChE. Furthermore, tacrine-like core is proposed to interacting at the bottom of the gorge, at the catalytic site of AChE.

In the search of new targets of potential interest for the pathogenesis of AD, muscarinic receptors have gained great importance in the development of AD drugs.[256] Neurotransmission via M_1 receptors can be stimulated either directly by muscarinic agonists or indirectly by allosteric agents acting as ACh enhancers.[256] M_1 stimulation has been targeted as a symptomatic treatment. In addition, M_1 stimulation can reduce $A\beta_{42}$ and τ pathologies via activation of protein kinase C (PKC), which led to the production of soluble APP among other pathways.[257,258] Heterodimer **37** (Fig. 9) was developed in a program launched by Fang et al.[259] linking the tetrahydroacridine moiety from tacrine, and xanomeline, a functionally selective M_1 muscarinic agonist with promising in vivo antidemential properties.[260] The lead compound **37** (AChE $IC_{50} = 6.6\,nM$) was significantly more potent than tacrine, and inhibited BuChE with a similar potency. Its M_1 muscarinic potential affinity was investigated using the affinity of the compound for unliganded receptors labeling them with the orthosteric radioligand [^{3}H]–*N*–methylscopolamine. Compound **37** showed enhanced M_1 allosteric affinity ($\log Kx = 6.35$) exceeding the binding affinity of xanomeline by a factor of 3. Structure–activity relationships suggest that compound **37** prefers a purely allosteric binding topography even in orthosterically free receptors, inhibiting binding of the endogenous neurotransmitter ACh. In vivo studies of scopolamine-induced memory impairments in rats showed a significant reduction in scopolamine impairment activity in the presence of **37**.

Another target widely used for drug development is the SERT,[261,262] expected to bestow antidepressant efficacy, as a symptomatic treatment of the psychiatric behavior of AD patients. Balanced inhibition of AChE and SERT is considered to be a promising dual-target for the treatment of AD, due to a potential improvement in cognitive deficits. After years of development, Kogen et al.[263] summarize results in this area. Compound **(*R*)-38** (Fig. 10) was characterized as a potent AChE inhibitor ($IC_{50} = 14\,nM$) and SERT antagonist ($IC_{50} = 6\,nM$). **(*R*)-38** was 155 times more potent antagonist of SERT than the correspondent **(*S*)-38** enantiomer. However, no further experiments with **(*R*)-38** were performed due to a poor penetration across the BBB. Related compounds were evaluated showing interesting properties, leading to compound BGC20-1259,[263] the most promising inhibitor of AChE and SERT that has completed phase I clinical studies in healthy volunteers.

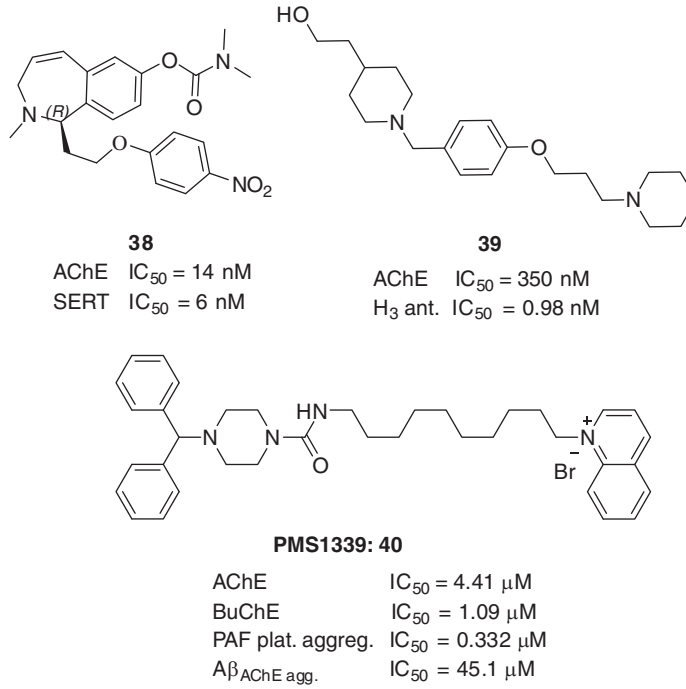


Figure 10. Chemical structure of AChE inhibitors including activity over SERTor histaminic or PAF receptors as potential target for AD treatment.

The H_3 receptor (H_3R) is an attractive G protein-coupled receptor drug target that regulates neurotransmission in the CNS and plays a role in cognitive and homeostatic functions.[264] H_3R antagonists improve cholinergic neurotransmission in the cortex via different mechanisms. When an H_3R antagonist is present, ACh release is improved. A combination of AChE inhibition with H_3R antagonism may induce an increased memory enhancing effect. Combination of both activities in one molecule has been achieved by Bembenek et al.[265] Based on virtual screening toward both (AChE and H_3) targets,[266] led to the discovery of family type **39** (Fig. 10). Compound **39** was a potent anti-cholinesterase agent ($IC_{50} = 350$ nM) including an interesting H_3R antagonist profile with an IC_{50} of 0.98 nM. QM/MM calculations of the interaction mode of **39** with AChE suggest a dual-inhibition mode interacting at the PAS including a possible Aβ antiaggregating effect.

