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Enhancing the Therapeutic Effect in Alzheimer's Disease Drugs: The role of Polypharmacology and Cholinesterase inhibitors

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Alzheimer's disease (AD) is a devastating syndrome that accounts for 60–70% of all dementia cases, putting an enormous burden on global healthcare and economy. Unfortunately, there is no cure for AD, and the currently approved drugs are limited in their effects. Given the various pathological mechanisms behind AD, the "one-target, one-drug" paradigm for drug design became obsolete, and a new paradigm, polypharmacology, emerged. Consequently, a greater focus has been put towards multi-target directed ligands (MTDLs), as these can regulate several targets operating in the disease network. Parallel to that, cholinesterase inhibitors have regained popularity after decades of being considered only symptomatic agents with no disease-modifying properties. In this review, the current AD hypotheses and therapeutic targets, the concept of polypharmacology in AD pathology and the importance of cholinesterases in the pathogenesis and biochemical processes of AD are discussed, with a final overview of the current development in cholinesterase-based MTDLs.

1. Introduction

Alzheimer's disease (AD) constitutes the most common form of dementia, contributing to 60–70% of all cases.[1] Clinically, AD is the consequence of premature neuronal death due to genetic and environmental factors that lead to deterioration in memory, thinking, behaviour and the ability to perform everyday activities. AD is commonly divided into sporadic AD (SAD), i.e., without apparent familial aggregation, and familial AD (FAD), i.e., with Mendelian disease transmission.[2] FAD represents only 1–5% of all cases, and is often observed before the age of 65, consequently being referred as early onset AD, while SAD represents around 95% of all AD cases, and usually occurs later in life (65 years or older), hence also called late-onset AD. While FAD has been linked to genetic mutations in the amyloid-β protein precursor (AβPP) or in presenilin (presenilin-1 (PSEN1) or presenilin-2 (PSEN2)), SAD is believed to be caused by a combination of genetic and environmental factors. Multiple risk factors are believed to be involved, which include age, genetic, lifestyle, medical, metal exposure, among many others.[3]

Although the pathogenesis of AD has remained elusive, two principal neuropathological hallmarks have been defined: the accumulation of amyloid beta (Aβ) peptides and the neurofibrillary tangles (NFT) containing hyperphosphorylated neuronal tau protein (PTau), consequently inducing neurotoxicity in the brain of AD patients.[2a,4] In addition, AD is characterized by a low level of the neurotransmitter acetylcholine (ACh) and loss of cholinergic neurons, excitotoxicity, impairment of other neurotransmitter systems, extensive oxidative stress, chronic neuroinflammation, mitochondrial dysfunction, calcium, metal dyshomeostasis, and others.[5] Consequently, many AD pathogenesis hypothesis have been formulated throughout the years, seeking to find potential strategies for therapeutic interventions. These hypotheses include the cholinergic hypothesis, amyloid hypothesis, tau propagation hypothesis, metal ion hypothesis, mitochondrial cascade hypothesis, calcium homeostasis hypothesis, inflammatory hypothesis, among others. The lead hypothesis in the past 20 years has been the amyloid hypothesis, corresponding to 22.3% of all clinical trials, followed by the cholinergic, mitochondrial/oxidation and tau propagation hypotheses.[6] For many years, only four drugs have been approved by the Food and Drug Administration (FDA)[6] and the European Medicines Agency (EMA)[7] for the treatment of AD: donepezil, galantamine and rivastigmine, developed following the cholinergic hypothesis, and later, memantine, developed as a *N*-methyl-D-aspartate (NMDA) receptor antagonist. The chemical structures of these drugs are presented in Figure 1.

Aducanumab and Lecanemab, monoclonal antibodies working under the hypothesis of amyloid clearing therapy, are the most recently approved drugs by the FDA, with Aducanumab being announced as the first disease-modifying drug approved for the treatment of AD.[8] Still, the amyloid hypothesis is becoming less relevant due to consecutive failures in clinical trials[9] and, while cholinesterases have been reemerging again as promising targets,[10] none of these drugs alone are considered enough to address a multifactorial disease such as AD. In this context, polypharmacology, and, particularly, (multi-target directed ligands) MTDLs, have emerged as the

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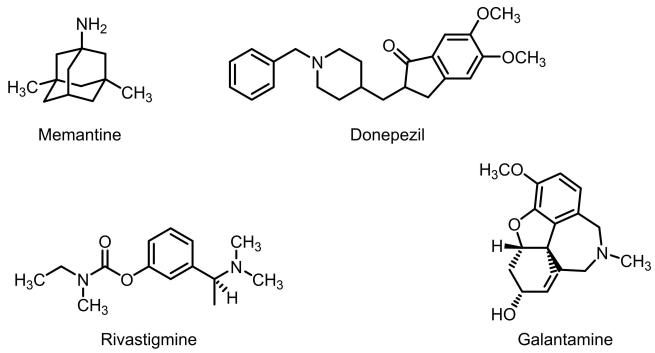

Figure 1. Approved drugs for the treatment of AD.

next therapeutic approaches to combat the disease, by addressing several pathological mechanisms simultaneously.

In this work, the current AD hypotheses and therapeutic targets that are most commonly explored in drug design are described. The concept of polypharmacology, which englobes both combination therapy (CT) and MTDL approaches is discussed in the context of AD, with an emphasis on MTDLs. Following, the recent reemergence of cholinesterase inhibitors (ChEIs) as promising targets to treat AD is discussed. Cholinesterases' structure and physiological roles are presented and their role in the pathogenesis and biochemical processes associated with AD is discussed. *In silico* strategies in the design of cholinesterase inhibitors are briefly introduced. Finally, an overview of the current development in cholinesterase-based MTDLs is performed, highlighting some relevant examples and scaffolds commonly seen in these molecules.

2. Current AD hypotheses and therapeutic targets

2.1. Cholinergic hypothesis

The first hypothesis ever proposed for the pathology of AD was the cholinergic hypothesis, described in 1976 by Peter Davies and A. J. F. Maloney.[11] This hypothesis states that reduction of ACh in presynaptic cholinergic terminals of the hippocampus and the neocortex regions, caused by degeneration of cholinergic neurons of basal forebrain nuclei, plays a key role in AD pathology.[12] Thus, inhibition of cholinergic enzymes would increase the levels of ACh in AD patients and alleviate cognitive impairment. The cholinergic hypothesis has been one of the most studied and impactful hypotheses, resulting in three approved drugs for the treatment of AD. Tacrine, a ChEI, was the first drug to be approved by the FDA for the treatment of AD, but was later discontinued due to its hepatotoxicity. Currently, donepezil, galantamine and rivastigmine constitute

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the three ChEIs used in the treatment of AD patients.[6] Generally, ChEIs are viewed as having limited therapeutic effect and regarded as only modestly treating the symptoms of AD.[2a] But further evidence is emerging that points to a more complex mechanism behind the cholinergic system, one that can interact with other pathophysiological features of AD, including abnormal Aβ and tau cascade, inflammation, apoptosis, and imbalanced neurotransmitter and neurohormonal systems.[13]

2.2. Amyloid hypothesis

The amyloid hypothesis was first proposed in 1991 by John Hardy and David Allsop[14] and later reformulated[15] to focus on soluble Aβ aggregates as the prime pathogenic agent in AD.[16] These oligomers are formed by aggregates of peptides of 40 to 42 amino acids which accumulate inside the brain, forming the known senile plaques. One extensively researched target for the treatment of AD based on the amyloid hypotheses is β-secretase (BACE-1).[17] In AD, Aβ peptides are generated by the hydrolysis of the AβPP and mediated by the sequential action of integral membrane β- and γ-secretases.[18] BACE-1 is the primary enzyme that determines the rate of AβPP cleavage and also the one that demonstrated activity in both *in vitro* and *in vivo* conditions, thus making it a promising target.[19] The degradation process of β- and γ-secretases originates both Aβ40 and Aβ42 peptides, with Aβ42 being the self-aggregation prone form of Aβ.[20] Thus, another common approach is to use anti-aggregation agents. Other approaches to reduce amyloid oligomers formation include neutralization of oligomeric species using immunotherapy and catalytic Aβ antibodies.[21]

On June 2021, aducanumab, was approved by the FDA, and announced as the first disease-modifying drug to be approved for AD.[8] Aducanumab is a monoclonal antibody that removes the amyloid plaques that are formed in the brains of AD patients. The approval, however, comes with much controversy,[22] as many question if there is actual cognitive benefit given the years of clinical trials with amyloid hypothesis derived drugs resulting in failure.[9] Various researchers have stated that no drug targeting amyloid alone can work, no matter how early it is given, as they believe amyloid does not play a direct role in AD pathogenesis, while others consider that it could be a promising preventing therapy for early-onset AD patients.[23] On the other hand, on December 2021, the EMA declined the approval of aducanumab for the treatment of AD patients on the basis of the lack of linkage between the reduction of amyloid plaques and clinical improvement.[24]

