
Multifunctional Ligand Approach: Search for Effective Therapy Against Alzheimer's Disease

Anna Pasieka • Dawid Panek • Barbara Malawska

Department of Physicochemical Drug Analysis, Faculty of Pharmacy, Jagiellonian University Medical College, Kraków, Poland

Author for correspondence: Barbara Malawska, Department of Physicochemical Drug Analysis, Faculty of Pharmacy, Jagiellonian University Medical College, Kraków 30-688, Poland. Email: mfmalaws@cyf-kr.edu.pl

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Abstract: Alzheimer's disease is a progressive, incurable, and complex neurodegenerative disease. Currently, an effective treatment that can slow down or stop the damage and death of neurons, which is a characteristic of Alzheimer's disease, is lacking. Taking into account the complex nature of the disease, a multitarget design approach has been developed for the production of new potential anti-AD agents. The goal of this approach is to create a single molecule that can interact selectively with several desired molecular targets relevant to the disease. This strategy was successfully developed two decades ago and has been improved in recent years. This chapter describes the progress made in the discovery and design of selected multitargeted drugs based on molecular targets, which can be used for treating Alzheimer's disease. The most promising among these drugs are the molecules having properties that are valuable not only in the symptomatic therapy but also in the causal treatment of the disease. The main hypotheses of Alzheimer's disease, such as β -amyloid (A β), tau, and cholinergic, suggest that compounds capable of inhibiting the aggregation of neurotoxic A β -amyloid peptide and tau protein, and improving the cholinergic neurotransmission, may possess such properties.

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Examples of such multifunctional molecules, which have been recently reported in the literature, are presented in this chapter.

Keywords: Alzheimer's disease; disease-modifying strategies; multimodal compounds; polypharmacology; symptomatic strategies

INTRODUCTION

Alzheimer's disease (AD) is a progressive, incurable, and complex neurodegenerative disorder. The two main neuropathological hallmarks of AD are extracellular amyloid plaques composed of β -amyloid ($A\beta$), and intracellular neurofibrillary tangles (NFTs) containing the tau protein, leading to nerve cell death. In addition, AD is characterized by a low level of the neurotransmitter acetylcholine and loss of cholinergic neurons, excitotoxicity, impairment of other neurotransmitter systems, extensive oxidative stress, chronic neuroinflammation, mitochondrial dysfunction, calcium and metal dyshomeostasis, and other factors (1). This complex and unclear pathogenesis of AD has led to the creation of several theories. The oldest one is the cholinergic hypothesis, followed by others such as the $A\beta$ hypothesis, the tau hypothesis, the oxidative stress theory, the metal imbalance theory, the mitochondrial cascade, and the inflammation hypothesis were developed. The available AD therapy is based only on medications that are capable of treating the cognitive symptoms: three cholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and one *N*-methyl-D-aspartic acid (NMDA) receptor antagonist (memantine). Currently, an effective treatment that may slow down or stop the damage and death of neurons in AD is lacking.

Complex diseases such as AD require a more intricate treatment (2, 3), and hence, agents acting on only one particular target or modulating only one process are not sufficient. This problem can be overcome by two possible approaches. The first, polypharmacy, involves combination therapies with the use of two or more drugs (4). An alternative way is to use multifunctional compounds that exhibit many effects by interacting with more than one biological target (5, 6). Such molecules, which are referred to as multifunctional or multidirectional compounds, multitargeted ligands (MTLs), or multitarget-directed ligands (MTDLs), provide additive or synergistic effects, called polypharmacology (7).

The issues related to the use of combination therapies for AD have been recently described by Cummings et al. (8). Pharmacodynamic combination therapies are based on two or more symptomatic agents that can improve the behavioral and cognitive symptoms of AD and/or disease-modifying therapies (DMTs) that affect the causes of the disease. In 2014, Namzaric, a combination of two symptomatic medications—the cholinesterase inhibitor, donepezil and the NMDA receptor antagonist, memantine—was approved for the treatment of AD patients with moderate-to-severe dementia (9). Currently, various clinical trials are ongoing on combination therapies involving a standard-of-care medication like a cholinesterase inhibitor or memantine combined with another agent. Selected add-on clinical trials of combination treatment for AD, using a small molecule with a standard-of-care medication, are presented in Table 1.

TABLE 1

Selected add-on clinical trials of combination treatment for AD using a small molecule with a standard-of-care medication

Agent	Biological target	Phase	Baseline Therapy	Clinical trials
Masitinib	Selective tyrosine kinase inhibitor	III	Rivastigmine and/or memantine	NCT01872598
Telmisartan	Angiotensin II receptor antagonist	II	AChE inhibitor and/or memantine	NCT02085265
Saracatinib	Src/Abl kinase family inhibitor	II	AChE inhibitor and/or memantine	NCT02167256
UE2343	β -Hydroxysteroid dehydrogenase inhibitor	II	AChE inhibitor and/or memantine	NCT02727699
ANAVEX2-73	Sigma-1 chaperone agonist	II	AChE inhibitor and/or memantine	NCT02244541
Neflamapimod	P38 MAPK α inhibitor	II	AChE inhibitor or memantine	NCT03402659
CT1812	Antagonist of the sigma-2 receptor	II	AChE inhibitor or memantine	NCT02907567
Idalopirdine	5-HT ₆ receptor antagonist	III	Donepezil or donepezil and memantine	NCT01955161
Citalopram	Selective serotonin reuptake inhibitor	III	AChE inhibitor and/or memantine	NCT00898807
Rasagiline	MAO-B inhibitor	II	AChE inhibitor or memantine	NCT02359552
Piromelatine	Melatonin and serotonin receptor agonist	II	AChE inhibitor or memantine	NCT02615002

AD, Alzheimer's disease; AChE, acetylcholinesterase; MAO, monoamine oxidase; MAPK, mitogen-activated protein kinase.

MULTITARGET LIGANDS APPROACH

Combination therapy has some disadvantages resulting from the possible accumulation of drug side effects, pharmacokinetic complexity, drug–drug interactions, and decreased compliance. Moreover, older patients might feel that using one tablet is more convenient than using two or more. Multifunctional compounds are free of the above-mentioned disadvantages. Since these agents can be considered as a form of multicomponent therapy, the creation of new molecules is based on similar principles. Multifunctional agents can be designed by combining two or more structures of active agents or their pharmacophore fragments that interact with the desired targets. Possible strategies for designing multifunctional agents include combining molecules that can interact with two symptomatic targets or symptomatic targets and disease-modifying targets or with two disease-modifying targets. The targets of DMT are neurotoxic aggregates of A β and tau

protein, as well as various processes associated with neuroprotection or neuroinflammation. The targets of symptomatic therapy are the cholinergic system, including acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) enzymes, NMDA neurotransmission, G protein-coupled receptors (GPCRs), and monoamine oxidase (MAO) enzymes (Table 2).