Converging lines of evidence suggests that platelet-activating factor (PAF), a potent pro-inflammatory mediator, is implicated in the inflammatory events,[267,268] promoting neuronal death in demential disorders.[269] Previous developments in dual PAF and AChE targets include series of 2,5-disubstituted tetrahydrofuran derivatives.[270] Among them PMS777 was shown to inhibit AChE, reverse scopolamine-induced dementia in mouse models, prevent PAF-induced neurotoxicity,[271] and LPS-induced oxidative/inflammatory disturbances in human neuroblastoma cell lines.[272] Recently, Ezoulin et al. reported a new improved candidate, **40** (Fig. 10) (PMS1339),[273] a piperazine derivative with improved AChE inhibitory activity ($IC_{50} = 4.41$ μM) and inhibition of PAF-induced platelet aggregation ($IC_{50} = 332$ nM). Compound **40** reduced the AChE-induced Aβ-aggregation ($IC_{50} = 45.1$ μM) by direct interaction at the PAS of AChE. Finally, compound **40** reversed the scopolamine-induced memory impairment, a proof of the benefits provided by the biological target included in this molecule.

8. NON-AChE-DIRECTED MULTITARGET DRUG DEVELOPMENTS

Non cholinergic-based drug discovery has been extensively investigated due to the implication of different mechanisms and pathways in AD pathology.[9] Great efforts have been devoted to the investigation of the Aβ processing targets,[69] τ hyperphosphorylation and aggregation,[5] and metal chemistry[274] related to AD. APP function is not completely understood; the extracellular domain has been suggested to serve as neurotrophic factor, while the intracellular domain may have a role as gene transcription regulator.[275,276] APP has been also linked to axonal transport inside neurons, helping the transport of different vesicles through kinesin-1.[277] APP can be proteolytically processed by three aspartic acid proteases, named α-, β-, and γ-secretase.[278] Subsequent cleavage by β- and γ-secretase generates a group of peptides which differ in length at their C terminus, $A\beta_{40}$ being the dominant species and $A\beta_{42}$ being the second major peptide. $A\beta_{42}$ readily aggregates to form the seed for larger oligomers and fibrils, eventually generating macroscopic amyloid plaques.[279,280] As referred before, this process can be catalyzed by the interaction of Aβ peptides at the PAS of AChE.[10] Thus, BACE-1 inhibition and γ-secretase modulation are two areas of great interest for drug development in AD. Aspartic acid protease inhibitors have been discovered in the past and some of them have entered in early clinical trials, for example, CTS-21166.[281] On the other hand, it should be noted that the proportion of APP that is processed by α-, β- and γ-secretase depends on the equilibrium processes inside the cell. Activation of G-protein-coupled-type receptors such as M_1 ACh receptor or 5-HT_4 serotonin receptor can induce an increase in the α-secretase–APP processing pathway, thus reducing the production of Aβ peptides. In this sense, it is worth to note that two partial agonists of these receptors, SL-65.0155[282] (Sanofi-Aventis, Paris, France), and PRX-03140[283] (EPIX Pharmaceuticals, Lexington, MA) have reached phase II clinical trials for the treatment of AD,[284,285] although none of them have progressed to more advanced phases. Their mechanism may be mediated by modulation of PKC, since activators of PKC have similar effects. Surprisingly, shifting between APP processing pathways was observed with some NSAIDs such as ibuprofen and indometacin.[286] This action is unrelated to their inhibition of COX-1 and -2 enzymes. The shifting effect is related to the peroxisome proliferator-activated receptor γ (PPARγ) agonist effect, included in NSAIDs.[287,288] Evidence indicates that PPARγ agonists may have multiple beneficial effects in AD. These benefits can be both on core pathological processes in brain and on peripheral factors, such as serum glucose levels and insulin sensitivity.[289]

In 2010, a new molecule trying to integrate α-secretase modulation activity and PPARγ activity was reported by Hieke et al.[290] Its development was based on the 2-(bis-(phenetoxy)pyrimidine-2-ylthio)-hexanoic acid that displayed γ-secretase modulation and effective PPARγ modulation. SAR studies and lead optimization yielded compound **41** (Fig. 11). Optimized activity over γ-secretase (inhibition of $A\beta_{42}$ production: 6 μM; activation of $A\beta_{38}$: 1.8 μM) and improved PPARγ activation (EC_{50} of 11.0 μM) was observed. Most NSAID type PPARγ–modulators are inhibitors of COX enzymes at the same time, resulting in side effects in their long-term clinical use. In order to minimize potential side effects, a decrease in the activity over these enzymes was needed. Thus, compound **41** was a weaker inhibitor of both enzymes providing a promising strategy to address the increased dementia risk in AD patients.

