# Dual Inhibitors of β-Amyloid Aggregation and Acetylcholinesterase as Multi-Target anti-Alzheimer Drug Candidates

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**Abstract:** Notwithstanding the functional role that the aggregates of some amyloidogenic proteins can play in different organisms, protein aggregation plays a pivotal role in the pathogenesis of a large number of human diseases. One of such diseases is Alzheimer's disease (AD), where the overproduction and aggregation of the β-amyloid peptide (Aβ) are regarded as early critical factors. Another protein that seems to occupy a prominent position within the complex pathological network of AD is the enzyme acetylcholinesterase (AChE), with classical and non-classical activities involved at the late (cholinergic deficit) and early (Aβ aggregation) phases of the disease. Dual inhibitors of Aβ aggregation and AChE are thus emerging as promising multi-target agents with potential to efficiently modify the natural course of AD. In the initial phases of the drug discovery process of such compounds, *in vitro* evaluation of the inhibition of Aβ aggregation is rather troublesome, as it is very sensitive to experimental assay conditions, and requires expensive synthetic Aβ peptides, which makes cost-prohibitive the screening of large compound libraries. Herein, we review recently developed multi-target anti-Alzheimer compounds that exhibit both Aβ aggregation and AChE inhibitory activities, and, in some cases also additional valuable activities such as BACE-1 inhibition or antioxidant properties. We also discuss the development of simplified *in vivo* methods for the rapid, simple, reliable, unexpensive, and high-throughput amenable screening of Aβ aggregation inhibitors that rely on the overexpression of Aβ42 alone or fused with reporter proteins in *Escherichia coli*.

**Keywords**: Aggregation, Alzheimer's disease, Amyloid, Bacterial inclusion bodies, Dual inhibitors, *In vivo* assays

## INTRODUCTION

Many distinct proteins and peptides can undergo under certain (patho)physiological conditions conformational rearrangements from the native unordered and soluble states into misfolded β-sheet rich conformers with exposed hydrophobic surfaces, which enable intermolecular attractive forces leading to aggregation into increasingly ordered and sized oligomeric and polymeric species, the so-called amyloid fibrils, which can eventually deposit within or around cells [1,2]. Notwithstanding the diverse amino acid sequences, size and *in vivo* distribution of amyloidogenic proteins, their aggregation seems to proceed through a similar process, namely a nucleation-dependent polymerization, which involves the initial formation of small oligomeric ordered nuclei as the rate-limiting step when the concentration of the amyloidogenic protein reaches a critical point, followed by the amyloid fibril growth by the thermodynamically favorable successive addition of monomers [3,4]. Moreover, the complex multi-step aggregation process of the different amyloidogenic proteins seems to result in very similar highly ordered structures which share a number of common features such as high insolubility and stability as well as similar morphological and tinctorial properties [4,5].

Increasing evidence suggests that amyloid fibers or some intermediate aggregated species might display specific physiological nontoxic roles [1,2,6]. Thus, several functional amyloids have been described in bacteria, including the *Escherichia coli* curli fibers, which play a role in bacterial growth in biofilms, the *Streptomices coelicolor* chaplins, which are involved in the development of aerial hyphea, or the *Klebsiella pneumoniae* microcin E492 and the harpins produced by plant pathogens, with a role in defense mechanisms. Also, functional amyloids have been described in eukaryotic cells, for example some yeast prion proteins such as Ure2p, involved in the regulation of the use of poor nitrogen sources, or Sup35p, with a role as the translation release factor in *Saccharomyces cerevisiae*, or the cytoplasmic polyadenylation element binding (CPEB) protein of the sea slug *Aplysia californica*, which has been found to act as a prion when expressed in yeast and has been suggested to play a role in the formation of long-term memory [6], or F-actin microfilaments, which participate in numerous cellular processes such as muscular contraction or cell motility and division, among others [2].

However, amyloid aggregation has been mostly associated with the pathogenesis of a number of human diseases. Indeed, aggregation of disease-specific amyloidogenic proteins and subsequent deposition seems to be the root cause of a broad range of disorders, called amyloidoses, which encompass neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases or spongiform

encephalopathies or systemic amyloidoses such as type II diabetes, cataracts, lysozime and fibrinogen amyloidosis, amyloid light-chain amyloidosis, amyloid A amyloidosis, or amyloid transthyretin amyloidosis [1,7]. Again some similarities have been proposed to exit in the mechanisms underlying the cellular toxicity of the amyloid aggregates of the different amyloidogenic proteins [3,5].