A multitarget design approach was successfully developed two decades ago and has been improved in recent years. There are basically three ways to combine pharmacophores to form an MTL structure, which can interact with appropriate biological targets (10). The simplest way is to connect two pharmacophores through a linker. Another way is to combine two pharmacophore fragments without a linker, forming condensed molecules. The third and best way is to create merged pharmacophores that result in small molecules with low molecular weight and thus with good drug-like properties. Over the years, various potential multifunctional anti-AD agents have been designed, synthesized, and developed (11–13). Based on the structures of currently used anti-AD drugs or their

TABLE 2 Therapeutic strategies of AD applied for the creation of multifunctional agents

Therapeutic strategies for AD treatment	Target for AD treatment	Agents
Disease modifying therapies	A β aggregates	β -Secretase inhibitor A β aggregation inhibitor Metal chelator
	Tau aggregates	GSK-3 β inhibitor Tau aggregation inhibitor
	Neuroinflammation Neuroprotection Various	COX-2 inhibitor, LOX inhibitor, Antineuroinflammatory agent, S1R modulator, Antioxidant agent, PDE4 inhibitor
	Symptomatic therapies	Cholinergic neurotransmission
NMDA neurotransmission		NMDA receptor antagonist
GPCRs		5-HT ₄ receptor agonist
Serotonin receptor		5-HT ₆ receptor antagonist
GPCRs		H ₃ receptor antagonist/inverse agonist
Histamine receptor		
GPCRs		CB ₁ receptor antagonist
Cannabinoid receptor		CB ₂ receptor agonist FAAH enzyme
Oxidative damage		MAO-A inhibitor MAO-B inhibitor

AD, Alzheimer's disease; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; COX, cyclooxygenase; FAAH, fatty acid amide hydrolase; GPCR, G protein-coupled receptors; GSK, glycogen synthase kinase; LOX, lipoxygenase; MAO, monoamine oxidase; NMDA, N-methyl-D-aspartic acid; PDE4, phosphodiesterase 4.

pharmacophoric fragments, multimodal compounds are produced. Thus, ligands containing fragments of tacrine (as a strong inhibitor of cholinesterases), donepezil, or rivastigmine are the most numerous. Of these, only ladostigil has entered phase II/III clinical trials in patients with mild cognitive impairment. Its structure represents a merged pharmacophore, which is formed by combining a propargylamine moiety derived from an MAO-A/B inhibitor rasagiline with a pharmacophoric carbamyl moiety derived from rivastigmine. Ladostigil is a dual AChE/BuChE and a brain-selective MAO-A/B inhibitor possessing *in vivo* neuroprotective properties (14) and, as such, is an example of a multifunctional agent created by combining two pharmacophores that act on the symptomatic targets of AD.

Among the various multifunctional agents, the largest group that focuses on the targets of symptomatic therapy is cholinesterase inhibitors combined with other targets (15). Dual binding site cholinesterase inhibitors capable of interacting with the catalytic active site and the peripheral anionic binding site of AChE can inhibit A β aggregation. Based on this action, many such ligands were obtained, which showed potency toward both symptomatic targets (AChE/BuChE) and disease-modifying targets (A β aggregation) (16–20). Herein, the recent advances made in the design of multifunctional agents for AD treatment based on molecular targets and selected examples of novel multimodal ligands are presented.

MULTIFUNCTIONAL AGENTS FOCUSING ON DISEASE-MODIFYING TARGETS: A β AND OTHERS

The A β hypothesis assumes that the primary cause of neuron loss is the formation of senile plaques due to abnormal processing of amyloid precursor protein (APP) (21). The key role in this process is played by the β -secretase enzyme (BACE-1, A β precursor protein-cleaving enzyme 1), which together with γ -secretase cleaves APP, producing A β peptides consisting of 38–42 amino acids. Because of their fibrillogenic and hydrophobic nature, A β peptides accumulate easily. These peptide aggregates induce oxidative stress, neuroinflammation, hyperphosphorylation, and the aggregation of tau protein, ultimately resulting in loss of neurons and dementia. Based on this theory, some biological disease-modifying targets have been identified and are used in the search for new anti-AD agents. Inhibition of BACE-1 and amyloid aggregation is considered as the most important objective of these agents. Currently, 38 (29%) A β -targeting agents are undergoing clinical trials, of which four BACE-1 inhibitors are gaining continued interest as biological targets in the field of drug discovery and development (22). BACE-1 inhibition has also been applied for the generation of MTDLs having other beneficial properties.

BACE-1 inhibitors with other properties

The structure of donepezil is widely used as a pharmacophoric moiety for developing multifunctional agents (19). Examples of compounds formed using the donepezil structure are *N*-benzylpiperidine analogs acting as BACE-1 and AChE inhibitors with antioxidant and antiaggregating properties, which were described

by Sharma et al. (23). As a core group, *N*-benzylpiperidine moiety present in donepezil and capable of inhibiting BACE-1 was selected. Virtual screening led to the emergence of the hit compound SEW06622. Based on its structure, a series of new anti-AD agents was designed. The compounds differed in the substitution of benzylamine and the presence of a double bond close to the nitrogen atom. The design of these compounds is presented in Figure 1. Among them, compound 1