Furthermore, on January 2023, the FDA also granted accelerated approval for Lecanemab,[25] a humanized monoclonal antibody with the same purpose of clearing amyloid plaques.[26] Marketing authorization in Europe has been submitted and up to the writing of this review it is still not known the decision from EMA.[27] Once again, the FDA accelerated approval comes with concerns regarding the actual cognitive benefit versus the adverse effects.[28]

2.3. Tau hypothesis

Proposed in 2009, the tau hypothesis is intimately related with the amyloid hypothesis, as the later suggests that the pathological cascades in AD are Aβ deposition, tau phosphorylation, NFT formation, and neuronal death.[6] However, with the failure of anti-amyloid therapy, many researchers questioned whether Aβ is necessary for tau neurotoxicity and if AD pathogenesis is not instead driven by tau, independently of Aβ.[29] The tau protein is a microtubule-associated protein (MAP), very abundant in neuronal axons, that plays a key role in microtubules stabilization.[30] According to the tau hypothesis, in pathological conditions, an increase in hyperphosphorylation of tau will promote its aggregation and reduce its affinity for microtubules, which will then promote aggregation into insoluble NFTs and result in cell death.[28] Consequently, strategies to target tau involve blocking of tau aggregation, utilizing tau vaccinations, stabilizing microtubules and manipulating kinases and phosphatases that govern tau modifications.[31] One particular target that has been widely popular is the threonine-serine kinase GSK-3β, as this enzyme is responsible for tau phosphorylation. Furthermore, increased levels of GSK-3β might regulate γ-secretase to induce Aβ formation, resulting in toxicity to cultured neurons.[12]

2.4. Other relevant targets

Many other relevant targets for AD have been identified that are currently explored in the search for more therapeutic strategies. For example, the approved drug for the treatment of AD, memantine, is a NMDA receptor antagonist, developed according to glutamatergic hypothesis. The later states that, in AD, there is glutamatergic overstimulation from glutamate, the principal excitatory neurotransmitter in the brain, resulting in excitotoxicity, which ultimately leads to neuronal calcium overload and neurotoxicity.[32] The NMDA receptor is more sensitive for glutaminergic stimulation, so memantine works by blocking the open ion channel of NMDA, inhibiting the Ca^{2+} ions influx, which would, in normal pathology, be regulated by Mg^{2+} ions.[33]

Another commonly explored target are metal ions in the brain. Some metal ions (biometals) such as copper, zinc, and iron, are naturally present in the brain and play essential functional roles in enzymatic activity, mitochondrial function, myelination, neurotransmission, among others.[34] However, high levels or dysregulation of any of these biometals is associated with AD pathology, particularly in the mechanism of Aβ aggregation and oxidative stress.[35] Increased concentrations of these metals have been observed in the brain of AD patients, which was not observed for normal individuals, as well as within Aβ plaques, neurons and NFTs.[36] Furthermore, these metals can promote oligomerization and aggregation of Aβ, and the resulting metal adducts can lead to the formation of reactive oxygen species (ROS), which are the main culprits in oxidative damage.[37] Molecules possessing metal chelating activity are one of the most explored ways to potentially neutralize the effects of metal accumulation in AD brains.[38]

In the context of addressing the extensive oxidative stress found in AD patients, drugs with radical scavenging properties (antioxidants) are also commonly explored.[39] Furthermore, inhibitors of monoamine oxidases (MAOs) have also emerged. MAOs are metabolic enzymes present in the superficial membrane of mitochondria that catalyze the oxidative deamination of a range of monoamines and metabolize released neurotransmitters, therefore playing an important role in neurodegenerative diseases, such as AD.[40] MAOs exist in two isoforms, MAO–A and MAO–B, distinguished by their difference on specificity of substrates and inhibitors. MAO–B, however, appears to play a more relevant role in neurodegenerative decline, as various researchers have observed that MAO–B is overexpressed in the brain of AD patients, leading to the production of hydrogen peroxide and ROS as toxic by-products.[41] Therefore, MAO–B inhibitors that can reduce the production of these species are a potential therapy to ameliorate oxidative damage in AD brains. More recently, it was observed that MAO–B activity can regulate Aβ production in neurons via γ-secretase, potentially providing another target to reduce the production of Aβ42.[42]

Another relevant target addressed in AD treatment are serotonin and serotonin receptors. Serotonin, or 5-HT, is a neurotransmitter involved in brain cognition, including short- and long-term memory, via direct activation of fourteen 5-HT-specific receptors and indirect modulation of other neurotransmission systems, namely, cholinergic, glutamatergic, dopaminergic, and gamma-aminobutyric acid-ergic (GABAergic).[43] These fourteen 5-HT receptors are divided into seven families, from 5-HT1 through 5-HT7. Receptors 5-HT1A, 5-HT4 and 5-HT6 have been identified as the primary receptors with potential for the treatment of AD, as decrease expression of these receptors and low levels of serotonin have been observed in the brain of AD patients.[43–44] Evidence suggests cognitive improvement in 5-HT4 agonist modulation. In addition, 5-HT4 agonism leads to non-amyloidogenic metabolism of APP. 5-HT6 antagonism has encounter more issues, and might not be adequate for clinical practice due to low efficacy in clinical trials.[45] Furthermore, serotonin has a neuroprotective role against Aβ toxicity, inhibition of Aβ fibrillization and destabilization of Aβ fibrils, having a role in inhibition of Aβ aggregation.[46]

Histamine H3 receptors have also been a target for AD treatment for some years. Histamine is another neurotransmitter associated to several relevant functions in the context of AD, such as memory, learning, and neuronal hyperexcitability.[47] Some reports indicate that brain histamine levels are significantly decreased in the hypothalamus, hippocampus, and temporal cortex of AD patients, as compared with controls. *In vivo* studies showed that H3 receptor antagonists indirectly modulate signaling pathways, resulting in symptomatic alleviation in AD patients.[48] However, clinical studies on H3 antagonists have demonstrated no improvement or only minor beneficial effects on cognitive performance in AD patients, which has diminished the exploration of targets.[49]

Finally, although not having a particular target, neuroinflammation is also a recurrent feature of AD. Despite still unknowing whether this response is a protective or harmful mechanism, there might be therapeutic benefit in reducing inflammatory responses as part of a wider treatment strategy.[35b,49c]

3. Polypharmacology's role in tackling AD

Nowadays, it is generally accepted that single-target drugs from the "one-target, one-molecule" paradigm present limited efficacy towards complex, multifactorial diseases, such as AD.[50] To address this issue, researchers shifted the paradigm towards the currently known polypharmacoly, i.e. the design/use of pharmaceutical agents that can act on multiple targets or disease pathways.[51] Polypharmacology can be achieve via two pathways: multiple drugs that bind to different targets are administrated in a "cocktail", known by CT; or a single drug binding to multiple targets within a given network is developed, the MTDL approach. CT is a well-established regimen for AD and has proven beneficial effects when compared to some traditional monotherapies.[52] Combination of the acetylcholinesterase inhibitor (AChEI) donepezil, with the glutamate NMDA antagonist memantine, was proven to lead to a synergistic effect, and is currently administered as a fixed-dose combination of both drugs (Namzaric®), approved by FDA, for the treatment of patients with moderate-to-severe AD.[53] On the other hand, EMA has refused the authorization for marketing of this CT, claiming that the study presented did not show significant improvement in patients.[54]

CT has also potentiated drug repositioning, allowing for new potential therapeutics to advance faster to the clinical development setting.[55] Still, the simultaneous administration of various drugs presents a higher risk for drug-drug interactions (DDIs), toxicity, and problems with patient compliance. In this context, a MTDL approach can reduce pharmacokinetic issues and DDIs by using a single molecule, which also translates into simplified therapeutic regimens easier to follow by patients. Given the nature of AD, these factors seem to be of critical importance, as AD patients might have difficulties following more complex therapies and the majority of patients are of geriatric nature, a population particularly susceptible to DDIs.[56]

The design of MTDL's can follow three different routes[57] (Figure 2): (1) linked pharmacophores, which consist of connecting bioactive compounds using spacers, and results in a molecule with both structures of the initial bioactive compounds intact, thus, a higher molecular weight. Linked pharmacophores can be further distinguished into cleavable and non-cleavable, according to the linkage's ability to be hydrolyzed *in vivo;* (2) fused pharmacophores, that result from the combination of moieties of the selected bioactive compounds in one molecule, thus the final structure has a lower molecular height in comparison to linked pharmacophores; and (3) merged pharmacophores, in which the overlap of the bioactive compounds is extreme, the final molecule contains only significant fragments of the selected pharmacophores and presents the lowest molecular height.