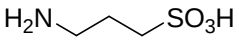
In the particular case of Alzheimer's disease (AD), two amyloidogenic proteins are involved in the onset and progression of the neurodegenerative cascade, namely the β-amyloid peptide (Aβ) and the tau protein, whose aggregates accumulate extra- and intra-cellularly, respectively, giving rise to the two main hallmarks of AD, the senile plaques and the neurofibrillary tangles. Even though the protein tau plays an important role in the neurodegenerative process, its processing, hyperphosphorylation and aggregation seem to take place downstream of Aβ formation and aggregation, as it seems to be the case of other important events such as inflammation and oxidative stress [8-10]. Aβ is a short peptide from 38 to 43 amino acids that is formed upon cleavage of a transmembrane glycoprotein, the amyloid precursor protein (APP), by the sequential action of the enzymes β-secretase (or BACE-1) and γ-secretase. The most abundant form of Aβ is 40 residues in length (Aβ40), whereas the form of 42 amino acids (Aβ42) is the one that most readily aggregates and the most neurotoxic. The credence that abnormally increased Aβ production and subsequent aggregation in brain is the real culprit of the neurodegenerative cascade of AD constitutes the core principle of the prevailing hypothesis on the etiology of AD, the amyloid cascade hypothesis [10-17]. Thus, the increased brain levels of Aβ resulting from an imbalance between its production and its clearance, and its aggregation under the increased concentration conditions trigger a cascade of deleterious events that ultimately lead to dysfunction and death of neurons, neurotransmitter deficits, and dementia.

Compelling evidence from genetic studies strongly support the amyloid cascade hypothesis, inasmuch as all familial cases of AD are associated with mutations or polymorphisms in genes that result in increased production of Aβ or of its amyloidogenicity (either increased levels of Aβ42 or increased ratio Aβ42/Aβ40), namely the genes of APP, apolipoprotein E, presenilin 1, and presenilin 2, in chromosomes 21, 19, 14, and 1, respectively [18]. Similarly, modulation of Aβ production or degradation by the action of external factors might be the cause of the much more common sporadic cases of AD [19]. Also, the fact that in transgenic mice overexpressing human mutant APP gene many pathological hallmarks of AD are recapitulated gives also support to this hypothesis.

The most crucial factor determining Aβ toxicity seems to be the aggregation state [20]. Thus, it was initially thought that mature Aβ fibrils deposited in senile plaques were the neurotoxic species in AD pathogenesis. However, brain Aβ plaque load does not correlate well with clinical progression of AD, and many AD patients with severe memory impairment do not show Aβ plaques at post-mortem analysis, whereas normal individuals can have Aβ plaques without cognitive deficits [15,21-23]. Increasing evidence indicates that soluble Aβ oligomers or prefibrillar aggregation intermediates are indeed the major culprit for neurotoxicity [24,25]. Indeed, the levels of Aβ oligomers equate with AD severity, and soluble Aβ oligomers have been found to be able to inhibit long term potentiation in the hippocampus, and, therefore, to disrupt synaptic plasticity [26]. Notwithstanding the currently widely accepted role of Aβ oligomers as the neurotoxic species responsible of triggering the neurodegenerative cascade of AD, the final Aβ fibrils seem also not to be devoid of toxicity [10,27].

The amyloid cascade hypothesis has provided the rational framework for the development of therapeutic interventions, labelled as "disease-modifying" because they are expected to be capable of preventing or delaying the onset of AD and slowing or halting its progression, thereby going far beyond the currently available anti-Alzheimer drugs that are regarded as merely symptomatic [28]. Hence, the discovery of anti-Alzheimer drug candidates aimed at reducing Aβ production through inhibitors of β- and γ-secretases, at reducing Aβ aggregation, or at increasing Aβ clearance by active or passive immunotherapies, has been very actively pursued in the past decades. Even though the predominant processing pathway of APP, which involves the initial action of α-secretase followed by γ-secretase, does not lead to amyloidogenic peptides, Aβ is continuously secreted under normal physiological conditions and might play a functional role, as it has been found for other amylodogenic proteins (see above). Indeed, provocative evidences for a physiological role of Aβ in synaptic transmission and neuronal viability have been reported [29,30]. Because Aβ might play physiological roles, inhibition of Aβ aggregation, without having to entirely remove Aβ from brain, is emerging as a very promising strategy in the search for effective and safe medications for AD. In most cases, Aβ aggregation inhibitors are discovered through screening campaigns [2,4,5,9,20,24,31-33], but important efforts are being carried out to understand the mechanisms involved in Aβ aggregation and in its inhibition by small molecule drug candidates and to develop suitable computational approaches that enable the rational design of novel Aβ aggregation inhibitors [34-38].

Disappointingly, despite huge research efforts and financial investments different Aβ-directed anti-Alzheimer drug candidates have failed in advanced clinical trials, including tramiprosate (Alzhemed, 3-amino-1-propanesulfonic acid, Fig. **1**), the most prominent Aβ aggregation inhibitor, which failed to demonstrate cognitive benefits in phase III trials.