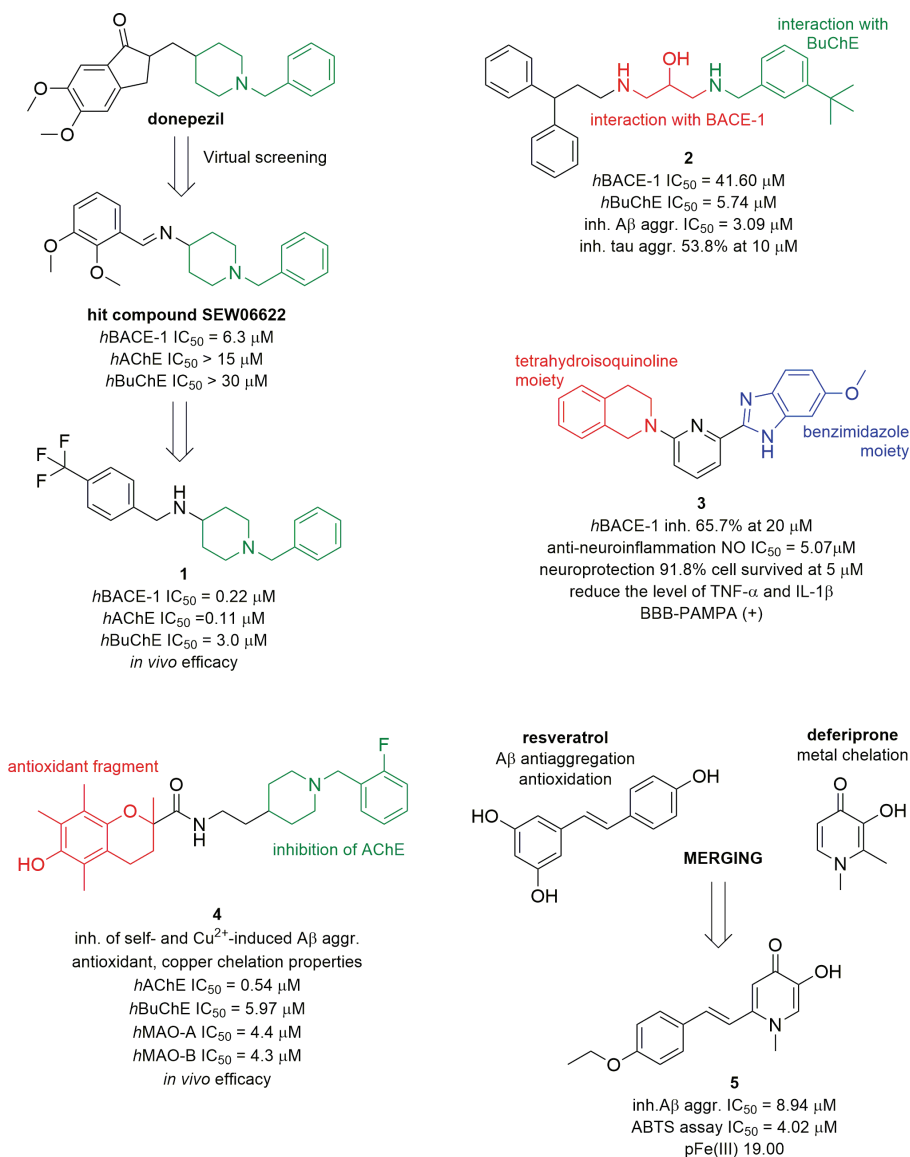


Figure 1. Multifunctional agents focusing on disease-modifying targets: BACE-1, Aβ, and others.

was found to be the most promising. Besides inhibiting both BACE-1 and AChE enzymes with IC_{50} values in the submicromolar range, it showed blood–brain barrier (BBB) permeability in the parallel artificial membrane permeability assay (PAMPA) and inhibited self-induced and AChE-induced $A\beta$ aggregation, while proving to be safe in SH-SY5Y neuroblastoma cell lines at a concentration of up to 80 μ M. Most importantly, it alleviated cognitive impairment in rat models of scopolamine-induced amnesia and by continuous intracerebroventricular infusion of a pathogenic dose of $A\beta$ peptides. In addition, compound **1** displayed good pharmacokinetic parameters after oral administration. Therefore, it can be considered as a potential drug candidate for AD treatment (23).

The approach to obtain disease-modifying and symptomatic multifunctional ligands inspired the design and synthesis of a group of 1-benzylamino-2-hydroxyalkyl derivatives (24). The new multifunctional agents contained substituted benzylamine, responsible for inhibiting BuChE, connected to aromatic fragments: benzyl, phenyl, and benzhydryl through a 2-hydroxyethyleneamine linker. This linker was suggested to confer the agents with BACE-1 inhibitory activity. Among these ligands, the diphenylpropylamine derivative **2** (Figure 1) was the most interesting, which was capable of inhibiting BACE-1 and BuChE, as well as $A\beta$ and tau aggregation. Due to its broad biological profile, it is regarded as a potential multifunctional compound (24).

Multifunctional agents combining $A\beta$ antiaggregation effects with other activities

A series of tetrahydroisoquinoline–benzimidazole derivatives was developed, which represent multifunctional anti-AD agents focusing only on the disease-modifying effects (25). To design this group, two well-known pharmacophores, benzimidazole, present in BACE-1 and inflammation inhibitors, and a tetrahydroisoquinoline moiety possessing anti-inflammatory, antioxidation, and neuroprotection properties, were chosen. Benzene or pyridine ring was used as a linker. Among the obtained derivatives, compound **3** showed the most balanced profile (Figure 1). It inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-induced BV2 microglia cells with an IC_{50} value of 5.07 μ M and BACE-1 activity by 65.7% at a concentration of 20 μ M. Additionally, it showed a strong neuroprotective effect against glutamate-induced cell death at 5 μ M (91.8% cell viability) and reduced the level of proinflammatory cytokines such as TNF- α and IL-1 β . Furthermore, the ability to penetrate the BBB demonstrated that compound **3** is a promising multiple anti-AD ligand.

Suppressing the formation of senile plaques is feasible not only by the inhibition of enzymes involved in the pathological $A\beta$ cascade but also by direct inhibition of $A\beta$ aggregation. Kong's group (26) designed compounds that displayed this property as well as other anti-AD activities, based on two structures: donepezil and Trolox, an antioxidative analog of vitamin E. The Trolox fragment was fused with a differently substituted *N*-benzylpiperidine moiety derived from donepezil by an amide bond. The designed compounds were supposed to bind to both active sites of AChE and inhibit AChE, $A\beta$ aggregation, and MAOs, while also acting as antioxidants. The biological evaluation of the effect of these compounds on all proposed targets revealed that compound **4**, which had a fluor

atom in position 2 of benzene ring, was a promising MTDL (Figure 1). This compound inhibited both cholinesterases (IC_{50} hAChE = 0.54 μ M, IC_{50} hBuChE = 5.97 μ M). Moreover, it displayed nonselective inhibitory activity against MAO-A and MAO-B at a micromolar range. Its antioxidant property compared to Trolox was confirmed in three different experiments. Furthermore, compound **4** exhibited a metal-chelating effect, especially with copper ions, which are associated with AD pathogenesis. The results of self-induced and metal-induced A β aggregation assays also indicated that compound **4** can inhibit both types of A β aggregation. The promising outcomes of the *in vitro* assays were translated successfully to *in vivo* experiments, in which multifunctional ligand **4** was found to significantly improve cognitive decline in scopolamine-, D-galactose-, and AlCl₃-induced memory deficit models. All these results suggested that this compound is an excellent anti-AD candidate.

Another antioxidant and antiaggregating molecule that can serve as a multifunctional compound is the well-known natural derivative resveratrol. For designing new hybrid structures, deferiprone, metal-chelating drug was used (27). These two structures were merged by replacing one of the aromatic rings of resveratrol by deferiprone moiety (Figure 1). Among the newly synthesized hybrids, compound **5** possessing an ethoxy group at position 4 showed triple anti-AD functions—inhibition of A β aggregation, antioxidation, and metal chelation. Such properties make it a potential disease-modifying drug candidate.