As pointed out before, 5-HT_4 agonists are of special interest in AD drug development; their benefits are inherent to the pathways of this G-protein-coupled receptor. The structure of ML10302,[291] a partial agonist with a benzoate base structure, was found to selectively enhance soluble APPα release in the hippocampus and cortex of mouse. This was the starting point for the development of derivative **51** (Fig. 11).[292] This compound is a potent 5-HT_4 receptor agonist with an EC_{50} of 9 nM and, as expected, in vivo studies showed an increased

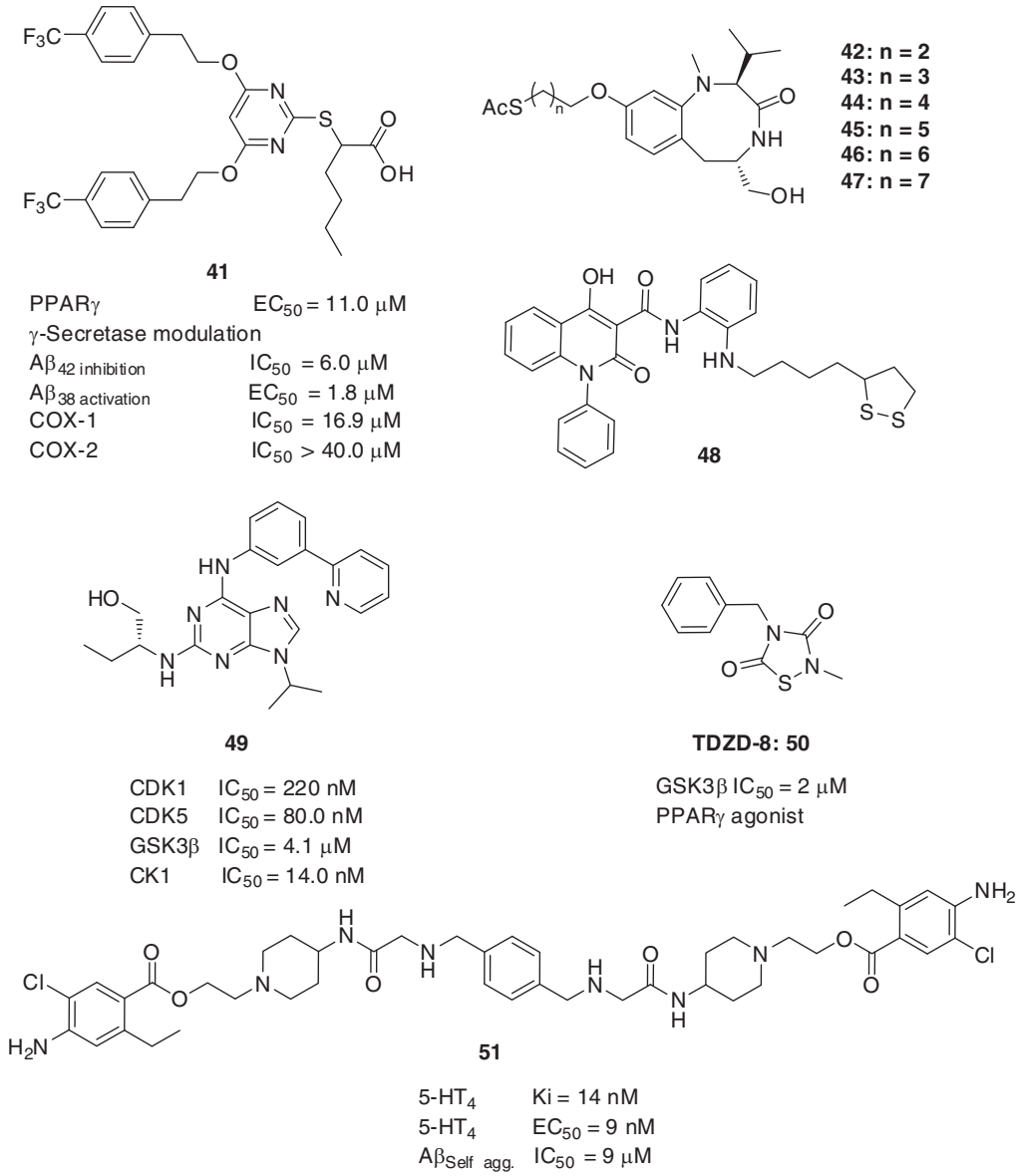


Figure 11. Chemical structure of noncholinergic multitarget-directed ligands developments.

concentration of soluble APPα in hippocampus and cortex in mouse. Moreover, **51** displayed an inhibitory effect on Aβ fibril formation ($IC_{50} = 9\,\mu M$) being, to the best of our knowledge, the first designed molecule exhibiting these two activities together.