In the context of AD, special considerations in the design of multi-target drugs must be taken, as these molecules need to

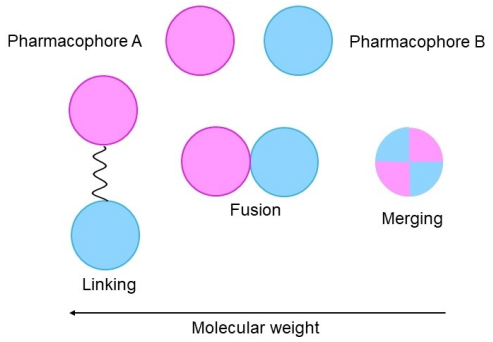

Figure 2. Design strategies for multitarget drugs with two pharmacophores. Adapted from.[58]

pass the blood-brain barrier (BBB) to reach their targets. In an extensive review,[57] Sun and co-workers realized that most of the MTDLs being developed in basic research present MWs above 500 Da, usually due to the combination of pharmacophores with extremely different biological targets (translating into structurally different pharmacophores) making it much more difficult to integrate into a compact compound with low MW. Although it has been shown that larger molecules are not completely excluded from passing the BBB, and technologies to reengineered molecules to facilitate their delivery to the brain exist, developing molecules with MW $<$ 400 Da, high lipid solubility and low hydrogen bonding will afford a greater chance of success in reaching the central nervous system (CNS).[59]

4. Cholinesterases as targets in AD

Notwithstanding the need for disease-modifying agents to be developed and stop the progression of the disease, symptomatic agents continue be of extreme importance in the treatment of AD patients to ameliorate symptoms and delay nursing home placement.[60] Additionally, in recent years, the number of clinical trials on amyloid clearing therapy, the leading hypothesis for the past 20 years, have considerably decrease due to the lack of success observed. The realization that the amyloid hypothesis may not be completely feasible might be a turning point for other targets to be explored.[61] Although with limitations to their therapeutic effect, the majority of the currently approved drugs for the treatment of AD are symptomatic agents based on cholinesterase inhibition, validating the cholinergic system as an important therapeutic target in the disease.[62] Furthermore, a recent study on the long-term effects of ChEIs containing over 17000 patients provided evidence that the use of ChEIs is associated with modest cognitive benefits that persist over time and with reduced mortality risk, with galantamine demonstrating a significant reduction in the risk of developing severe dementia.[63] In fact, cholinesterases have very important physiological roles and ChEIs might extend beyond the augmentation of central cholinergic neurotransmission and act on the disease mechanisms responsible for AD.[10,64]

4.1. Structure and physiological roles

Mammalian brains contain two cholinesterases, AChE and butyrylcholinesterase (BuChE), which share ~50% amino acid sequence homology within species.[65] AChE is the predominant ACh hydrolyzing enzyme in the nervous system and erythrocytes, while BuChE is present in the blood plasma at high concentrations. The physiological role of BuChE is still not completely understood, but it is able to hydrolyze exogeneous butyrylcholine and compensate for ACh hydrolysis if AChE is completely inhibited. It might also have a role as a protective bioscavenger of bioactive esters derived from foods sources.[66]

X-ray crystallographic studies have demonstrated that both AChE and BuChE present similar active sites, with a catalytic triad composed of serine, histidine, and glutamate, located near the bottom of a 20 Å deep gorge (Ser203, Glu334, His447 in human AChE, *h*AChE, and Ser198, Glu325, His438 in human butyrylcholinesterase, *h*BuChE).[67] It is within this region that ACh is hydrolyzed under the following mechanism: glutamate deprotonates the adjacent histidine residue, which, in turn, deprotonates the adjacent serine, activating the latter. Subsequently, a nucleophilic attack from the activated serine on ACh occurs, resulting in the acylated enzyme. Finally, the covalent bond is hydrolyzed, releasing acetic acid and choline.[67] Before a substrate can reach the catalytic triad, it needs to enter the active site gorge, passing through a region known as the peripheral anionic site (PAS). The residues that compose the PAS of AChE and BuChE differ considerably, with AChE presenting several aromatic residues (Tyr72, Tyr124, Trp286 and Tyr341 in *h*AChE) while BuChE only has one (Tyr332 in *h*BuChE). Still, in both enzymes, an aspartate residue in the PAS has a central role in facilitating the transfer of the substrate into the active site of the enzymes (Asp74 in *h*AChE and Asp70 in *h*BuChE).[68] This process occurs via ionic interactions with the aspartate residue and cation $-\pi$ interactions with tryptophan residues (Trp86 and Trp82 in *h*AChE and *h*BuChE, respectively) present near the active site.[69] In AChE, the latter residue is part of the "hydrophobic subsite", which also contains the aromatic residues Tyr337 and Phe338 (*h*AChE) and accommodates the quaternary ammonium moiety of choline.[70] Also, within this region of the active-center gorge, is found the acyl-binding pocket, constituted by Phe295 and Phe297 in *h*AChE.[70] The acyl-binding pocket of BuChE presents aliphatic residues (Leu286 and Val288 in *h*BuChE) in place of the aromatic ones of AChE, resulting in a larger active site that allows BuChE to tolerate bulkier ligands.[71]

Two other subsites of the active gorges that are also relevant to address are the oxyanion hole and the omega-loop (Ω-loop). The oxyanion hole accommodates a negatively charged carbonyl oxygen (e.g. the carbonyl from acetylcholine), stabilizing the tetrahedral transition enzyme-substrate complex and facilitating the nucleophilic attack of serine from the catalytic triad on the partially positive carbon atom of the substrate's carbonyl.[71–72] In *h*AChE the oxyanion hole is comprise of peptidic NH groups Gly121, Gly122, and Ala204[72] while in *h*BuChE these are Gly116, Gly117 and Ala199.[71] The Ω-loop contains two cysteine aminoacids that form a disulfide

loop (Cys69–Cys96 in *h*AChE and Cys65–Cys92 in *h*BuChE) and flanks the active site gorge of cholinesterases, with experimental and theoretical studies showing that this loop is flexible and contributes to transient gorge enlargements that could facilitate ligand binding and release.[73] Representations of the active site gorges of *h*AChE and *h*BuChE are presented in Figure 3, depicting the various subsites referred above.

In the context of AD, the compensatory role of BuChE for ACh hydrolysis begs the question if BuChE should also be targeted to maximize ACh levels in AD patients, and if selective acetylcholinesterase inhibitiors (AChEIs), such as donepezil, would perform worst in comparison with nonselective cholinesterase inhibitors, like rivastigmine, that also acts on BuChE. On this regard, contradictory evidence emerges. Studies comparing the therapeutic effect of donepezil and rivastigmine show no significant differences in improvement of cognition and behavior between the two, and the hierarchies of efficacy are essentially still unclear.[75] Some *in vitro* studies have shown that BuChE activity increases when AChE is inhibited[76] while others found the increase in BuChE barely reaching significance.[77] *In vivo* studies are scarce, but one has stated no compensatory increase of BuChE activity in cholinergic terminals.[78] Various explanations have been suggested to explain these contradictory results, mainly regarding the positron emission tomography (PET) imaging conditions used.[79] Still, studies with AChE knockout mice showed no compensatory increase in the distribution of BuChE, but revealed that BuChE contributes to normal cholinergic transmission in the absence of AChE, thus suggesting its potential as a target to modulate levels of ACh.[80] Overall, there are still many questions to unpack regarding the role of BuChE inhibitors in AD pathogenesis, but the general consensus seems to be that BuChE is a promising biomarker/target of AD because of its association with Aβ plaques and NFTs in human AD.[79,81] In fact, both enzymes can interact with Aβ fibrils, but it appears that only AChE can lead to Aβ fibrils formation. There is evidence that AChE promotes Aβ fibril formation via a tryptophan residue namely, Trp279 in *Torpedo* acetylcholinesterase, (Trp286 in *h*AChE) that belongs to the PAS of AChE. As BuChE lacks this essential residue in its PAS, it does not produce the same effect.[82] The development of ligands that can bind to the PAS would possibly reduce the rate of Aβ peptide binding with AChE and, therefore, reduce AChE-induced Aβ aggregation, whilst also hindering the entrance to the enzyme's gorge.[66b] Because of this, the design of dual-binding site AChE drugs, that can simultaneously bind to the catalytic site and PAS, became popular.[83]