MULTIFUNCTIONAL AGENTS FOCUSING ON DISEASE-MODIFYING TARGETS: TAU PROTEIN AND OTHERS

Besides the presence of senile plaques in the brain of patients suffering from AD, the occurrence of NFTs consisting of hyperphosphorylated tau protein aggregates is discerned as the second hallmark of the disease. Based on this observation, the tau hypothesis was formulated to explain the development of AD (28). Tau is a microtubule-associated protein, which, in the physiological condition, stabilizes the microtubules and takes part in axonal transport. During the pathological process, kinases, especially glycogen synthase kinase (GSK-3 β) and GSK-3 α , excessively phosphorylate this protein, resulting in a loss of function. Hyperphosphorylated tau proteins aggregate and easily create intracellular lesions—primarily paired helical filaments and further NFTs. As indicated by the tau theory, the presence of these aggregates contributes to all the processes related to the AD pathomechanism leading to dementia, including A β aggregation. Therefore, in drug discovery and development, the tau-centered approaches, which involve the interaction with kinases, mainly the GSK-3 β enzyme, and the direct inhibition of tau aggregation process, are especially important (29).

Tau and A β inhibitors

One of the valuable strategies applied in the search of new anti-AD agents is the dual inhibition of A β and tau aggregation for reducing simultaneously the level of both lesions. An example of these agents is 1,2,3,4-tetrahydro-1-acridone

analogs, which were designed by combining the structure of tacrine with quinolone moiety, and the antiaggregating compound cinnamaldehyde containing α , β -unsaturated carbonyl fragment (30). Among the derivatives that differed in the length of linker, degree of saturation, substitution of cinnamaldehyde moiety, and methylation of the nitrogen atom of tacrine, five compounds inhibited $A\beta_{1-42}$ and tau aggregation by 84.7–99.5% and 71.2–101.8%, respectively, at the screening concentration of 20 μM . All these compounds had a quaternary amine considered crucial for the observed inhibitory activities. Compound **6** with a naphthalene residue (Figure 2), which was identified as a noncovalent inhibitor of both neurotoxic proteins and predicted to penetrate the BBB as well as prevent tau aggregation in living cells, was selected as the most promising dual agent.

Due to its rich biological properties, the curcumin scaffold is highly preferred in the design of multiple antiaggregation agents. It is a natural polyphenol, reported to have antioxidant, antiaggregating, and anti-inflammatory activities, and is used as a yellow spice. Because of its poor pharmacokinetic parameters, especially bioavailability, the scaffold is not applied in the treatment of AD. However, its structure was included in a series of potential multifunctional ligands (31). Compound **7** (PE859) is a perfect example of such ligands (32) (Figure 2), which was found to display higher dual antiaggregating properties compared to the parent compound. Moreover, it penetrated the BBB, improved memory *in vivo*, reduced the amount of both aggregated lesions in mice brains, and showed a promising pharmacokinetic profile.

Dual $A\beta$ and tau antiaggregating properties were also combined with anticholinesterase activity in order to achieve disease-modifying and symptomatic effects. However, it should be noted that such hybrid molecules were designed as dual binding site cholinesterase inhibitors with potential $A\beta$ antiaggregating properties. The hybrids described by Muñoz-Torreo et al. (33) were formed by the fusion of 6-chlorotacrine, a strong AChE inhibitor, and the previously described tetrahydrobenzonaphthyridine derivatives capable of interacting with both active sites of AChE and displaying weak $A\beta$ aggregation inhibitory activity. This combination allowed obtaining a group of compounds that can target tau, $A\beta$, and cholinesterases. The most potent derivative **8** (Figure 2) inhibited hAChE with an IC_{50} value of 2.06 nM and hBuChE with 0.286 μM , as well as $A\beta_{42}$ and tau aggregation by 77.5% and 68.7%, respectively, in recombinant *Escherichia coli* cells at a concentration of 10 μM . However, a major disadvantage of this compound is its weak drug-likeness. Similarly, a series of shogaol–huprine hybrids displaying antioxidant properties, in addition to anticholinesterase and antiaggregating activities, was developed, which was characterized by poor drug-likeness (34).

GSK-3 β inhibitors with other activities

The inhibition of tau protein phosphorylation by interaction with GSK-3 β is an important tau-centered approach. A group of researchers from the University of Bologna (35) developed a series of 2,4-thiazolidinedione derivatives, which were capable of inhibiting not only GSK-3 β but also directly the tau aggregation process. Compound **9** possessing a substituted indole moiety (Figure 2) was the most potent GSK-3 β inhibitor (IC_{50} = 0.89 μM). It inhibited the AcPHF6 aggregation peptide (306VQIVYK311) up to 80% in a model system at 10 μM concentration.