tramiprosate, **1**

**Fig. (1).** Chemical structure of the Aβ aggregation inhibitor anti-Alzheimer drug candidate tramiprosate.

The clinical failures of advanced supposedly disease-modifying Aβ-directed drug candidates might be ascribed, however, to a number of factors other than their apparent lack of efficacy, such as confusion of clinical outcomes by the occurrence of adverse effects (for example the meningoencephalitis produced by the Aβ vaccine AN1792 or the side effects derived from Notch inhibition by γ-secretase inhibitors), pharmacokinetic issues (poor brain penetration in the case of tramiprosate), and especially the timing of the therapies [28], as these drug candidates are clinically assayed when AD patients become symptomatic and are diagnosed as such, which occurs years or decades after the amyloid pathology started and progressed, which leaves no much room for a disease-modifying intervention because at that point neurodegeneration is too widespread. With this in mind, and while awaiting the development of suitable biomarkers and technologies that allow an early diagnosis of AD and, hence, an advanced therapeutic intervention with more chances of success, Aβ aggregation and production remain viable targets to derive effective medications. However, there is an increasing perception that these targets should be approached in a different way. Human disease, in general, and evidently complex multi-factorial diseases as AD in particular, are increasingly believed not to result from an abnormality in a single protein target but to consist of a complex pathological network of interconnected protein targets endowed with compensatory signaling and redundant pathways that render ineffective the modulation of a single target of the network [39]. This conception challenges both the relevance of overly simplistic *in vitro* assays of drug candidates against a particular protein target isolated from its complex physiological environment and the effectiveness of single-target therapies, and boosts the use of phenotypic *in vivo* tests and the

development of multi-target therapies, particularly multi-target single-molecule drugs, i.e. the so-called multi-target drugs, multiple ligands or multi-target-directed ligands [40,41].

In the case of AD, the notions that it is not a straightforward process, that Aβ is *one* of the causative factors, and not *the* sole factor contributing to AD neuropathogenesis [15], and that AD should be better confronted with drugs that simultaneously hit Aβ formation and/or aggregation as well as other targets are gaining credence [42-46], and important endeavors are being done for charting the protein-protein interactions network of AD [47] to rationally select those targets and pathways to be hit by multi-target drugs that may overcome the robustness of the pathological network of AD.

Among the different targets involved in AD neuropathogenesis that can be hit together with Aβ formation and/or aggregation by multi-target drug candidates, the enzyme acetylcholinesterase (AChE) deserves a special attention. AChE is the target of four out of the five marketed anti-Alzheimer drugs, namely the AChE inhibitors tacrine, donepezil, galantamine, and rivastigmine (Fig. **2**), which, together with the NMDA receptor antagonist memantine, are used to alleviate the cognitive and functional decline of AD patients in mild to moderate or moderate to severe symptomatic stages of the disease.

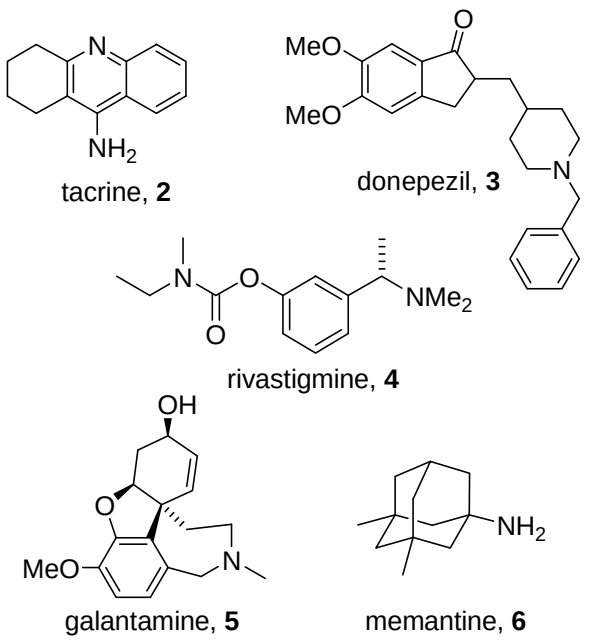



**Fig. (2).** Chemical structures of marketed anti-Alzheimer drugs.

Despite the primary involvement of AChE at the level of the neurotransmitter deficits that appear as a downstream event in the neurotoxic cascade of AD, the finding of Inestrosa one decade ago that AChE can promote Aβ aggregation upon binding to form an Aβ-AChE complex and increase its neurotoxicity as an early event in AD [48-50] shifts AChE as an upstream element in AD neuropathogenesis, thereby increasing the interest in the development of multi-target compounds simultaneously directed to Aβ and to AChE, especially taking into account that: i) the binding site of Aβ within AChE has been identified, and ii) this site, the so-called peripheral anionic site (PAS) of AChE, is placed at the mouth of a narrow 20 Å-deep gorge at the bottom of which is placed the catalytic anionic site (CAS) of AChE [51], which enables the rational design of both peripheral site AChE inhibitors (AChEIs) and dual binding site AChEIs, able to simultaneously bind to both PAS and CAS [52-62]. Apart from a high AChE inhibitory activity, dual binding site AChEIs should be endowed, by definition, with the ability to inhibit the AChE-induced Aβ aggregation. Also, a number of dual binding site AChEIs have been screened and found to be active against Aβ self-aggregation. Herein, we review recent reports on the development of new multi-target anti-Alzheimer drug candidates that hit both AChE and Aβ aggregation, induced by AChE and/or spontaneous.