4.2. In silico studies for cholinesterase inhibitors

In recent years, *in silico* studies have become extremely useful in drug design, as these offer the chance to understand the mechanisms by which small-molecule ligands recognize and interact with relevant macromolecules in a virtual manner, without resorting to experimental approaches, which are commonly difficult and expensive to perform. Techniques such as X-ray crystallography, nuclear magnetic resonance (NMR), among others, have enabled the resolution of thousands of three-dimensional protein structures throughout the years, providing structural information about key macromolecular drug targets.[84] This information, combined with other resources (such as homology modeling), is now used by the two most

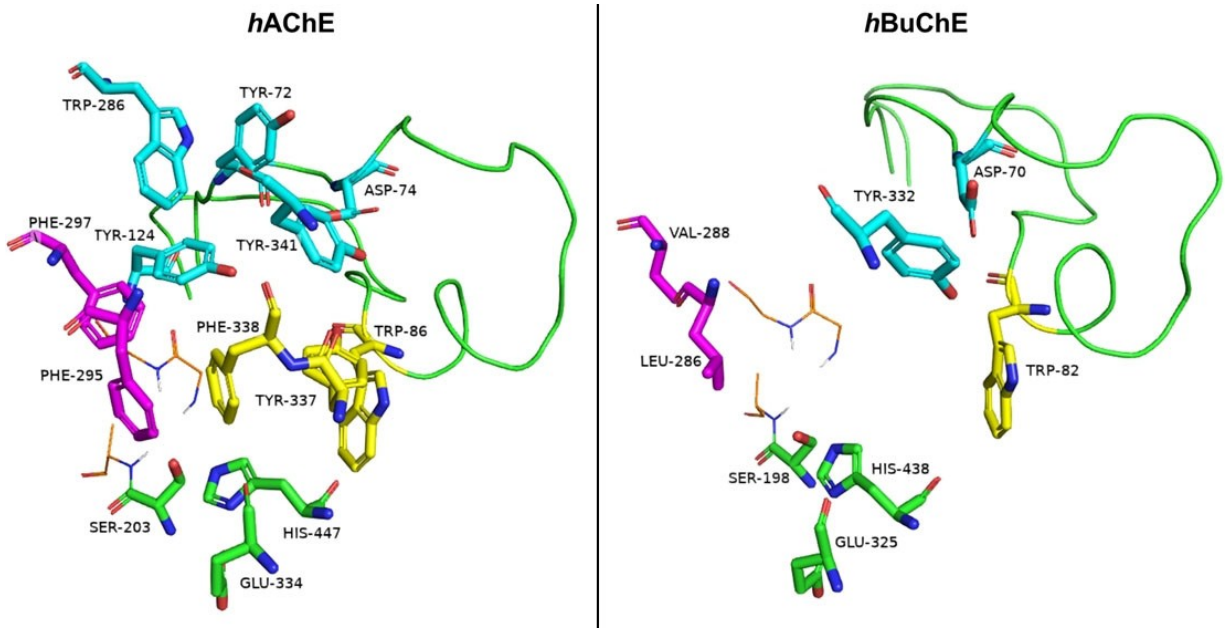

Figure 3. Active site gorges of *h*AChE (pdb: 4EY4) and *h*BuChE (pdb: 1P0I). The main residues important in the active sites are represented. The catalytic triad is presented in green. The acyl-binding pocket is presented in magenta. The hydrophobic pockets are in yellow. The PAS residues located at the rim of the gorge are in cyan. The oxyanion hole residues are represented as orange lines with the respective polar hydrogens and the Ω-loop is represented as a green ribbon. Illustrations were made using the program PyMOL 2.5.2 (Schrödinger, Mannheim, Germany).[74]

important toolboxes for *in silico* drug discovery: molecular docking and molecular dynamics (MD) simulation.[85]

Molecular docking is an approach which predicts the binding modes and affinities of small molecules or macromolecules in contact with protein receptors, at the atomic level. Docking protocols are advantageous by offering fast results with little computational power, but this results from the many approximations done, including lack of protein flexibility.[86] Protein conformation is one of the biggest approximations in ligand design, as proteins are dynamic and undergo various conformational changes that can affect the complementarity between the ligand and the binding site of a protein, of which docking cannot access.[87] With MD simulations techniques, arrangements can be monitored and probed at different timescales, allowing studies from fast internal motions and slow conformational changes to complex processes such as ligand binding to an active site or protein folding.[86] Thus, MD simulations provide important information on the dynamic character of the target with regard to drug design, providing significant complementary information to docking, further validating the results obtained by the latter.[87]

In the design of acetycholinesterases inhibitors, docking must be performed carefully, since acetylcholinesterase has been observed through multiple X-ray structures to present a large mobility in the aromatic residues that compose the PAS and the hydrophobic pocket, especially Trp286 and Tyr337 in *h*AChE, in order to accommodate ligands. MD studies can overcome this issue. In the case of butyrylcholinesterase, a more robust system is found, with no mobile gorge residues found upon ligand binding. The most evident change in conformation is observed in the acyl-binding pocket only when the catalytic serine is phosphorylated with bulky substrates, thus making docking in butyrylcholinesterase much more reliable.[88]

Besides docking and MD, another useful *in silico* approach is the prediction of ADME(T) parameters, (absorption, distribution, metabolism, excretion (ADME) and toxicity (T)). Computational prediction of these parameters can accelerate the drug discovery process by filtering the most promising compounds, contributing to hamper the passage of drug candidates that will ultimately fail in later stages of development.[89] It is becoming very common within research groups to combine these *in silico* approaches, docking and MD with ADMET predictions, in the development of small AD cholinesterase inhibitors and cholinesterase-based MTDLs. The prediction of ADME(T) properties helps to filter an initial library of compounds or evaluate the ADME(T) profile of potential candidates that were found after docking and MD. If the former approach is firstly applied, then the most promising compounds according to their ADME(T) profile are subjected to docking studies and the best complexes found are more thoroughly evaluated using MD studies.[90]

For a more in depth discussion, two recent reviews by Adeowo *et al.*[91] Miles *et al.*[92] discuss the use of these *in silico* approaches and others in the design, development and discovery of novel cholinesterase inhibitors for AD treatment.

4.3. The current development in cholinesterase-based MTDLs

ChEIs have proven their increasing relevancy in the treatment of AD, with various groups urging for its reappraisal. Hence, combination of ChEIs with several other scaffolds with interesting biological activities for AD is a promising therapeutic tool widely explored. Furthermore, the design of MTDLs based on ChEI approved drugs, such as donepezil and tacrine, is very promising since these scaffolds have already been studied and approved for treatment of AD, increasing the efficacy of new compounds, at least for targeting cholinesterase, and reducing the failure risk of developing new drugs.[12] Previous reviews have extensively address developments in MTDLs for AD.[12,93] In this section, it is intended to give an overview of the current development in cholinesterase-based MTDLs, highlighting recent and representative examples of combinations and scaffolds commonly seen.

Combination of ChEIs with anti-Aβ therapy has been the most explored route because of the potential combination of symptomatic and disease-modifying properties. For this purpose, inhibition of Aβ self-aggregation, AChE-induced Aβ aggregation and BACE-1 are the most common approaches. In the context of Aβ self-aggregation inhibition, one of the most explored molecular scaffolds appears to be N- and O-heterocyclic indene derivatives and analogues,[94] such as indole,[95] indoline,[96] benzoxazole,[97] indanone,[98] among others. This might be due to the structural similarities they share with the neurotransmitter serotonin, its precursor tryptophan, and the derived hormone, melatonin, which are all implicated in AD (Figure 4) through alterations in the serotonergic system and, in particular, in the disruption of Aβ42 aggregates.[46]

Additionally, combining good AChE inhibition and Aβ anti-aggregation properties in one molecule appears easier since anti-aggregation agents exert their actions by forming covalent bonds and/or non-covalent interactions (i.e. π–π interactions, hydrogen bonding, or charge–charge interactions) with the amyloid peptide or protein.[99] On the other hand, combining good AChE and BACE-1 inhibition properties can be more challenging. This requires the MTDL to interact with the binding pockets of two different enzymes and, since AChE and BACE-1 are very distinct biological targets, with the gorge of AChE being much narrower than of BACE-1(which presents a distinctly large active site), this process becomes tricky.[17b,100] Because of this, it is not unusual to observe linking of pharmacophores through spacers to design this type of MTDLs,

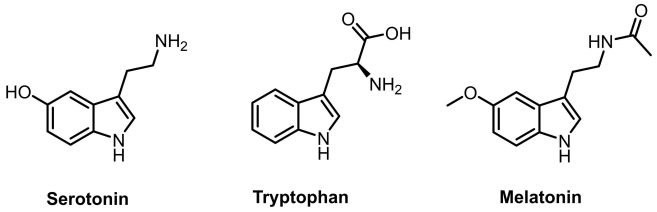

Figure 4. Chemical structures of the neurotransmitter serotonin, its precursor tryptophan, and the hormone melatonin.