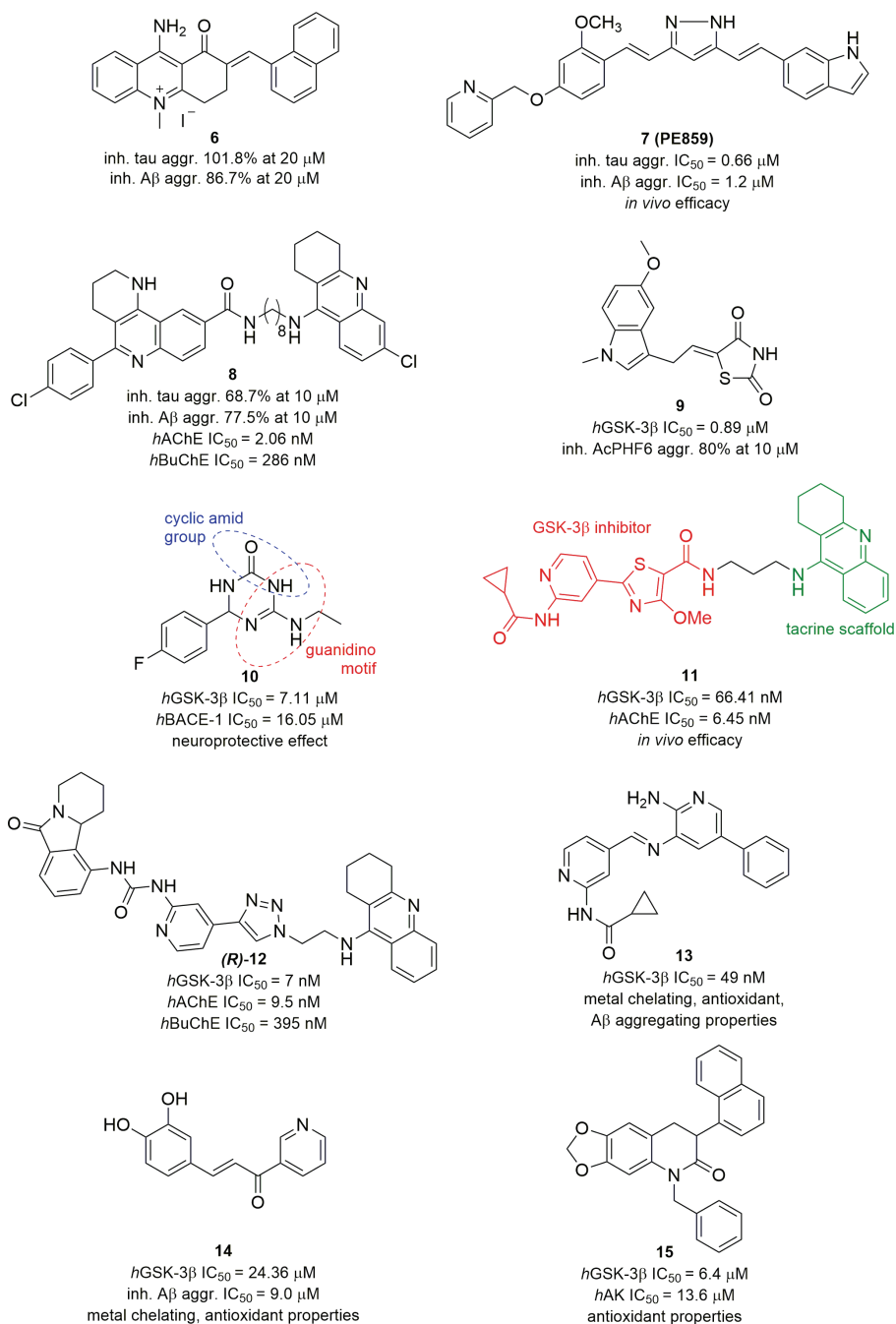


Figure 2. Structures of small molecules targeting tau protein and other targets.

The BBB permeability of the derivatives revealed by PAMPA, their safety, and ability to inhibit truncated and full-length tau aggregation allow regarding them as tau-centric multitarget agents.

A new disease-modifying strategy that suppressed both amyloid and tau cascades by the inhibition of two enzymes, GSK-3 β and BACE-1, was proposed by Bolognesi's group (36, 37). A series of triazinones was designed based on two fragments: a guanidine motif interacting with BACE-1 and a cyclic amide group binding to GSK-3 β . Among the obtained derivatives, compound **10** (Figure 2) was recognized as a novel well-balanced multiple anti-AD agent (IC₅₀ GSK-3 β = 7.11 μ M, IC₅₀ BACE-1 = 16.05 μ M) because it showed neuroprotective and neurogenic effects and a good ADME (Adsorption, Distribution, Metabolism, Excretion) profile. Thus, it seems to be a promising lead structure for further development.

The inhibitory activity against GSK-3 β , together with AChE inhibition, was proposed as a new strategy by Chinese researchers (38). Figure 2 presents the most interesting example (compound **11**) for simple combination of tacrine scaffold with the structure of a selective GSK-3 β inhibitor using an alkyl linker. Among the ligands developed by the above team, compound **11** exhibited a well-balanced biological profile with the IC₅₀ values in the nanomolar range (IC₅₀ hAChE = 6.5 nM, IC₅₀ hGSK-3 β = 66 nM). The efficacy of this dual strategy was verified in the animal model where compound **11** ameliorated the cognitive decline. Moreover, the examined compound did not demonstrate any hepatotoxicity for tacrine.

Oukoloff et al. (39) also used the same AChE inhibitor fragment for creating hybrids, which showed a similar mechanism of action. They connected tacrine pharmacophore by a linker containing 1,2,3-triazole with the structure of the GSK-3 α/β inhibitor valmerin. The *in vitro* results revealed that compound **12** in the form of an *R* enantiomer (Figure 2) was the most potent, which exhibited inhibitory potential with an IC₅₀ value of 9.5 and 7 nM for AChE and GSK-3 α/β , respectively. Additional advantages of the developed novel tacrine-valmerin multifunctional ligands were low cytotoxicity and good BBB permeability.

The metal hypothesis of AD indicates that excess levels and dysregulation of biometal ions, such as Cu²⁺, Fe²⁺, Zn²⁺, and Ca²⁺, in the brain cause A β aggregation, generation of reactive oxygen species (ROS), and oxidative stress, leading to cell death (40). Metal chelators are potential anti-AD agents and are also used in the development of multifunctional agents (41). A novel approach based on combining GSK-3 β inhibitors with agents exhibiting metal-chelating and A β -antiaggregating properties was recently presented. The newly formed compounds included 2,3-diaminopyridine derivatives (42) and hydroxy-substituted *trans*-cinnamoyl derivatives (43). The most promising representative compound **13** from the first group (Figure 2) inhibited the GSK-3 β enzyme with an IC₅₀ value of 49 nM and acted as a selective Cu²⁺ and Al³⁺ chelator, showing antioxidant and A β -antiaggregating properties. On the other hand, compound **14** from the second series (Figure 2) exhibited lower inhibitory potency against GSK-3 β (IC₅₀ = 24.36 μ M), but displayed a broad profile of anti-AD activities.

In turn, two series of compounds, benzoxazinone and indole derivatives, were designed as dual kinase inhibitors (44). Besides inhibiting GSK-3 β , they targeted human adenosine kinase (hAK), which also induces the development of AD by

regulating the level of adenosine and cytoprotective effects. Based on three well-known *hAK/hGSK-3 β* inhibitors, the structures of novel agents were designed and developed. Among them, **15** (Figure 2) displayed dual kinase inhibitory activity (IC_{50} *hAK* = 13.6 μ M, IC_{50} *hGSK-3 β* = 6.4 μ M) with antioxidant and neuroprotective properties, and thus identified as an appropriate lead structure for the development of multifunctional ligands that can exhibit an innovative mechanism of action.

MULTIFUNCTIONAL AGENTS FOCUSING ON SYMPTOMATIC THERAPIES WITH ADDITIONAL BENEFICIAL PROPERTIES

In the course of Alzheimer's disease, it is important to both treat the causes and symptoms of the disease. Dementia changes in patients are also associated with other disease symptoms, such as depression, anxiety, psychosis, and personality changes. According to the oldest theory of AD, dementia changes and memory disturbances are associated with damage to cholinergic neurotransmission. Other symptoms result from disturbances in other neurotransmitter systems, including GPCRs, such as serotonin, H_3 histamine receptors, and the cannabinoid system or with impaired functioning of enzymatic systems such as MAOs. Hence, the design of multifunctional molecules aimed at the treatment of disease symptoms is based on combining the inhibitory activity of cholinesterase with the influence on the other molecular targets mentioned earlier.

Multifunctional ligands influencing serotonin and cholinergic neurotransmission

Among all GPCRs, serotonergic receptors are the most attractive as they are targeted for designing MTDLs as potential treatment of AD. Their particularly desired actions are the activation of 5-HT₄ receptors (5-HT₄Rs) and inhibition of 5-HT₆ receptors (5-HT₆Rs) (45). The first action leads to the modulation of APP metabolism and redirects the protein to a nonamyloid pathway. In turn, 5-HT₆ blockage improves cognitive performance by increasing the release of glutamate, acetylcholine, and catecholamine in the cortical and limbic areas.