Whereas the *in vitro* determination of cholinesterase inhibitory activity, usually carried out by the method of Ellman [63], is a quite well established and reproducible standard procedure, *in vitro* determination of the Aβ anti-aggregating activity is rather troublesome. Apart from the artificiality and over-simplicity of *in vitro* assays, Aβ aggregation is extremely sensitive to experimental conditions [9,15], thereby challenging the reliability of the obtained results regarding their physiological relevance or making it difficult the comparison between studies by differents groups. Herein, we also review on novel simplified *in vivo* models of Aβ aggregation that allow an easy, rapid, inexpensive, and more physiological screening of Aβ aggregation inhibitors.

## DUAL INHIBITORS OF Aβ AGGREGATION AND AChE

### Tacrine-based dimers and hybrids

Tacrine is the pharmacophoric moiety most consistently found in the chemical structures of heterodimeric or hybrid dual inhibitors of AChE and Aβ aggregation developed so far, likely because of the ease of synthesis of the intermediates bearing the tacrine scaffold and also because of the outstanding multi-target pharmacological profile of its heptamethylene-linked dimer, *bis*(7)-tacrine or *bis*(7)-cognitin, the first

rationally designed dual binding site AChEI [53,57,64-66]. The development of novel classes of multi-target anti-Alzheimer tacrine derivatives has been recently reviewed [67,68]. In this section, we summarize recent reports on novel tacrine-based dimeric or hybrid compounds that exhibit a multi-target profile, encompassing AChE and Aβ aggregation inhibitory activities, among others.

The research groups of Bolognesi and Minarini have recently reported the synthesis and evaluation of novel derivatives of *bis*(7)-tacrine where the heptamethylene linker was replaced by more rigid aromatic spacers or by a unit of the neuroprotective and antioxidant agent cystamine, respectively [69,70]. The rationale behind the rigidification of the linker that connects both tacrine units by introduction of oligo(*p*-phenylene) groups, as in the biphenyl-linked tacrine dimer **8** (Fig. **3**), was triple, as improvements in the interactions with human AChE (hAChE), Aβ and BACE-1 were pursued. On the one hand, the presence of the aromatic rings in the linker might allow to gain additional π-stacking interactions with the aromatic residues that line the active site gorge of AChE, thereby enabling a three-site (CAS, PAS, and mid-gorge) interaction, and hence a strong interaction, with AChE. On the other hand, the planar aromatic surfaces of these aromatic rings of the linker might lead to a better interaction with Aβ, thereby interfering with the aggregation process. Also, the high molecular hindrance provided by the linker might allow a better fit into the extended substrate binding site of BACE-1. Indeed, tacrine dimer **8** turned out to be more potent than the parent compound *bis*(7)-tacrine as inhibitor of Aβ42 self-aggregation (76% *vs* 51% inhibition at 10 μM) and as inhibitor of BACE-1 (90% *vs* 27% inhibition at 10 μM), but less potent for hAChE inhibition (Table **1**). Even though the interactions of one tacrine unit at the CAS and of the biphenyl linker with mid-gorge aromatic residues of AChE were suggested by molecular docking experiments, a lack of interaction with Trp286 (hAChE numbering), the characteristic PAS residue, as a consequence of the rigidity imposed by the biphenyl system might account for the 4-fold lower hAChE inhibitory activity of **8** relative to *bis*(7)-tacrine. Despite the missing interaction with the PAS of AChE, protrusion beyond the PAS of one tacrine unit to the solvent-exposed gorge entrance seems to account for the significant inhibitory activity of **8** against the AChE-induced aggregation of Aβ40. Worthy of note, compound **8** was more potent than the parent compound as inhibitor of human butyrylcholinesterase (hBChE; $IC_{50}$=0.86 *vs* 5.66 nM), an enzyme that exert a compensatory effect in response to a great decrease in brain AChE activity as AD progresses, thereby constituting a valuable target for anti-Alzheimer agents [71].

Replacement of the heptamethylene linker of *bis*(7)-tacrine by a cystamine unit was carried out with the hope that the resulting compound, the cystamine-tacrine dimer **9** (Fig. **3**) would retain the antioxidant,

radical scavenging, and neuroprotective properties of cystamine as well as the dual inhibition against cholinesterases and Aβ aggregation of *bis*(7)-tacrine [70]. Even though the cystamine-tacrine dimer **9** was less potent than *bis*(7)-tacrine against hAChE and AChE-induced and self-induced Aβ aggregation (Table **1**) and roughly equipotent for hBChE inhibition ($IC_{50}$ 4.23 *vs* 5.66 nM), **9** was clearly superior to *bis*(7)-tacrine in terms of neuroprotective activity and toxicity in human neuroblastoma SH-SY5Y cell cultures. Thus, compound **9** was able to protect SH-SY5Y cells against hydrogen peroxide insult even at a dose as low as 5 nM, the protective effect being complete at 0.5 μM. Apparently, this protective effect of **9** was related to its ability to scavenging oxygen peroxide, thereby reducing reactive oxygen species (ROS) production, likely by activation of the prosurvival kinases ERK1/2 andAkt [70].