rather than merging or fusion. Furthermore, the increasing number of atoms in the spacer usually results in increased inhibitory activity of the molecule, probably due to facilitating the entrance of the respective pharmacophore into the enzyme's gorge.[101] Nevertheless, several examples of fused MTDLs with AChE and BACE-1 activity have been reported.[101] One recent example of the combination of AChE and BACE-1 inhibitory activities in a merged MTDL has been provided by Tripathi and co-workers,[102] which combined four structural frameworks of already reported molecules, as illustrated in Figure 5. Verubecestat is a potent BACE-1 inhibitor that reached phase III clinical trials for the treatment of AD, before being discontinued.[103] The substituted 2-pyridyl ring moiety was reported to bind with the catalytic dyad of BACE-1. Compounds **A**, **B** and **C**, containing, respectively, a 1,3,4-oxadiazole, a piperazine and a terminal phenyl ring with an electron withdrawing group, were previously studied by the authors, and proved to possesses various multifunctional activities relevant for AD, with the main one being AChE inhibition.[104] The combination of these fragments afforded the most promising MTDL, compound **D**, an inhibitor of *h*AChE (IC_{50} = 0.054 μM), *h*BuChE (IC_{50} = 0.787 μM) and human BACE-1 (*h*BACE-1) (IC_{50} = 0.098 μM). The presence of halogens in the phenyl ring, in particular, the two fluor atoms, appears to be essential to increase both AChE and BACE-1 inhibition. Compound **D** also demonstrated to inhibit self- and AChE-induced Aβ aggregation at a level comparable with donepezil. Neuroprotective ability against Aβ -induced oxidative stress in SH-SY5Y neuroblastoma cell lines was also verified. Additionally, parallel artificial membrane permeation assay (PAMPA) was done to examine BBB permeability, affording great permeability potential. The authors performed *in vivo* behavioral studies in scopolamine- and Aβ-induced cognitive dysfunctions on Y-maze and Morris water maze tests, with results overall showing a significant improvement in learning and memory.

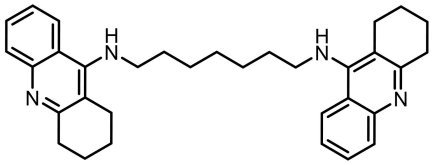

Figure 6. Chemical structure of bis(7)-tacrine that inspired the development of tacrine heterodimers with enhanced therapeutic effect.

Zha and co-workers also reported the synthesis of tacrine-benzofuran hybrids with good inhibitory activity of both enzymes through linking of these pharmacophores.[105] The development of novel heterodimers derived from tacrine has been potentiated by the discovery of the broad and complex pharmacological profile of bis(7)-tacrine (Figure 6). The latter has shown to be a highly selective dual-inhibitor of AChE (acting on both the catalytic site and PAS), and a potent inhibitor of other targets, such as BACE-1, nitric-oxide synthase, receptors NMDA, serotonin 5-HT3, GABA, nicotinic and also Aβ self- and AChE-induced aggregation.[106] Compound **E** (Figure 7) presented the most promising profile, being a subnanomolar selective inhibitor of *h*AChE (IC_{50} = 0.86 nM) and a good inhibitor of both BACE-1 (IC_{50} = 1.35 μM) and Aβ self- and AChE-induced aggregation (58.4%, 61.3% respectively). The optimal linker for anti-AChE activity was a seven-carbon chain. In addition, *in vivo* studies were carried out, in which compound **E** demonstrated cognitive improvement in scopolamine treated ICR mice. Furthermore, they achieved a significantly

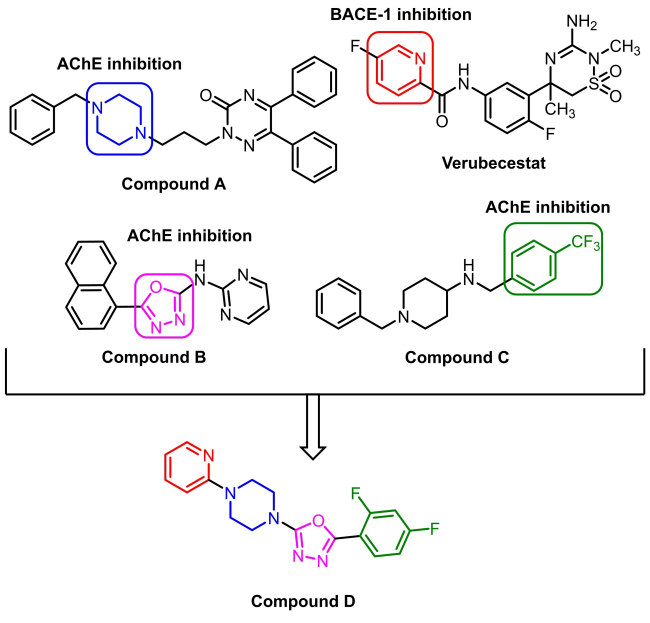

Figure 5. Combination of the four relevant fragments from Verubecestat, Compound **A**, **B** and **C** to afford the AChE and BACE-1 MTDL, Compound **D**.

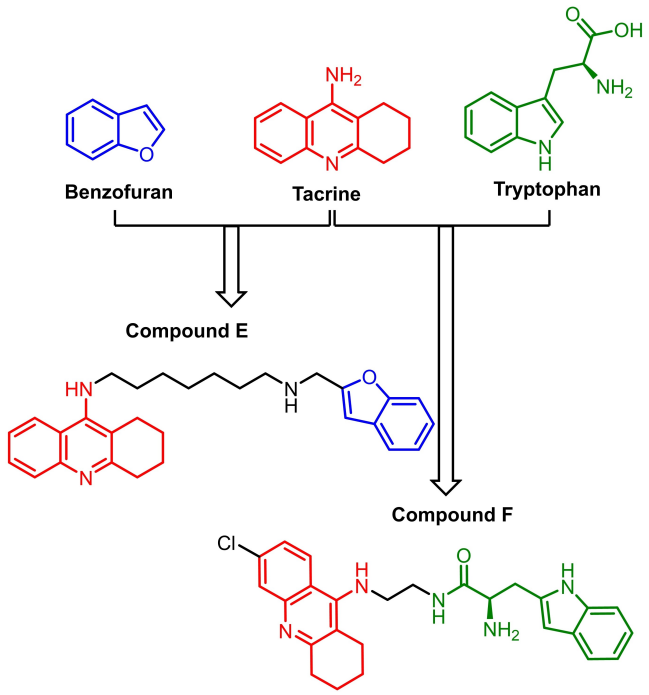

Figure 7. ChE and anti-Aβ multi-target strategy examples.

improved hepatotoxicity compared to tacrine. More recently, a library of also tacrine-benzofuran hybrids has been reported in which the linker size was reduced significantly, with a maximum of two carbon atoms between the tacrine and the benzofuran moieties.[107] Inhibition of AChE was in the range of 0.1 to 1 μM, significantly smaller, which may result from the linker's size, as a longer linker promotes simultaneous binding to the PAS and CAS and, consequently, stronger inhibition of ChEs.[108]

Similarly, Chalupova and co-workers, developed tacrine-tryptophan heterodimers capable of inhibiting AChE, BuChE, Aβ self-aggregation and AChE-induced Aβ aggregation.[95] Compound **F** (Figure 7) was found to be the most potent inhibitor of *h*AChE and *h*BuChE (IC_{50} = 6.3 and 9.1 nM, respectively).

For all the tacrine-tryptophan heterodimers, the optimal spacer length for favorable inhibitory effect of *h*AChE was between five to eight carbon atoms, similar to what Zha *et al.* observed.[105] Compound **F** also showed good ability to inhibit Aβ self-aggregation (58.6 ± 5.1 % at 50 μM) as well as *h*AChE-induced Aβ aggregation (48.3 ± 6.3 % at 100 μM). The authors also performed PAMPA to predict the capacity of these molecules to pass the BBB and reach the CNS. Compound **F** was amongst the most promising, with likeability to pass the BBB via passive diffusion. Again, *in vivo* assays in scopolamine-treated rats were performed, demonstrating beneficial effect when treated with compound **F**. Although BACE-1 inhibitory assays were not carried out, the similarity between this library of MTDLs and that of Zha *et al.*[105] begs the question if these tacrine-tryptophan heterodimers might also exhibit BACE-1 inhibition.