Similar effects have been described for PKC activators, due to their ability to modulate the α-secretase activity.[293] This modulation increases the nonamyloidogenic pathway to produce soluble APPα. Another emerging approach wich could affect multiple pathways to ameliorate AD pathophysiology and cognitive impairment is epigenetic remodeling through the inhibition of histone deacetylase (HDAC).[294] In 2009, Kozikowski et al.[295] included both targets in one molecule generating a small chemical library of benzolactam analogs. This scaffold has previously been found to have high-binding affinity for PKC.[296] Among all the

benzolactam derivatives generated, compound **43** (Fig. 11) induced a statistically significant elevation of soluble APPα at a concentration as low as 1 nM. This compound, however, was not the best ligand for PKC ($K_i = 15.8$ nM) with a medium potency among the library. Compound **47** (Fig. 11) was the most potent with a K_i of 2.8 nM. Compounds **42–47** (Fig. 11) induced an increase in histone H4 acetylation, confirming HDAC inhibition activity at 10 μM. These compounds were found to activate PKC and inhibit HDAC-inducing histone H4 acetylation. HDAC inhibition blocks the deacetylation process, increasing the global acetylation level of histones. Consequently, profound changes in gene expression are induced activating survival pathways. Compounds **42–47** protected cortical neurons upon exposure to homocysteate (oxidative stress model) with high potency.[295]

As discussed previously, oxidative stress is one of the most important pathological pathways of AD.[196] Oxidative stress has been widely investigated leading to the discovery of antioxidant molecules or MTDLs with antioxidant properties (discussed above).[73] In this line, a new antioxidant molecule was designed to include anti-inflammatory activity. Inflammation is a well-known process related to AD pathology.[73] In the past, several NSAIDs have been investigated as potential drugs targeting glia inflammation in AD.[73] In this line of research, several unsuccessful clinical trials have been reported;[98] nevertheless, inflammation continues to be a key target in AD drug development. The quinolinone structure includes motifs that are characteristic of numerous natural products and synthetic analogs, exhibiting a wide variety of biological activities.[297–300] Linomide (*N*-phenylmethylpz-1,2-dihydro-4-hydroxy-1-methyl-2-oxo-3-quinoline carboxamide) is a synthetic immunomodulator used as a starting point for lead optimization of several analogs.[297,301–306] Rebamipide,[307] another example of quinolinone derivative, has effective antioxidant effect due to its ROS scavenging ability and over-expression induction of endogenous prostaglandin. Scavenger properties are due to the presence of the 3,4 double bond together with the 2-oxo functionality in the quinoline moiety.[307] On the basis of these observations, Detsi et al.[308] used this quinoline structure to generate hybrid molecules. This class of compounds included α-lipoic acid and the quinoline core. α-Lipoic acid is a very well-known antioxidant with anti-inflammatory activity.[188] Compound **48** (Fig. 11) as lead compound in this series showed good anti-inflammatory properties; thus, it reduced the carragenin-induced inflammation by 65% at low dosages in rat paw. It also proved to be a good antioxidant.

Formation of τ-protein aggregates has been mentioned throughout the text giving some hints about the importance of these aberrant structures in AD pathology. Much attention has been devoted to the destabilization of the neuron cytoskeleton; however, no drug has yet reached the clinic to directly interrupt this pathological process. The cytoskeleton consists of microtubules, polymers of tubulin. Microtubules bind microtubule-associated proteins, which regulate their stability. The most important stabilizing protein in the axon is τ-protein, a very hydrophilic protein which contains at least 25 potential phosphorylation sites. τ-Protein is phosphorylated by kinases CDK5, GSK3β, ERK2, and CK1 (casein kinase 1). Phosphorylation can detach τ from microtubules, inducing neuronal damage in AD.[309] Phosphorylation of τ under normal conditions induces equilibria between bound and unbound protein. This dynamic equilibrium helps the axonal transport of vacuoles and integrity of the cell. Thus, hyperphosphorylation of τ, as a result of an imbalance in the kinase and phosphatase activities, generates aberrant hyperphosphorylated τ. These aberrant structures induce toxicity and axonal transport dyshomeostasis previously mentioned. In AD brains, total expression of τ is around 8-fold higher than in controls, and it is abnormally hyperphosphorylated.[310] Implication of τ protein in AD pathology is supported by the existence of tauopathies such as amyotrophic lateral sclerosis, Pick's disease, progressive supranuclear palsy, and frontotemporal dementia with Parkinsonism.[311] Drug development based on the τ hypothesis has been focused on the screening of protein kinase inhibitors.