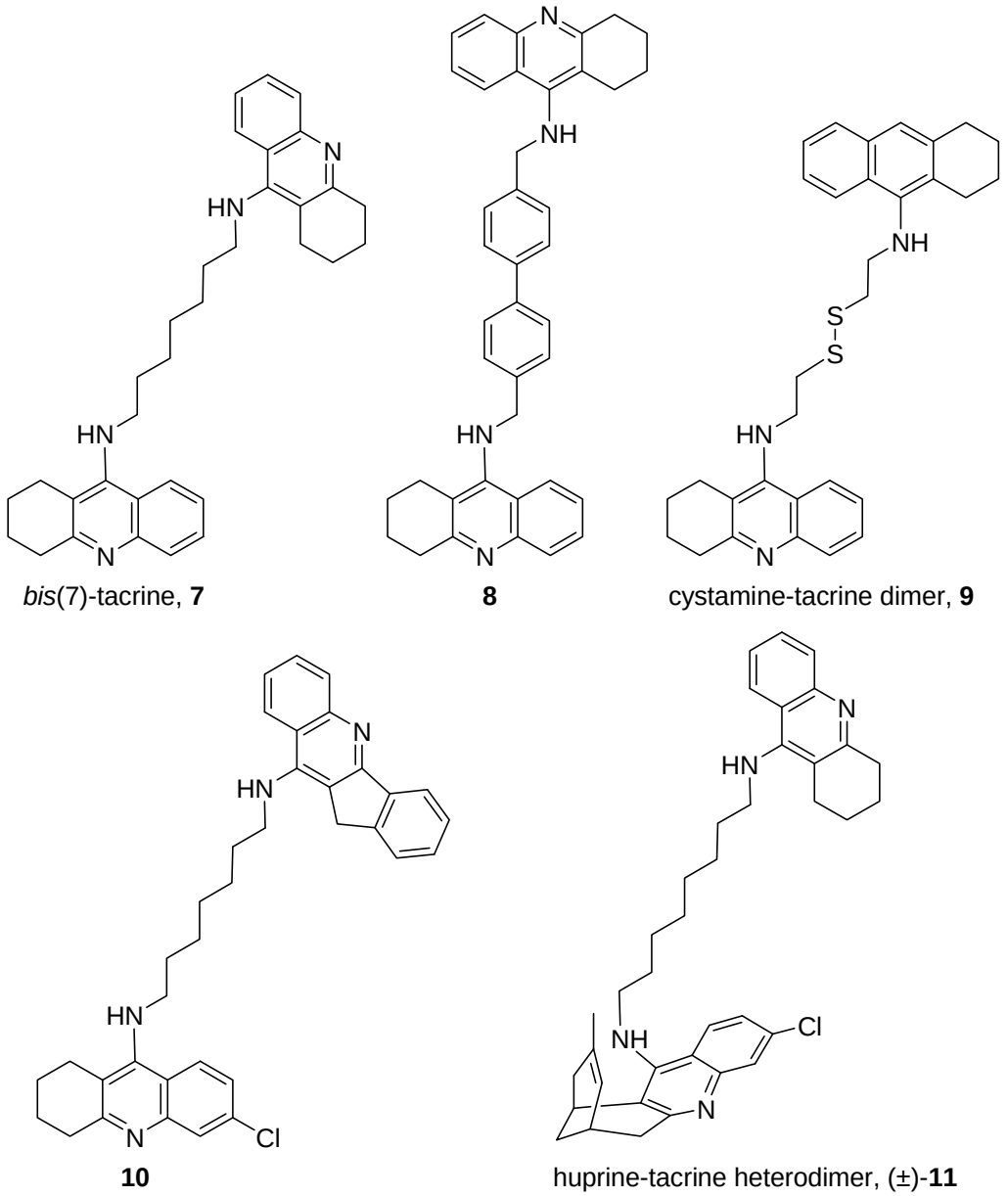



**Fig. (3).** Chemical structures of tacrine dimers and tacrine-4-aminoquinoline heterodimers.

**Table 1. *In Vitro* Dual Inhibitory Activities of Tacrine Dimers and Tacrine-4-Aminoquinoline Heterodimers Against AChE and Aβ Aggregation**

| Compound | hAChE ($IC_{50}$ nM) | AChE-induced Aβ40 aggregation (% inhibition at 100 μM)[a] | self-induced Aβ42 aggregation (% inhibition at 10 μM[b] and/or $IC_{50}$ μM) |
|---|---|---|---|
| *Bis*(7)-tacrine, **7** | 0.81 | 68 | 51 / $IC_{50}$ 8.4 μM |
| **8** | 3.61 | 62 | 76 |
| **9** | 5.04 | 53 | $IC_{50}$ 24 μM |
| **10** | 1.05 | 46 | |
| (±)-**11** | 1.38 | 67 | 64 |

[a] [inhibitor (I)]=100 μM, [Aβ40]=230 μM, [AChE]=2.30 μM, Aβ40:AChE ratio 100:1. [b] [I]=10 μM, [I]:[Aβ42] ratio 1:5.