The first class of multifunctional ligands formed by combining 5-HT₄Rs with cholinesterases was presented by Dallemagne's research team (46). They described a compound named donecopride (compound **16**, Figure 3) as a new preclinical multifunctional drug candidate showing both *in vitro* AChE inhibitory activity and 5-HT₄R agonistic potency. Moreover, donecopride also displayed *in vivo* procognitive and antiamnestic activities in mice. The same research group (47) described novel MTLs with an interesting biological profile, which were capable of restoring cholinergic transmission through the activation of 5-HT₄R, blockage of 5-HT₆R, and inhibition of AChE. This class of compounds was developed by merging the previously developed dual compound with the benzyl analog of donecopride displaying *in vitro* 5-HT₄R agonist and 5-HT₆R antagonist properties. The most interesting compound was **17** (Figure 3), a fumaric acid salt exhibiting well-balanced activities toward all the three mentioned targets. Compound **17** was found to act

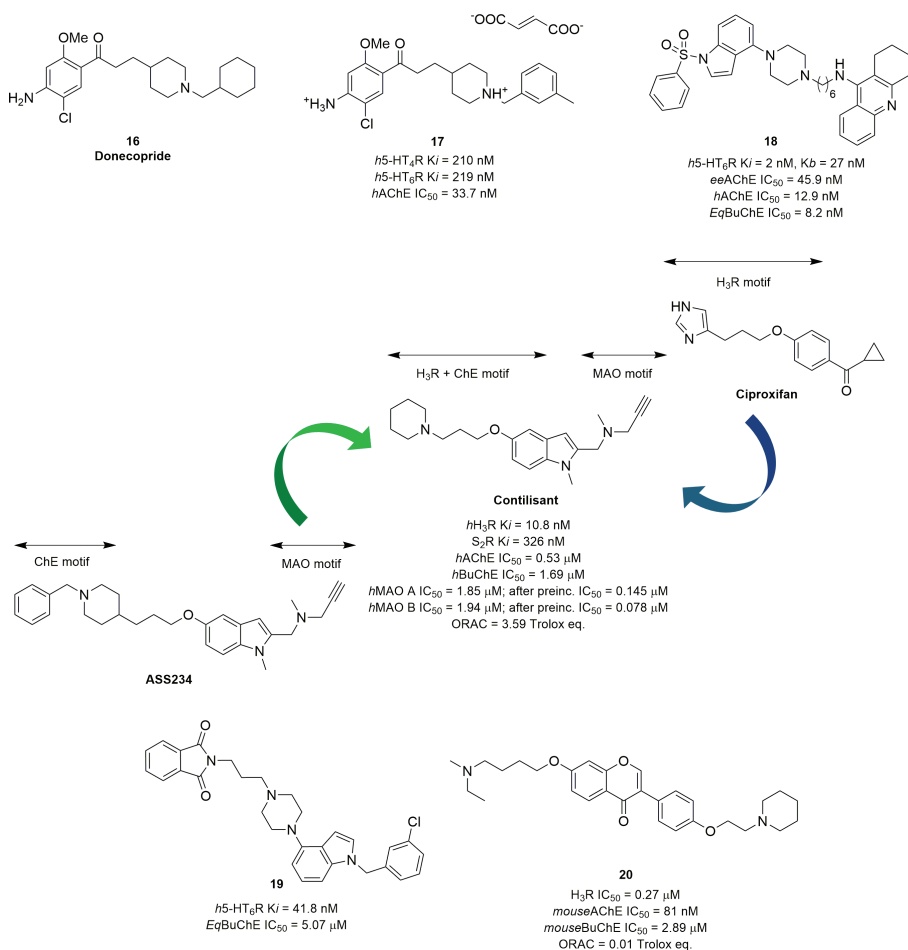


Figure 3. Multifunctional ligands influencing serotonin or histaminergic neurotransmission and cholinesterases.

as a partial agonist of $h5\text{-HT}_{4R}$ (K_i = 210 nM), an inverse agonist of $h5\text{-HT}_{6R}$ (K_i = 219 nM), and an inhibitor of $hAChE$ (IC_{50} = 33.7 nM). Additionally, the compound was tested *in vivo* in a model of scopolamine-induced working memory deficit, where it displayed anti-amnesic effects at a dose of 0.3 mg/kg.

Several multifunctional ligands based on the combination of pharmacophores that are dedicated to 5-HT_{6R} blockage and cholinesterase inhibition were developed by Więckowska et al. (48, 49). These novel hybrid ligands were obtained by combining the pharmacophores directed against 5-HT_{6R} (1-(phenylsulfonyl)-4-(piperazin-1-yl)-1H-indole) and cholinesterases (tacrine or *N*-benzylpiperidine analogs). Among them, compound **18** (Figure 3) was the most interesting as it displayed potent and balanced antagonist activity toward 5-HT_{6R} (K_i = 27 nM) and inhibitory effect against both cholinesterases (IC_{50} AChE = 12 nM, IC_{50}

BuChE = 29 nM). The compound also showed good *in vitro* BBB permeability (proved by PAMPA). Additionally, an *in vivo* study with the use of a scopolamine-induced hyperlocomotion rat model confirmed the central cholinomimetic activity of the compound (48). The further development of this series of hybrids resulted in multifunctional ligands with A β antiaggregation properties (49).

Indole-based multifunctional ligands were designed, synthesized, and evaluated for the treatment of AD as 5-HT₆R blockers with inhibitory abilities toward *eq*BuChE and antioxidant properties (50). Based on the biological screening of the 5-HT₆R antagonists against BuChE, two compounds were selected. In order to improve their BuChE inhibitory activity, structural modifications were introduced in the next stage in the two series of compounds. Of them, ligand **19** (Figure 3) displayed beneficial, dual activity targeting 5-HT₆R (K_i = 41.8 nM) and *eq*BuChE (IC_{50} = 5.07 μ M), in addition to favorable antioxidant properties that were comparable with the reference ascorbic acid.

Multifunctional ligands combining H₃R antagonism and cholinesterase inhibition

Histamine H₃ receptor (H₃R) belongs to the GPCRs family. In the central nervous system (CNS), H₃R acts as a presynaptic autoreceptor and is involved in inhibiting the release of histamine and the modulation of other neurotransmitters such as acetylcholine. Numerous *in vivo* studies have proven that both antagonist and inverse agonists of H₃R improve cognitive deficits, memory, and spatial orientation (51). Based on their results, H₃R is frequently chosen as a biological target for anti-AD multifunctional ligands (52–54).