GSK3β is the most widely investigated, believed to be the most important τ-phosphorylating agent.[312] Early developments on kinase inhibitors pursued high selectivity against only one of the kinases. This inhibitors were used to study the, not yet very well understood, phosphorylation–dephosphorylation equilibria of τ-protein.[313] Recently, kinase inhibitors developments pursue mainly multitarget kinase inhibitors. These new inhibitors target several protein kinases simultaneously, with the right balance of inhibition of different kinases, to stop the pathological phosphorylation of τ-protein. Oumata et al. reported the synthesis and biological evaluation of dual-specificity inhibitors of CDKs and CK-1[314] based on roscovitine[315] (CYC202 or seliciclib), a purine-based inhibitor.[316] Roscovitine has finish phase IIb of clinical trials against nonsmall-cell lung cancer,[317] and starting phase I against advanced solid tumors,[318] and several others diseases. Frequently considered as highly selective for CDKs, roscovitine has been shown to interact with several other kinases (DYRK1A, CK1, pyridoxal kinase).[319] Purines are a large family of biologically active molecules, constituting a scaffold of a wide variety of promising drugs, including kinase inhibitors as 2,6,9-trisubstituted purines.[320] Tri-substituted purine **49** (Fig. 11) was one of the most potent inhibitors of CK1 ($IC_{50} = 14\,nM$).[321] Compound **49** inhibited also with high potency CDK5 ($IC_{50} = 80\,nM$), GSK3β ($IC_{50} = 4.1\,\mu M$), and CDK1 ($IC_{50} = 0.22\,\mu M$).[320] On the other hand, proliferation assays were performed in order to elucidate its possible toxic effect; in general, biarylamine type compounds were highly potent CDK/CK1 inhibitors but displayed low antiproliferative effect. As a general rule, CK1 inhibition inversely correlated with antiproliferative activity; thus, compound **49** can be described as a protective agent. Some evidence suggests a regulatory effect of CK1 on the production of Aβ as CK1 inhibitors were able to inhibit the production of Aβ.[322] Compound **49** was the most effective agent reducing the production of Aβ in the N2A-APP_{695} cellular model.

Noscira (Madrid, Spain), a pharmaceutical company, has developed an interesting family of thiadiazolidindinones exemplified by compound **50**[323] (Fig. 11). Noscira has finished a safety study with NP031112 for AD treatment.[324] NP031112 is a potent GSK3β inhibitor developed as selective inhibitor. More importantly, it is one of the first non-ATP competitive inhibitor molecule of this important kinase. Compound **50** was developed as a selective kinase inhibitor; however, NP031112 was found to be a nuclear receptor PPARγ agonist,[325] showing effective anti-inflammatory and neuroprotective properties. Both properties could constitute a significant advance in further developments of multitarget GSK3β inhibitors.

9. MULTITARGET BIOAVAILABLE METAL CHELATORS

As mentioned above, oxidative stress is considered to play a central role in AD pathogenesis.[193] Recently, different drug development programs have pursued different strategies in order to combat increased levels of ROS species in AD patients. Strategies included interference in ROS production,[243] development of antioxidants,[107] metal chelators,[274] and others. Multitarget drug development programs have implemented some of these targets in one molecule. Cholinergic neurotransmission, Aβ antiaggregating effect and/or clearance facilitation, and neuroprotection activity against ROS species have been included. Several studies have indicated that cerebral biometal (Fe, Cu, and Zn) dyshomeostasis and oxidative stress are intimately associated with the formation of Aβ plaques and NFTs. For instance, iron increases production and translation of APP via activation of APP mRNA iron-responsive element. Consequently, it induces Aβ plaque formation.[326] Dyshomeostasis of biometals and their interactions with Aβ cause Aβ aggregation and deposition.[274,327] Metal chelators have the ability to attenuate a broad spectrum of oxidative stress as well as APP translation, Aβ production/aggregation, and NFTs formation. Metal chelators as desferrioxamine®[328] and

clioquinol[329] were under investigation as potential drugs for AD. However, they are not brain specific chelators, and possibly will display undesirable interactions with beneficial biometals, affecting the normal physiological function of essential metal-requiring metalloenzymes. With the aim to overcome these problems, some chelators or pro-chelators with improved target specificity have been developed. Franz et al. reported in 2006 one of the first molecules based on the prodrug approach.[330] Compound **52** (Fig. 12) is a pro-chelator that has almost no affinity for metal ions until the boronic pinacol ester mask is removed by ROS species. In the absence of oxidative stress, the masked molecule is a poor ligand and thus cannot alter healthy metal ion distribution. When ROS are elevated in pathogenic conditions, compound **52** is unmasked and is revealed as a potent iron chelator, inhibiting the iron ability to generate $OH^{\bullet}$ species. It includes a boronic ester as a latent phenolic oxygen, a key donor of the aroylhydrazone class chelators.[331] As expected, experiments showed the ability of compound **53** to inhibit the formation of ROS via effective iron chelation. Although **52–53** pair is not considered as a multitarget drug, we felt it was interesting to mention in this review. It was the first example of a pro-chelating drug that open a new line of research to new target-directed drugs aiming to diminish nondiscriminating biometal chelation.