The group of Rampa reported the synthesis of compound **10** (Fig. **3**), closely related structurally to *bis*(7)-tacrine, bearing a chlorine atom at position 6 of a tacrine unit to increase the affinity toward the CAS of AChE and an indenoquinoline moiety to interact with the PAS [72]. Compound **10** was found to be equipotent to *bis*(7)-tacrine as inhibitor of hAChE (Table **1**). In agreement with kinetic studies that suggeted a mixed-type inhibition of hAChE by **10**, i.e. the ability to interact with both CAS and PAS, this compound was able to inhibit the AChE-induced aggregation of Aβ40, albeit with lower potency than *bis*(7)-tacrine [72]. Moreover, indenoquinoline-tacrine heterodimer **10** turned out to be a potent inhibitor of hBChE ($IC_{50}$ 64 nM) and, very interestingly, it also displayed a potent BACE-1 inhibitory activity ($IC_{50}$ 1.0 μM), being 7.5-fold more potent than *bis*(7)-tacrine ($IC_{50}$ 7.5 μM), likely as a consequence of its greater molecular size, whereas none of its monomeric constituents, neither tacrine nor the indenoquinoline fragment, were active for BACE-1 inhibition.

The group of Muñoz-Torrero recently developed a family of heterodimeric compounds that are also structurally related to *bis*-tacrines, namely the huprine-tacrine heterodimers [73], in which a unit of tacrine was connected through different linkers to a unit of huprine Y, a high affinity inhibitor of the CAS of AChE that was designed by merging the structures of the natural AChE inhibitor huperzine A and

tacrine [74]. Among these compounds, heterodimer (±)-**11** (Fig. **3**) exhibited a very interesting multi-target profile, encompassing a highly potent inhibitory activity toward hAChE, resulting form its dual site binding to CAS and PAS as predicted by molecular dynamics simulations and kinetic studies, as well as moderately potent inhibitory activities toward AChE-induced and self-induced aggregations of Aβ40 and Aβ42, respectively (Table **1**). Worthy of note, this compound was found to be able to block the aggregation induced by AChE of another amyloidogenic protein, namely the prion peptide PrP106-126, one of the key domains involved in the conformational change and aggregation of the prion protein (87% inhibition at 100 μM, $IC_{50}$ 69 nM). The facts that AChE can act as a pathological chaperone promoting the aggregation of distinct amyloidogenic proteins such Aβ and PrP106-126 [75], and also of PrP82-146 [76], the main component of the amyloid plaques in patients with Gerstmann-Sträussler-Scheinker disease, and that huprine-tacrine heterodimers as (±)-**11** can inhibit both processes support the increasingly accepted notion that amyloidoses share common mechanisms and might also be confronted with common therapeutic interventions [77]. Regarding the multi-target profile of (±)-**11**, it is also a potent inhibitor of hBChE ($IC_{50}$ 74 nM) but it was found to be inactive against BACE-1, contrary to its analog bearing a chlorine atom at position 6 of the tacrine unit ($IC_{50}$ for BACE-1 inhibition 5 μM). Finally, *ex vivo* experiments in OF1 mice confirmed the ability of the huprine-tacrine heterodimer (±)-**11** to cross the blood-brain barrier (BBB) after intraperitoneal administration and inhibit brain AChE.

Other tacrine-based hybrid compounds with dual action on AChE and Aβ aggregation in which the tacrine unit is linked to an heteroaromatic system different from 4-aminoquinoline have been also recently developed. The group of Wang and Kong has designed a series of hybrids that consisted of a tacrine unit connected to a coumarin system through a linker containing a piperazine ring. Unlike most tacrine-based dimeric or hybrid compounds developed so far, where the nitrogen atom at position 9 of the tacrine unit is an amino group, in these coumarin-tacrine hybrids, exemplified by compound **12** (Fig. **4**), the exocyclic nitrogen atom of the tacrine unit was introduced in an amide functionality with a triple purpose, namely enabling metal ions chelation, promoting choline uptake, and decreasing toxicity [78]. Moreover, the piperazine moiety of the linker, which should be protonated at physiological pH, was expected to endow the coumarin-tacrine hybrids with the ability to establish cation-π interactions with AChE mid-gorge aromatic residues, which would add to the expected interactions of the tacrine and coumarin moieties at the CAS and PAS of AChE, respectively. Indeed, kinetic and molecular docking studies confirmed the expected three-site binding mode of **12** to AChE. The tight binding of **12** to AChE

was also evidenced by its potent inhibition of electric eel AChE (eeAChE, $IC_{50}$ 92 nM), clearly higher than that of the parent monomeric tacrine ($IC_{50}$ 269 nM, in the same assay conditions). Also, compound **12** at 20 μM, was able to inhibit by 68% the aggregation of Aβ42, more potently than the positive reference compound curcumin (42% inhibition at 20 μM). Also, as expected, compound **12** was able to chelate $Cu^{2+}$ and $Fe^{2+}$, which might be important for lowering ROS production, and was found to be nontoxic to human neuroblastoma SH-SY5Y cells at concentrations up to 50 μM [78].