An international multiteam group discovered new multifunctional ligands with a broad and well-balanced spectrum of activities against H₃R, AChE, BuChE, and MAO-A/B (55, 56). These new indole derivatives were designed by combining the neuroprotectant ASS234 with cholinesterase- and MAO-inhibiting motifs and ciproxifan containing H₃R-blocking and MAO-inhibiting pharmacophore fragments (Figure 3). Among the newly formed hybrids, contilisant was identified as the most promising multifunctional agent. It inhibited *h*MAO-A (IC_{50} = 1.85 μ M, after 30 min of preincubation IC_{50} = 0.145 μ M), *h*MAO-B (IC_{50} = 1.94 μ M, after 30 min of preincubation IC_{50} = 0.078 μ M), *h*AChE (IC_{50} = 0.530 μ M), and *h*BuChE (IC_{50} = 1.690 μ M), as well as blocked H₃R (K_i = 10.8 nM). Moreover, contilisant demonstrated *in vitro* antioxidant neuroprotective effects and the ability to penetrate the BBB in PAMPA. Due to its original *in vitro* biological profile, contilisant was selected for *in vivo* studies and tested using the novel object recognition test in mice with LPS-induced cognitive deficit. Based on the excellent *in vitro* data supported by positive *in vivo* activity, contilisant was studied further with the aim of exploring new pharmacological properties that may be potentially beneficial for AD therapy (57). The studies revealed another valuable activity of the compound: selective agonistic effect on Sigma 1 receptor (S1R) (K_i = 65.2 nM). S1R is associated with learning and memory processes, and hence, its agonists are used as anti-amnesic agents in a variety of pharmacological models, probably due to the improvement of glutamatergic and cholinergic neurotransmissions. Additional *in vivo* studies including Y-maze and radical arm-maze tasks in mice with cognitive impairment induced by A β ₁₋₄₂ oligomers showed that contilisant exhibited higher activity in comparison with the

commonly used anti-AD drug donepezil. Due to its multifunctional profile as well as *in vitro* activities, which were reflected in *in vivo* tests, contilisant thus seems to be an interesting multifunctional ligand for further development as a disease-modifying anti-AD agent (57).

A number of anti-AD multifunctional ligands have been developed based on naturally occurring substances. Wang et al. (58) synthesized a series of multifunctional ligands based on isoflavone, which was recently proven to exhibit inhibitory properties toward cholinesterases. *In vitro* studies revealed that some of these ligands showed a multifunctional profile including blockage of H₃R, neuroprotective effect, and antineuroinflammatory properties. Among them, compound **20** (Figure 3) is noteworthy since it displayed moderate H₃R antagonist property ($K_i = 270$ nM) and potently inhibited AChE ($IC_{50} = 80$ nM). In addition, the compound **20** demonstrated neuroprotective and antineuroinflammatory properties. The *in vivo* study also confirmed that it did not induce acute toxicity even at high doses (1000 mg/kg), but penetrated through the BBB into the CNS and caused significant improvements in mice with scopolamine-induced cognitive deficit, which were revealed by passive avoidance test (58).

Ismaili et al. (59) described new small molecules combining activities against three biological targets including hH₃R. The most promising lead showed high affinity toward hH₃R ($K_i = 0.565$ μ M), Ca²⁺ channel blockade activity ($IC_{50} = 21$ μ M), and moderate selective hBuChE inhibition ($IC_{50} = 7.83$ μ M), besides strong antioxidant properties and ability to restore cognitive impairment induced by LPS.

Multifunctional ligands targeting endocannabinoid system and cholinesterases

Since the discovery that the activation of cannabinoid receptors (CB₁R, CB₂R) causes a reduction in the production of neurotoxic factors (ROS, proinflammatory mediators) leading to decreased neuroinflammatory processes, the receptors were considered as another biological target in the search for anti-AD agents as well as multipotent ligands (60, 61). Consequently, Decker's group (62) developed a series of benzimidazole-based dual-acting ligands that can activate CB₂R and inhibit BuChE. Among them, the authors highlighted compound **21** as the most promising hybrid (Figure 4) as it activated hCB₂R with an IC_{50} value of 0.763 μ M and hBuChE with an IC_{50} value of 1.6 μ M. It is worth noting that this compound **21** was selective over hCB₁R and AChE. Compound **21** was further evaluated *in vivo* which revealed that it improved cognitive functions in mice showing neuroinflammation and cognition deficits after A β_{25-35} administration at doses ranging from 1 to 3 mg/kg.

In turn, Montanari et al. (63) synthesized multifunctional ligands that indirectly enhanced endocannabinoid signaling by inhibiting fatty acid amide hydrolase (FAAH), an enzyme responsible for the degradation of crucial endocannabinoid signaling molecules: *N*-arachidonylethanolamine and 2-arachidonoyl glycerol. In addition to FAAH, the compounds inhibited cholinesterases. Some of the compounds exhibited very high potencies toward single targets; however, the most interesting MTL was compound **22** (Figure 4). It potently inhibited FAAH ($IC_{50} = 157.2$ nM, after preincubation $IC_{50} = 27.9$ nM), hAChE ($IC_{50} = 922$ nM), and hBuChE ($IC_{50} = 42.7$ nM).

Enzyme inhibitors—MAO-A/B and cholinesterases

MAOs (MAO-A and MAO-B) are the enzymes responsible for the degradation and inactivation of monoamine neurotransmitters. The inhibition of MAOs leads to neuroprotective effects, not only due to an increase in monoaminergic neurotransmission but also due to limitations in the production of neurotoxic substances, which are by-products of a reaction catalyzed by these enzymes. Due to its multiactive nature, the chromone scaffold is commonly applied as a structural motive in the development of multimodal ligands. Based on its structure, Reis et al. (64) reported a series of dual MAO and cholinesterase inhibitors. Among the tested compounds, the most promising was found to be compound **23** (Figure 4), which selectively inhibited *hAChE* ($IC_{50} = 210$ nM) over BuChE and nonselectively inhibited both the isoforms of MAO (IC_{50} MAO-A = 0.94 μ M, IC_{50} MAO-B = 3.81 μ M). Moreover, the additional pharmacokinetic and toxicological studies of compound **23** showed its lack of cytotoxicity at a concentration below 25 μ M, and PAMPA predicted its penetration through the BBB. Thus, the established well-balanced activities, low toxicity, and predicted permeability of the tested compound make them interesting multifunctional ligands for AD therapy.

Another group of dual inhibitors of MAOs and cholinesterases was developed by connecting *N*-benzylpiperidine fragment derived from donepezil with di-*tert*-butylated hydroxytoluene, which is a fragment responsible for antioxidant, anticancer, and anti-inflammatory properties in numerous compounds (65). These inhibitors were analyzed by studies divided into two parts: the first aimed to find an appropriate linker to connect the two pharmacophores and the second aimed to decorate the aromatic ring of the benzylpiperidine scaffold. Among the tested compounds, **24** was recognized as a double AChE (IC_{50} *eeAChE* = 75 nM, IC_{50} *hAChE* = 750 nM) and MAO-B ($IC_{50} = 7.5$ μ M) inhibitor with noticeable antioxidant properties and ability to reduce both self-induced and *hAChE*-induced $A\beta$ aggregation (65).