On the basis of the same approach, Schugar et al. developed the **54–55** pair.[332] It is also a prodrug; however in this case, the metal chelator moiety is based on a bidentate

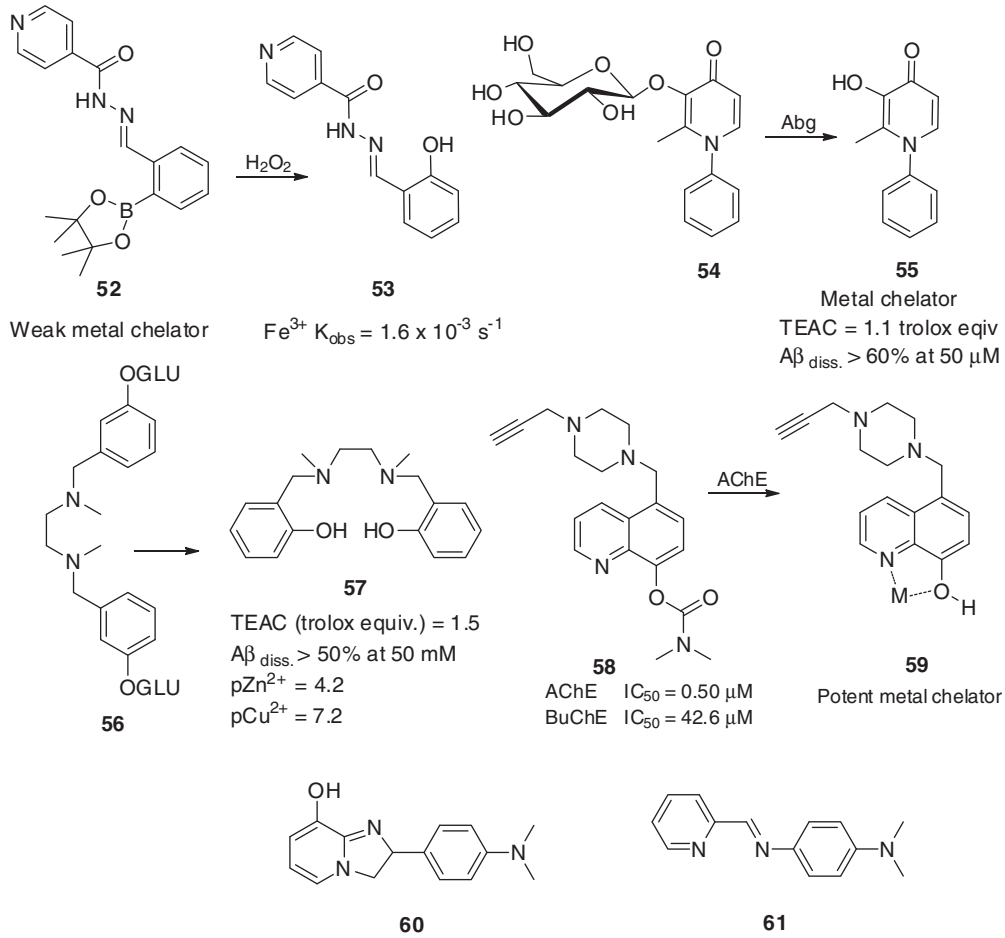


Figure 12. Metal chelator-based ligands with multiple activities included.

hydroxypyridinone. The presence of this moiety addresses both the metal ion and the oxidative imbalances linked to a glucose receptor targeting moiety. The carbohydrate moiety is expected to interact with the glucose receptor in the BBB. This receptor is present in the BBB to facilitate the entry of glucose across it. The trifuncionalized compound **54** successfully penetrated the BBB demonstrated by brain uptake of a radiolabeled hydroxypyridinone glucoconjugate. The presence of the carbohydrate moiety serves at the same time to inhibit systemic metal binding, thereby reducing side effects. Within the brain, compound **54** is hydrolyzed by glycoside hydrolysis. The authors used β-glucosidase (Abg), which successfully cleaves glucose from hydroxypyridinone. Hydroxypyridinones are known binders of di- and trivalent metal ions like Cu^{2+} and Fe^{3+}.[333,334] It is also known that free alcohols are scavengers of free radicals; thus, compound **55** was assayed in the TEAC test, proven to be a more potent antioxidant than vitamin E, known antioxidant, equivalent to trolox. The potential of compound **55** to dissolve Aβ plaque was assayed by turbidimetry; it reduced the preaggregated $A\beta_{1-40}$ by 60% at 50 μM. This trifunctional ligand combines metal sequestering and antioxidant properties with glucose conjugation, helping BBB crossing and minimizing systemic complexation of metal ions. The same hypothesis was used in the development of the pair **56–57** which demonstrated improved chelating properties toward Zn^{2+} and Cu^{2+}.[335] The **56–57** pair reduced the preaggregated Aβ by 50% at 50 μM. Furthermore, **57** proved to be a good antioxidant being, 1.5-fold more potent than trolox in the TEAC assay.