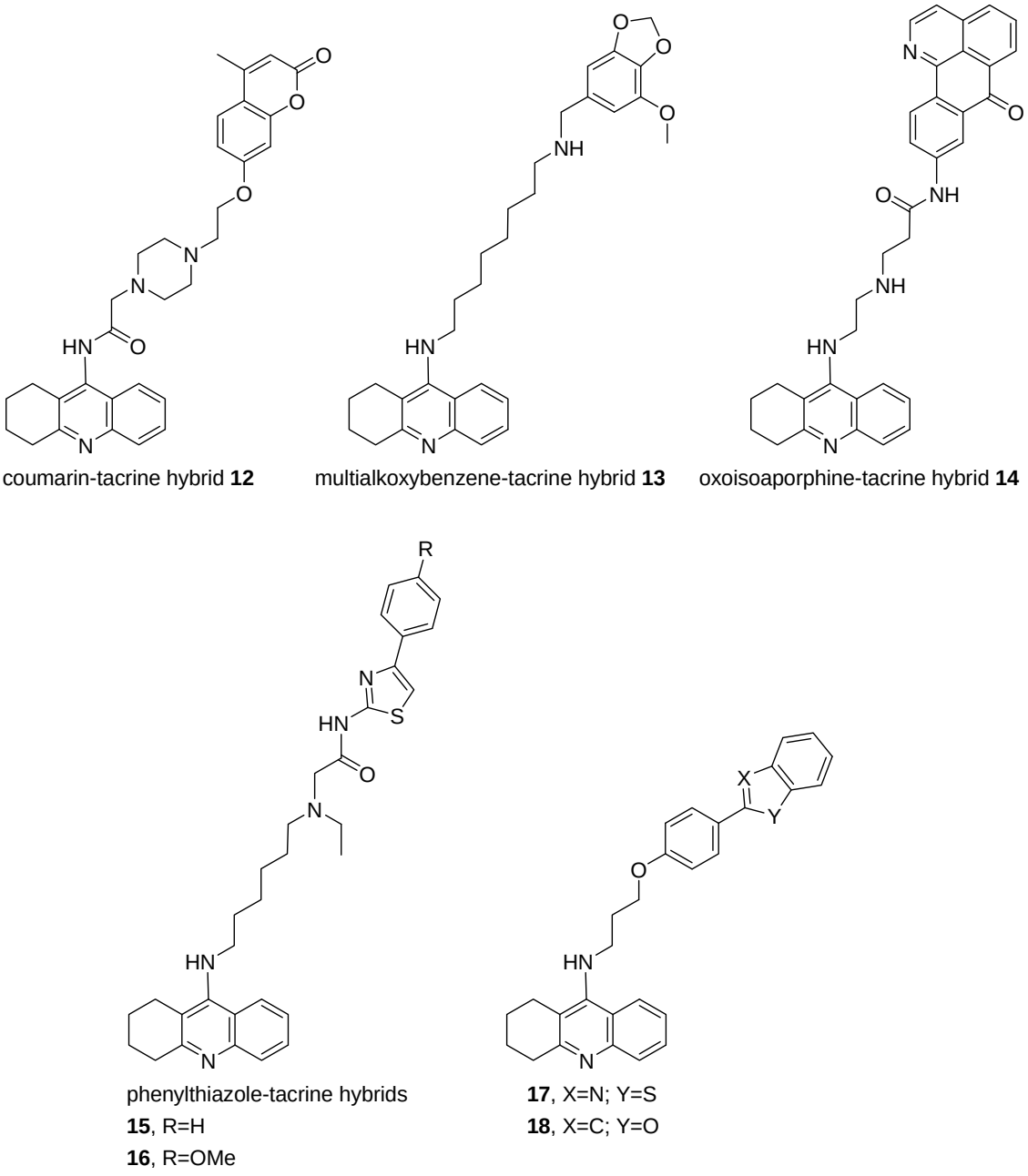



**Fig. (4).** Chemical structures of other tacrine-based hybrids.

On the basis of previous results from screening campaigns pursuing the discovery of Aβ anti-aggregating compounds, which suggested an important role of the methylenedioxybenzene moiety for Aβ anti-aggregating activity, and taking into account that this moiety seemed to be also important for the AChE inhibitory activity of a series of lignans, isolated from *Schizandra chinensis* [79], the group of Huang designed a series of hybrid compounds, such as **13** (Fig. **4**), which contained a tacrine unit linked to a methylenedioxybenzene or related multialkoxybenzene moieties. These multialkoxybenzene-tacrine hybrids were expected to act as dual binding site AChEIs with both AChE and Aβ aggregation inhibitory activities [80]. Both, kinetic and molecular docking studies suggested a dual site binding of **13** to AChE, with the tacrine unit occupying the CAS of AChE and the methylenedioxybenzene ring stacked against the characteristic Trp279 PAS residue (*Torpedo californica* AChE (*Tc*AChE) numbering). As expected from this binding mode, compound **13** exhibited a potent inhibitory activity of eeAChE ($IC_{50}$ 7.98 nM), it being equipotent toward equine BChE ($IC_{50}$ 7.94 nM). Also, **13**, at 20 μM and with a ratio [I]:[Aβ42] 1:1, inhibited Aβ42 self-aggregation by 65%. Worthy of note, compound **13** was more potent that the reference compounds for AChE and Aβ aggregation inhibition, tacrine ($IC_{50}$ eeAChE 193 nM, in the same assay conditions) and curcumin (52% inhibition of Aβ42 aggregation), respectively.

Starting from the knowledge that synthetic derivatives of oxoisoaporphine displayed a high AChE inhibitory activity, arising from an interaction of the 1-azabenzanthrone moiety with the PAS [81], Tang *et al.* designed a series of novel dual binding site AChEIs bearing a tacrine unit for the interaction with CAS and an oxoisoaporphine moiety for the interaction with PAS [82]. Among these compounds, **14** (Fig. **4**) emerged as a potent inhibitor of eeAChE ($IC_{50}$ 3.4 nM) and equine BChE ($IC_{50}$ 110 nM), as well as a potent inhibitor of both AChE-induced aggregation of Aβ40 (83% inhibition at 100 μM) and self-induced aggregation of Aβ42 (80% inhibition at 10 μM, with a ratio [I]:[Aβ42] 1:2), it being more potent than tacrine for eeAChE inhibition ($IC_{50}$ 104 nM, in the same assay conditions) and curcumin for AChE- and self-induced Aβ aggregation inhibition (35% and 42% inhibition, respectively, in the same assay conditions), but less potent than Congo red for inhibition of AChE-induced Aβ40 aggregation (97% inhibition) [82]. Disappointingly, an uncertain BBB permeation was predicted for the oxoisoaporphine-tacrine hybrid **14** in the PAMPA-BBB assay, thereby challenging its potential interest for AD treatment.