MULTIFUNCTIONAL AGENTS FOCUSING ON VARIOUS DISEASE-MODIFYING AND SYMPTOMATIC THERAPIES

The complexity of AD etiopathogenesis forced a search for new biological targets in the CNS and even beyond. Among the numerous molecular targets discovered, some have been used to create multifunctional ligands.

In pathological conditions, S1R modulates regulatory proteins, restores calcium homeostasis, and controls the production of ROS, thereby contributing to the overall neuroprotection effect (66). Based on their previous study, Rui et al. (67) developed multifunctional agents capable of modulating S1R and inhibiting AChE. The chemical structure of the new multifunctional ligands was developed through an interesting combination of RRC-33 (S1R agonist), curcumin (having antioxidant properties), and donepezil. The newly developed compounds exhibited a high affinity to S1R, but very weak AChE inhibitory properties. Among them, compound **25** (Figure 4) showed binding affinity to S1R with a K_i value of 15 nM; however, it inhibited AChE only by 64.80% at a concentration of 50 μ M. Its neuroprotective effect associated with S1R modulation was confirmed by the neurite outgrowth observed in the dorsal root ganglia in the *in vitro* model (67).

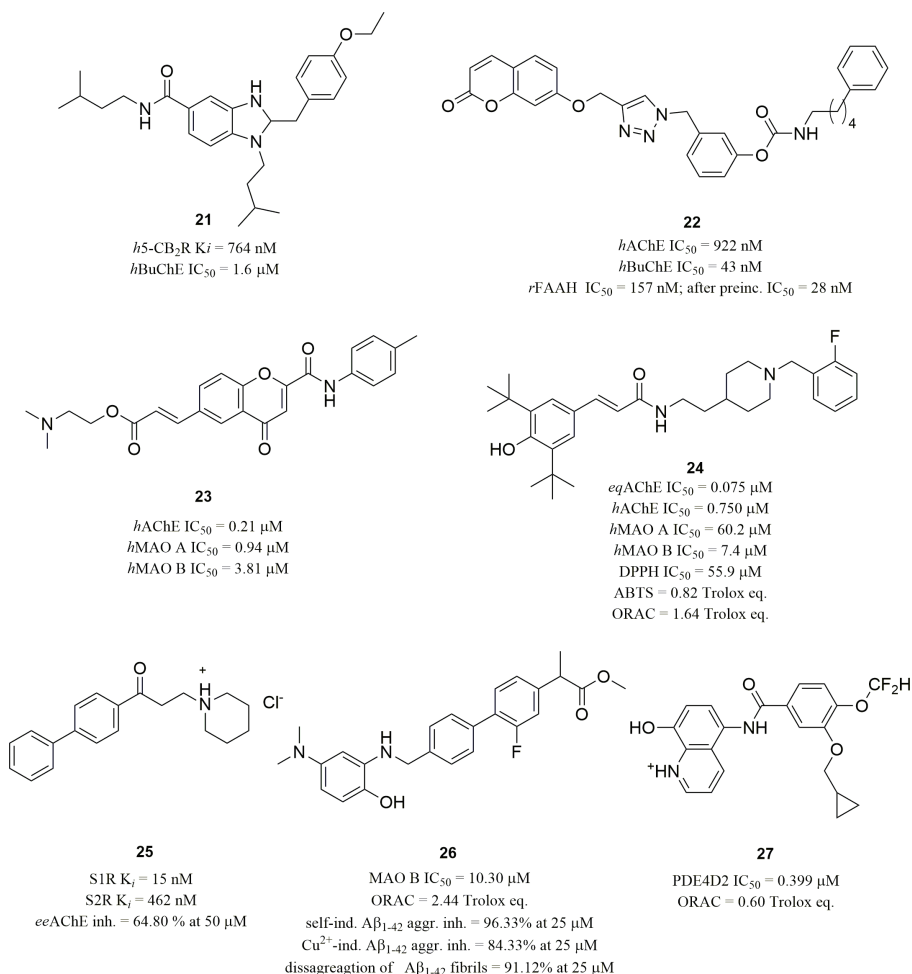


Figure 4. Multifunctional agents focusing on various targets.

Interesting biological activities were also presented by flurbiprofen–clioquinol hybrids (68). Flurbiprofen is a known potent nonsteroidal anti-inflammatory drug, which also inhibits platelet aggregation and Aβ aggregation and reduces tau phosphorylation. On the other hand, clioquinol is a metal chelator with proven antioxidant properties. Among the obtained hybrids, compound **26** (Figure 4) was able to inhibit both self-induced and Cu²⁺-induced Aβ aggregation and MAO-B. In addition, it presented biometal-chelating abilities as well as antioxidant and antineuroinflammatory activities and appropriate BBB permeability.

In turn, Hu et al. (69) developed a novel group of multifunctional compounds by combining clioquinol with rolipram or roflumilast. These compounds inhibited phosphodiesterase 4D (PDE4D), an enzyme participating in the process of memory consolidation and long-term potentiation. The most interesting of them

was compound **27** (Figure 4), which inhibited not only the PDE4D with an IC_{50} value of 0.399 μ M but also the metal-induced aggregation of A β , chelated metal ions, and exhibited antioxidant properties (ORAC [oxygen radical absorbance capacity] = 0.60 Trolox eq.). In addition to *in vitro* activity, compound **27** showed *in vivo* activity by demonstrating procognitive effects in the Morris water-maze test.

Another example of multifunctional compounds is hybrids formed by the combination of pharmacophore cyclooxygenase-2 and 15-lipoxygenase enzymes with the structure of tacrine (**70**). These compounds are endowed with antineuroinflammatory and anticholinesterase activities.

CONCLUSION

The lack of an effective treatment or DMT capable of influencing the causes of AD has led to a constant need to search for new solutions and drugs. Undoubtedly, an important issue here is the identification of new disease biomarkers, which can indicate early enough the pathological changes in the brain that may lead to irreversible processes and symptoms over time. The search for new anti-AD agents is challenging due to the complexity of the disease and the corresponding lack of appropriate animal models useful in preclinical studies. In addition, there is a need for properly planned clinical trials to demonstrate the effectiveness of the drugs. These problems concern small molecules targeting single biological target as well as combination treatment and multifunctional compounds. The clinical trials of combination therapy for AD focus on combining a cholinesterase inhibitor or memantine with other medications having various therapeutic indications. In this context, multifunctional ligands seem to be a much better strategy as they combine effects aimed at both causal and symptomatic treatment of the disease.

The examples presented in this chapter show that the search for new anti-AD drugs is based on the combination of pharmacophores acting not only on cholinesterases and A β aggregation but also on tau protein aggregation (GSK-3 β inhibitors), neuroinflammation, and antioxidation. The ligands that regulate the influence on GPCRs or MAOs are particularly interesting, taking into account that the symptoms of depression or other mental disorders often coexist in AD. This may indicate that the proper design of a multifunctional molecule can facilitate the discovery of an effective therapy for this devastating disease.

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