Focusing on the NMDA receptor antagonistic profile of tacrine, Remya and co-workers have recently reported improved tacrine-based MTDLs with high affinity towards AChE and NMDA receptor.[109] *In silico* structure-based modifications on tacrine lead the group to synthesized nineteen derivatives, of which two promising compounds, compound **G** and compound **H** were obtained (Figure 8). Compound **G** presented values of IC_{50} of 40.89 ± 4.82 nM for AChE, 0.85 ± 0.01 μM for BuChE and 15.17 ± 6.14 μM for the NMDA receptor, while compound **H** presented IC_{50} values of 75.07 ± 9.46 nM for AChE, 0.13 ± 0.02 μM for BuChE and 0.31 ± 0.09 μM for the NMDA receptor. The group observed that these aromatic/hetero aromatic substitutions on these positions of tacrine were key determinants for improved antagonistic potential towards NMDA receptor. Both compounds were able to protect the animals against mono sodium glutamate-induced behavioral impairment (in the Morris water maze test) thus, demonstrating their efficacy *in vivo*. Derivatization of tacrine reduced toxicity in all compounds, with compound **G** showing no hepatotoxicity *in vivo*, thus being a strong candidate for more preclinical studies. Although compound **H** presents stronger a NMDA receptor antagonistic profile, it showed mild hepatotoxicity.

In the search for MAOs inhibitors, propargylamines appear as particularly important motifs. Propargylamines are amine molecules that contain a propargyl moiety, which typically inhibits MAO–B. Compounds such as selegiline, rasagiline and PF9601N (Figure 9), known inhibitors of MAO–B, present this moiety, and have been clinically used for the treatment of Parkinson's disease. Furthermore, these compounds possess anti-apoptotic properties independent of their ability to inhibit MAO–B.[110]

Marco-Contelles and co-workers have developed a hybrid compound, ASS234, (Figure 10) through the combination of the *N*-benzylpiperidine group present in donepezil, for cholinesterase inhibition, and the *N*-propargylamine motif present in selegiline and PF9601N.[111] The group also resorted to a hydroxy-indole scaffold to connect both of these pharmacophores, which granted access to antioxidant properties. ASS234 has shown to effectively inhibit *h*AChE (IC_{50} = 0.81 ± 0.06 μM), *h*BuChE, (IC_{50} = 1.82 ± 0.14 μM), MAO–A and MAO–B, (IC_{50} = 5.44 ± 1.74 and 177 ± 25 nM, respectively) and also Aβ aggregation. *In vivo* studies have been carried out in scopolamine-treated rats, where a significant increase of the recognition index was observed, indicating reversal of memory impairment and beneficial therapeutic effect. This MTDL is ready to enter pre-clinical trials for AD treatment.

This hybrid also opened the gates for the production of propargylamine-derived MTDLs in the following years.[110] Recently, it was also developed a new hybrid, contilisant (Figure 10), from ASS234 modification, that presents a very interesting biological profile, being a tetratarget for cholinesterases and MAO, H3 and Sigma 1 (S1) receptors, all while presenting a smaller molecular weight.[112] For this purpose, the

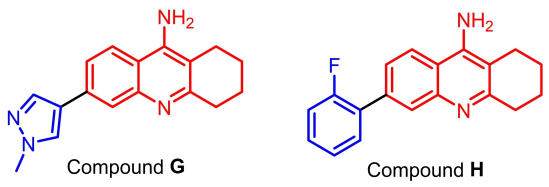

Figure 8. Compounds **G** and **H** developed as tacrine derivatives with improved hepatotoxicity and NMDA receptor antagonistic profiles.

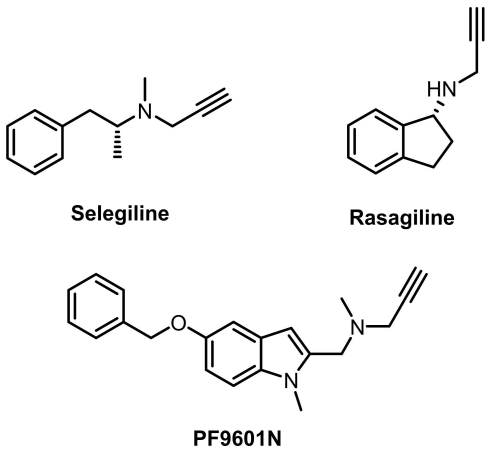

Figure 9. Chemical structures of known MAO–B inhibitors presenting the propargylamine moiety.

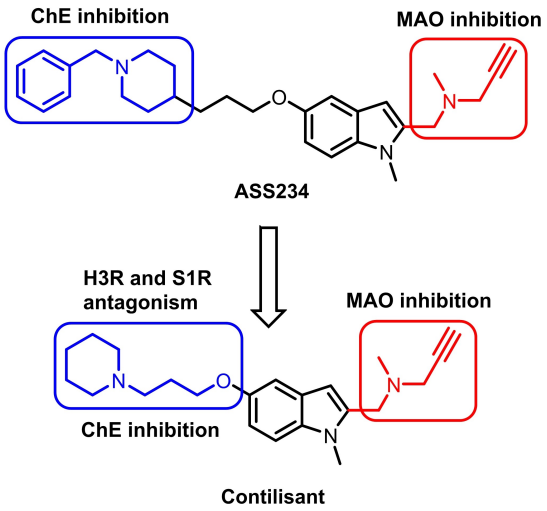

Figure 10. Structures of ASS234, a ChE and MAO inhibitor, and tetratarget contilisant, a ChE and MAO inhibitor with additional H3 and S1 receptors antagonism.

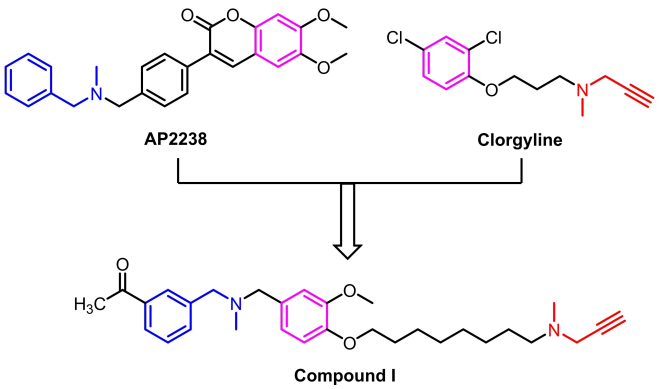

Figure 11. Chemical structure of Compound **I**, merged hybrid from AP2238 and clorgyline, with AchEI and selective MAO–B inhibition.

group substituted the *N*-benzylpiperidine moiety with a (piperidinopropyloxy)phenyl motif, which granted both ChEI and H3 and S1 receptors antagonism. The initial indole core with the propargylamine remained unchanged. This resulted in a smaller molecule, more hydrophilic than ASS234. Contilisant presents potent *h*AChE and *h*BuChE inhibition (IC_{50} = 0.53 μM and 1.69 μM, respectively), potent *h*MAO A and *h*MAO B inhibition (IC_{50} = 0.145 μM and 0.078 μM) and human H3 and human S1 receptor inhibition (inhibitory constant, Ki = 10.8 nM and 65.2 nM, respectively). PAMPA assay indicated that contilisant can pass the BBB by passive diffusion. In addition, the hybrid also presents good radical scavenging properties and significant neuroprotection. *In vivo* studies were performed using the novel object recognition test (NOR) in mice before and after administration of lipopolysaccharide (LPS) to impair NOR performance. Subsequent treatment with contilisant revealed better performance than with ASS234.

Very recently, Zhong and co-workers developed a library of AP2238-clorgyline hybrids with potential inhibition of ChEs and selectivity towards MAO–B.[108] For this purpose, the authors combined specific parts of the molecules AP2238 (a selective AChE inhibitor) and clorgyline (irreversible selective MAO–A inhibitor) to access their biological activity (Figure 11). Due to the shared benzene ring that the structures present, the authors were able to merge the two compounds in one that retained this moiety, so that the final compound could interact with aromatic residues present in the center of the ChEs. They first obtained a primary structure that, after optimization, led to the most promising compound, compound **I** (IC_{50} = 8.30 ± 0.04 μM, for *h*AChE, 1.91 ± 0.06 μM for *h*BuChE and 3.29 ± 0.09 μM for *h*MAO–B). Again, as previously observed, a linker of seven carbon atoms was optimal for AChE inhibition. Compound **I** was also shown to improve the memory function of mice with scopolamine-induced memory impairment and have an excellent ability to cross the BBB.