A further advance in the pro-chelator approach was made by Zheng et al. including several new ideas in its design.[336] As described before, by using masked chelators, the systemic chelation of healthy metal homeostasis is avoided. Masking the chelator as an AChE inhibitor included a new activity in the molecule. The pair pro-chelator **58**–chelator **59** was based on the structure of the bifunctional chelator 5-(4-propargylpiperazin-1-yl-methyl)-8-hydroxyquinol (HLA20)[337] and the AChEI rivastigmine (**4**, Fig. 2), and donepezil (**2**, Fig. 2). Compound **58** included moieties from these three structures, where the carbamoyl moiety of rivastigmine also acts as a protective group of the quinolinol OH. Its biological evaluation revealed the AChE inhibition properties. Thus, compound **58** was a time-dependent AChEI ($IC_{50} = 500$ nM) slightly more potent than rivastigmine and 85-fold more active against AChE than BuChE. After the anticholinesterasic properties were described, the potential chelating effect was investigated. Although compound **58** was unable to bind Cu^{2+} or Zn^{2+} ions, AChE successfully cleaves its carbamyl moiety, releasing the unprotected form **59**. Complexation experiments of Fe^{3+} and Cu^{2+} with chelator **59**, using absorbance spectroscopy, showed the formation of the different complexes. Complexation was confirmed by mass spectrometry studies. Among the benefits of the masked pro-chelators, activity against AChE can induce an antiaggregating effect. This together with the potential antioxidative properties makes these derivatives highly interesting for further preclinical[338] and clinical development.

A slightly different approach was reported by Rodríguez-Rodríguez et al.[339] using the structure of thioflavin-T as substructure for recognition/interaction with Aβ aggregates. In this proposal, instead of masking the metal chelator as performed in previous developments, they used molecular recognition molecules targeting Aβ aggregates. Based on thioflavin-T which exerts specific affinity for amyloid plaques, and clioquinol,[329] known methal chelator, the idea was to insert metal chelator properties into these models without important modifications of the core structure. In a extensive and exhaustive study, the authors described three new hybrids with highly encouraging capabilities as intercalation properties in amyloid fibrils and their potential use for radioisotopic detection of $A\beta_{1-42}$ deposits in the human brain.[339]

A similar approach has been described by Hindo et al. in 2009.[340] Structures of IMPY[341] and *p*-^{125}I-stilbene (two Aβ aggregate-imaging probes),[342] were used to provide the core structures. Derivatives **60** (Fig. 12) and **61** are the result of this development and they were

able to bind copper forming different stoichiometry complexes, 1:2 and 1:1 for **60** and 1:1 for **61**. NMR studies indicated the ability of both metal chelators to directly interact with Aβ-aggregates. Interaction is probably in positions close to where metal ions bind to Aβ-aggregate structures. These compounds exert a bifunctional ability, binding directly Aβ-aggregates and chelating metals as independent activities. Their potential to inhibit Cu^{2+}-induced Aβ-aggregation was therefore tested. Co-incubation experiments resulted in the inhibition of the high-molecular-weight aggregates in the presence of compounds **60** and **61**, an effect that was not observed in the presence of other metal chelators (EDTA) or the model substructures MPY and stilbene. This suggests the need for both activities, metal chelating and Aβ direct interaction, to be able to inhibit the aggregation effect of Cu^{2+}. Metal chelators are known to inhibit H_2O_2 production by Cu-bound Aβ.[274] Interestingly, compounds **60** and **61** are reduced by 70% H_2O_2 production. Finally, toxicity experiments proved compound **61** to be non toxic at concentrations as high as 200 μM, suggesting that it is a good candidate to be further studied in vitro and in vivo. These small compounds are neutral, lipophilic and able to cross the BBB. By mimicking them and inserting oxygen and/or nitrogen donors, these molecules should have low toxicity, and selective chelating properties.

10. CONCLUSIONS

During the last decade, substantial research efforts have been devoted to the development of multitarget drugs for different diseases. This change in the emphasis has been driven by the increase in the understanding of the pathogenesis of complex chronic diseases, and the fact that the paradigm one-target-one-molecule has not been as successful as expected. Complex diseases, such as cancer and neurodegenerative diseases, particularly AD, are composed of many different cross-linked pathological pathways, affecting a continuously increasing number of metabolic routes. AD pathogenesis is composed of many different mechanisms, interacting between them to generate a high complex network.[67] Relationships between different routes generate key nodes implicated in disease progression. Even now, this network is not well understood; however, there are some hints about important nodes that are known targets for disease modifying drugs.[107] Examples of these targets include APP pathogenic cleavage,[343] cytoskeletal destabilization,[344] axonal transport impairment, neurotransmitter and Ca^{2+} dyshomeostasis,[27] metal ion accumulation,[345] protein misfolding,[343] oxidative stress,[73] neuronal death, and gene mutations.[14] There are drugs effective against almost all of these targets; however, none of them are likely to provide an efficient treatment for AD. On the other hand, only a few marketed drugs are available. These drugs constitute only a symptomatic treatment, effective for a few months; afterwards, disease progression is unavoidable. Furthermore, compelling evidence is questioning the effectiveness of the treatment with AChEIs,[108] while other treatments are emerging such as memantine, the first non-AChEI marketed for AD.

Initial evidence for the multitarget approach was the discovery of multiple activities in some natural products. These have been used as starting points for further developments. Compounds such as curcumin,[346] resveratrol,[346] and some flavonoids[347] were studied, because of their interesting antioxidant and anti-inflammatory properties. Further studies demonstrated their ability to modify Aβ aggregation and metal dyshomeostasis. Although natural products are not discussed in this review, however, there is a plethora of compounds which may be important in multitarget drug design. These can also serve as templates to achieve new biological activities, which medicinal chemists should have in mind, during the discovery process.[348–350] Recent multitarget drugs have mainly been designed by studying the

3D structure of previous molecules with known activities and crystal structures of target proteins. This information is focused to the virtual design of new chemical entities that include more than one activity in a single molecule.[351]

Multitarget approach has been used to describe the benefits of the use of combination of drugs. It is now widely accepted in the treatment of complex diseases such as cancer, where the use of several one-target specific drugs in combination leads to better results, than the treatment with each one separately. This effect can be explained by the alteration of several interconnected pathological pathways, modifying the progression of the disease. This type of therapy has opened a broad line of research for scientist in different fields. Defining correlations and links among the pathological network, and the use of this information to design specific molecules. These molecules will include several targets which will be tested as new potential drugs, among many others.[46] Nevertheless, feedback currents of information between all different fields will be crucial for success in the search of a real disease modifying drug for AD. Future advances in AD treatment will summarize all knowledge about the physiopathological progress of the disease and the different relations among them, in order to achieve the right balance of biological activity in the optimal multitarget drug.

ABBREVIATIONS

Aβ	amyloid-β
ACh	acetylcholine
AD	Alzheimer's disease
AChEI	AChE inhibitor
AChEIs	AChE inhibitors
AICD	amyloid intracellular domain
AMPA	α–amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOE	apolipoprotein E
APOE4	apolipoprotein E 4
APP	amyloid precursor protein
APPα	amyloid protein precursor α fragment
BACE-1	β-secretase
BBB	blood–brain barrier
BuChE	butyrylcholinesterase
ChAT	choline acetyl transferase
CoQ10	coenzyme Q10
CDK5	cyclin-dependent kinase 5
EDTA	ethylene-diamino tetracetic acid
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERK2	extracellular signal-regulated kinase 2
GABA	γ-aminobutyric acid
GSK3β	glycogen synthase kinase 3β
H_3	Histamine 3
5-HT	5-hydroxytryptamine receptors
IDE	insulin-degrading enzyme
InsP3R	inositol triphosphate receptors
MCU	mitochondrial Ca^{2+} uniporter
mtPTP	mitochondrial permeability-transition pore
MTDLs	multitarget-directed ligands

nAChRs	nicotinic acetylcholine receptors
NET	norepinephrine transporter
NFTs	neurofibrilary tangles
NMDAr	*N*-methyl D-aspartate receptors
NQO1	NAD(P)H:quinone oxidoreductase 1
NSAIDs	nonsteroidal anti-inflammatory drugs
ORAC	oxygen radical absorbance capacity
PAS	peripheral anionic site
PS	presenilins
ROS	reactive oxygen species
RTC	tyrosine kinase
RyR	ryanodine receptors
sAPPα	APP fragment α
sAPPβ	soluble APP β
SERT	serotonin reuptake transporter
τ	tau-protein
VDCCs	voltage-dependent calcium channels
VEGF	vascular endothelial growth factor

ACKNOWLEDGMENTS

R.L. thanks Ministerio de educación y ciencia of the Spanish government and Marie Curie Foundation for fellowships. The authors gratefully acknowledge to Peter Wathen and Rhiannon Beard for helpful discussion. A.G.G. thanks Fundación CIEN (PI 016/09) and Agencia Laín Entralgo (NDG07/9) for financial support. J.M.C. thanks MICINN (SAF2006-08764-C02-01; SAF2009-07271), CSIC-GRICES (2007PT-13), ISCIII, MICINN (RD06/0026/1002 Retic "RENEVAS"), and Comunidad de Madrid (S/SAL-0275-2006) for financial support.

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