Coumarins are also commonly associated to MAOs, lipoxygenase, AChE inhibition and additional ROS scavenging activities.[113] Chromones, isomers of coumarin, are the primary scaffold present in the natural family of compounds, flavonoids, which, historically, have been found in many plants used in the therapeutic treatment of mental illness.[114] Chromones and coumarins share similar biological properties, with studies revealing no relevant differences in respect to the recognition of MAO–A and MAO–B.[115]

Jalili-Baleh and co-workers recently designed a new library of compounds based on coumarin and lipoic acid scaffolds.[116] They were inspired by Lipocrine (Figure 12), a tacrine-lipoic acid hybrid developed by Rosini *et al.*,[117] and a very promising candidate for AD that demonstrated potent inhibition of AChE, AChE-induced Aβ-aggregation and protection against ROS. Rather than resorting to the usual aliphatic linker, the use of amide and triazole moieties by Jalili-Baleh *et al.*[116] to connect

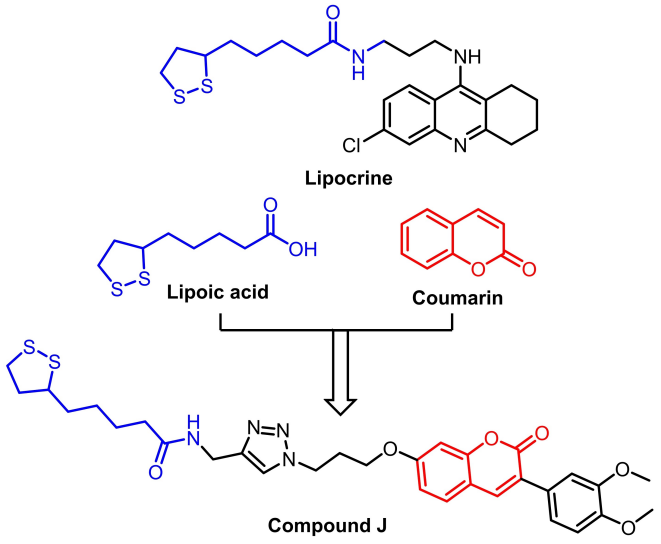

Figure 12. Chemical structure of Lipocrine and Compound **J**, hybrid from lipoic acid and coumarin scaffolds, with AChEI, inhibition of AChE-induced Aβ-aggregation and anti ROS formation.

the coumarin and lipoic acid pharmacophores, allowed for additional chelating properties in the resulting MTDLs. Compound **J** (Figure 12) was found to be the most promising, with good AChE inhibition (IC_{50} = 16.4 μM), good inhibitory effect on Aβ self- and AChE-induced aggregation (51.2% and 47.4%, at 100 μM, respectively) and intracellular ROS formation, as well as the ability of selective bio-metal chelation for Cu^{2+} and Fe^{2+} ions, and neuroprotection against H_2O_2- and Aβ42-induced cytotoxicity. The dimethoxyphenyl substituent in the coumarin scaffold proved relevant for AChE inhibition, has analogues without this moiety presented only weak activity. The high molecular weight in comparison to Lipocrine might hamper crossing onto the CNS, in which more insight could be given from *in vivo* and/or permeation assays. Also, inhibition of MAOs was not tested, but could also be present from the coumarin group and afford an even more interesting biological activity profile.

Reis and co-workers designed chromone-based hybrids with potent dual binding AChE and MAOs inhibition.[118] For the design strategy, the chromone scaffold was functionalized with a phenylcarboxamide moiety, with the intent to stabilize the complex formed with the MAO enzyme, and an acrylate containing a tertiary amine moiety, as this fragment is commonly present in ChE inhibitors. This type of fragment rational design fused the relevant fragments to afford a molecule with lower molecular weight, which will be favorable when evaluating drug-like properties. The most promising compound, compound **K** (Figure 13), displayed selective and potent AChE inhibition (IC_{50} = 0.21 μM), and dual MAO inhibitory activity (*h*MAO–A, IC_{50} = 0.94 μM, *h*MAO–B, IC_{50} = 3.81 μM). Compound **L** (Figure 13) was also interesting, as it showed selectivity towards MAO–B, with a potent inhibition (IC_{50} = 0.63 μM), while still displaying *h*AChE inhibition (IC_{50} = 3.69 μM), although losing the selectivity (*h*BuChE, IC_{50} = 4.32 μM). In the BBB permeation assays, the group found both compound **K** and **L** to present good potential for crossing to the CNS. Interestingly, the group also analyzed the putative metabolite of compound **K**, resulting from the hydrolysis of the acrylate side chain (compound **M**, Figure 13). Although retaining AChE and MAO inhibition, the compound was unable to cross the membrane. This can be justified by the increase in hydrophilicity due to the loss of a two-carbon chain and the formation of a carboxylic acid.

Not many reports of ChE and GSK-3β dual inhibitors for amelioration of NFT formation can be found, but pyridine derivatives such as pyrimidines and aminopyridines, appear as key pharmacophores for the latter effect.[119] In this framework, a promising library of compounds was reported by Yao *et al.*, by combining tacrine and pyrimidone scaffolds through linking.[119a] Tacrine was again used to afford AChE inhibition, which, upon linking to compound **N**, afforded the most promising hybrid (compound **O** – Figure 14). For this compound, an alkylamine linker of seven carbons was found to be the most appropriate, resulting in potent dual AChE and GSK-3β inhibition (IC_{50} = 0,05 and 0,09 μM, respectively). As previously discussed, the length of the linker used can be essential in order to allow for each pharmacophore to enter the respective enzyme active site. This was further confirmed when increased linker length resulted in increased activity, in both enzymes. Additionally, the group observed that a pyrimidone unit bearing fluorine-substituted pyridine was vital for the strong affinity of this pharmacophore with the kinase enzyme, as pyridine-substituted pyrimidones demonstrated relatively weak affinity. Again, this linking strategy is associated with large molecular weights that can influence the drug-like properties of the molecule. For evaluation of these properties,

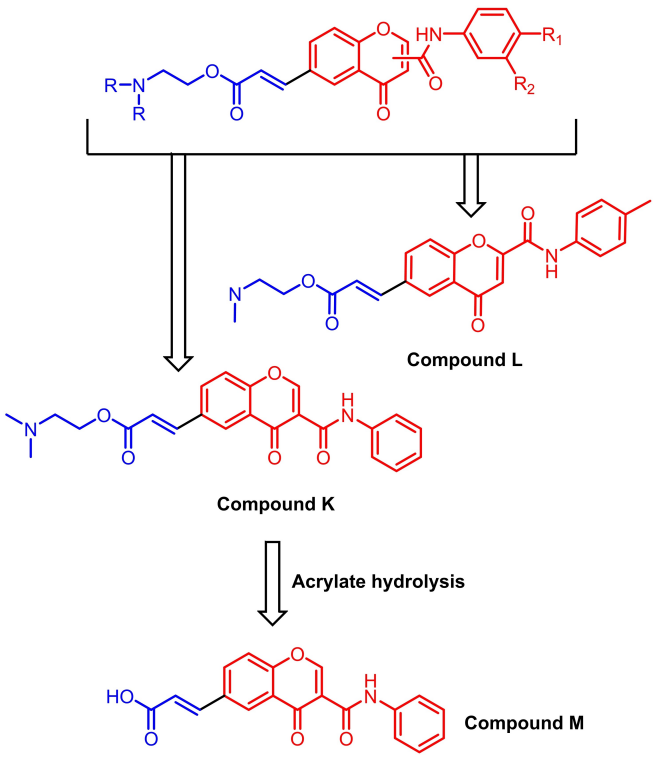

Figure 13. Coumarin based hybrids with AChE and MAOs inhibition.

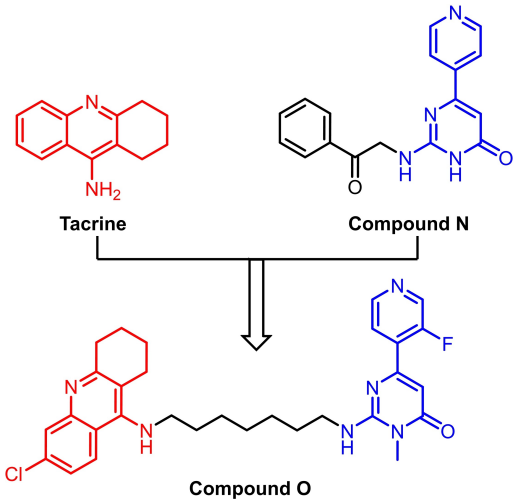

Figure 14. Tacrine-based MTDL with AChE and GSK-3β inhibition properties.

in vivo studies in ICR mice were performed, revealing to significantly ameliorate the scopolamine-induced impairment. However, some weak hepatotoxicity was observed. BuChE inhibition should also have been addressed, as tacrine is not selective towards AChE. Nevertheless, this library demonstrates promising substrates with potential symptomatic and disease-modifying proprieties.

Also in the development of ChE and GSK-3β dual inhibitors, Jiang and co-workers combined **G1**, a GSK-3β inhibitor with aminopyridine and thiazole moieties, with tacrine, in band-shaped compounds, as potential MTDLs for AD.[119b] Aided by docking studies in both enzymes' active sites, the group synthesized 28 compounds. The band-shape of the compounds was relevant to facilitate entrance into AChE's active site. The authors utilized a triazole linker, which according to their previous work favored hydrogen bond interactions within the active-site gorge of AChE.[119c] Additionally, in this work, longer linkers of this triazole were found to decreased the ability to inhibit GSK-3β. Thus, compound **P** (Figure 15) was found to be the most promising, demonstrating IC_{50} values of 1.2 ± 0.1 nM for *h*AChE and 22.2 ± 1.4 nM for *h*GSK-3β. In addition, compound **P** demonstrated high kinase selectivity for GSK-3, with exception of phospho-regulated kinase 1 (DYRK1), for which strong inhibition was also found ($IC_{50} = 28.3$ nM for DYRK1α and 119.7 nM for DYRK1β). Still, this can be beneficial since there is some evidence that DYRK1α may be involved in premature development of AD, by phosphorylating tau.[120] Interaction with DYRK1 may explain the compound's ability to inhibit phosphorylation of Ptau. Compound **P** also exhibited promising cognitive improvement in the scopolamine-induced cognitive deficit mice in the Morris water maze model, and good permeability across the BBB.

The final examples regard MTDLs for ChE inhibition and 5-HT receptor modulation. The combination of these two receptors is also less commonly studied, but recently one interesting approach was reported regarding the development of MTDLs as hybrid prodrugs. For this purpose, Toublet and co-workers combined AChE inhibition and 5-HT4 receptor agonism, inspired by the mechanism of the prodrug ladostigil (Figure 16),[121] which can bind to AChE covalently and release a hydroxy derivative of rasagiline (Figure 9), thus inhibiting MAO–B.

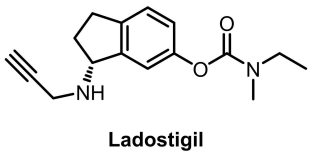

Figure 16. Chemical structure of ladostigil, a ChE and MAO dual inhibitor developed in 2000.[122]

In this work, the authors considered AChE as being both the therapeutic target and the activating enzyme for the prodrug. Upon inhibition of AChE through binding with the hybrid molecule, hydrolysis will take place and subsequently release the respective metabolite, which will target 5-HT4 receptor, while the remaining molecule still inhibits AChE (Figure 17).

For this purpose, a combination of rivastigmine for AChE inhibition and donecopride-analogues, which had been previously described as 5-HT4 receptor agonists,[123] were combined in a prodrug illustrated as compound **P** (Figure 17). The fumaric acid salt of compound **P** was then synthesized (compound **Q** – Figure 17), and biologically evaluated. Com-

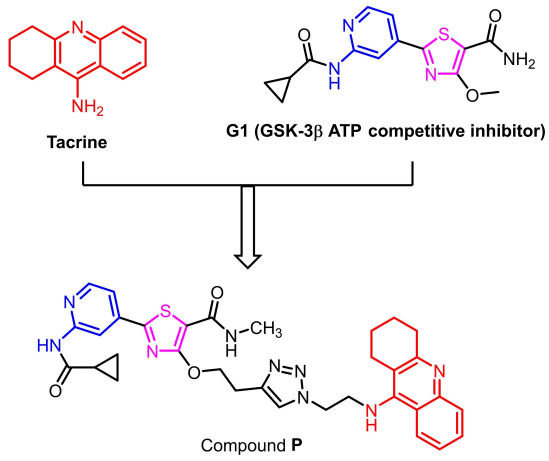

Figure 15. Cholinesterase-based MTDL by combination of tacrine and **G1**, a GSK-3β inhibitor.

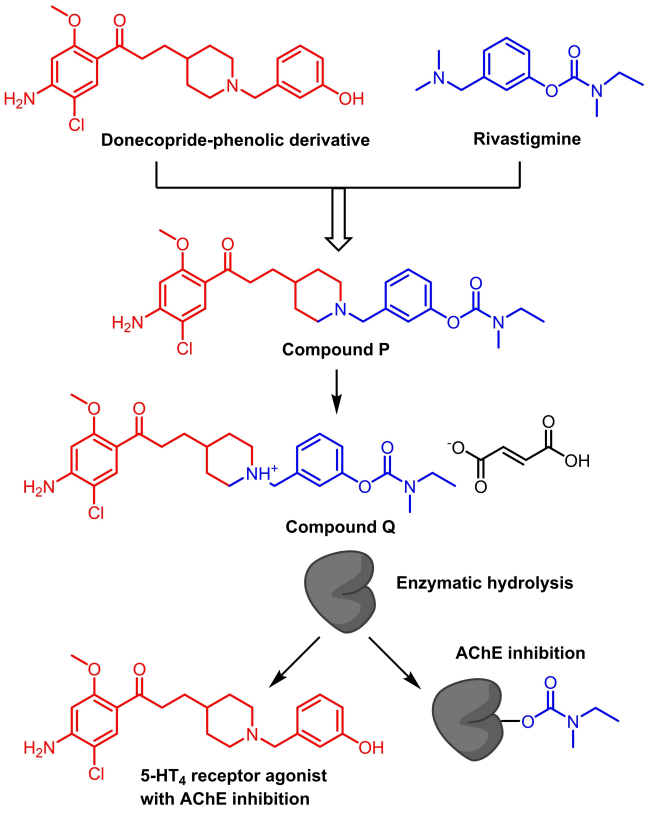

Figure 17. Hybrid prodrug with AChE inhibition and a 5-HT4 receptor agonist, with respective mechanism of action.

pound **P** presented the best AChE inhibition (IC_{50} = 4.1 μM), followed by compound **Q** (IC_{50} = 6.1 μM). No activity on the 5-HT4 receptor was observed for both compounds. Only their hydrolysis product, the donecopride-phenolic derivative, can act both as a potent AChEI (IC_{50} = 0.15 μM) and as a 5-HT4 receptor partial agonist (Ki = 5.1 nM). *In vivo* studies demonstrated that administration of compound **Q** at a low dose of 1 mg/kg produced antiamnesic effects towards cognitive deficits, resulting from either cholinergic or glutamatergic neurotransmission impairments in mice. Usage of these prodrug that covalently binds to the target and release active metabolites can possibly reduce peripheral side effects, being an interesting MTDL for AD.

5. Summary and Outlook

AD is a devastating condition with enormous social and economic costs worldwide. Its multifactorial facet has, for many years, question scientists regarding its underlining pathological mechanisms and hinder most attempts to develop effective disease-modifying drugs. There are currently seven drugs approved for the treatment of AD, arguably, none of which can effectively treat the disease. Scientists have also been shaken by the fact that the most studied and promising hypothesis of AD, the amyloid cascade hypothesis, has not translated well into clinical practice and is currently target of immense controversy regarding its veracity. On the other hand, this has also prompted researchers to look for other targets that might have been overlooked in the last decades. Nowadays, most researchers believe the key to unlock a drug with enhanced therapeutic effect for AD lies in the polypharmacoloy paradigm and, in particular, in MTDLs. To achieve this, they combine several scaffolds than can act on different targets relevant in AD to potentiate synergic effects. The primary targets and hypothesis addressed in AD were briefly discussed in this review, but cholinesterases are the main focus, since these enzymes are reemerging as important tools for the treatment of AD. An extensive review of the development of cholinesterase-based MTDLs in the context of AD is not the goal, as this has been previously accomplished in other elegant reviews. Instead, in this the review, it is intended to contextualize new researchers interested in the area and overview of the current development in cholinesterase-based MTDLs, through representative examples, recent works and major scaffolds explored.

It is a fact that most MTDLs developed for AD do not reach clinical trials and a rational drug design is crucial. But it is also important to mention that a MTDL approach cannot alone solve the puzzle of finding an effective cure for AD and avoid late-stage drug development failure. Efforts in other areas of research are also necessary. *In silico* studies, as briefly discussed in this review, can be key tools to find/filter the most promising compounds before synthesis and preclinical studies. Furthermore, although outside the scope of this review, we also want to acknowledge the importance of advanced *in vitro* models of AD, that accurately represent the disease. These appear critical to hamper the passage of drugs that ultimately fail in clinical trials, as most drugs developed for AD fail in late-stage drug development, albeit presenting themselves strong candidates in preclinical studies.[124]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Keywords: Alzheimer's disease · Biological activity · Cholinesterase inhibitors · Current hypotheses · Drug design · Multi-target directed ligands

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