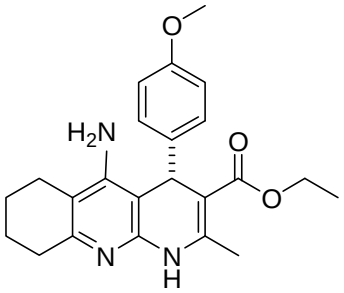
Because the phenylthiazole scaffold is present in compounds with inhibitory activity against the aggregation of tau protein [83], the group of Chen inferred that it might also afford Aβ anti-aggregating activity, and conjugated it with a unit of tacrine through different linkers [84]. The 2-amino-4-

phenylthiazole-tacrine hybrids **15** and **16** (Fig. **4**) emerged as multi-target compounds endowed with potent eeAChE inhibitory activity (81 nM and 158 nM, respectively), and moderately potent equine BChE inhibitory activity (467 nM and 588 nM, respectively), and, as expected, a significant inhibition of Aβ42 self-aggregation (70% and 72% inhibition at 20 μM, with a ratio [I]:[Aβ42] 1:1) [84]. Even though these compounds were less potent cholinesterase inhibitors than the parent tacrine (64 nM and 3 nM for eeAChE and equine BChE inhibition, respectively, in the same assay conditions), they were more potent Aβ42 aggregation inhibitors than the reference compound propidium iodide and the monomeric 2-amino-4-phenylthiazole and 2-amino-4-[(4-methoxy)phenyl]thiazole (57%, 11%, and 12% inhibition at 20 μM, respectively). Interestingly, the Aβ42 anti-aggregating activity of compound **16** was confirmed by atomic force microscopy.

The thiazole and other related heteroaromatic rings in the form of benzofused systems were also conjugated to tacrine by the group of He and Li in the search for multi-target anti-Alzheimer agents [85]. Compounds **17** and **18** turned out to be potent inhibitors of eeAChE ($IC_{50}$ 17 nM and 58 nM, respectively) and equine BChE ($IC_{50}$ 122 nM and 156 nM, respectively) and moderately potent inhibitors of Aβ42 self-aggregation (52% and 63% inhibition at 20 μM, respectively, with a ratio [I]:[Aβ42] 2:5), they being more potent AChEIs than the parent tacrine ($IC_{50}$ 311 nM, in the same assay conditions) and with similar Aβ42 aggregation inhibitory activity than the reference compound curcumin (52% inhibition at 20 μM, $IC_{50}$ 18.7 μM). Molecular docking experiments suggested that the tacrine-based hybrid **17** can simultaneously bind to both CAS (through the tacrine moiety) and PAS (through the benzothiazole moiety) of AChE, but its potential effect on the aggregation of Aβ induced by AChE was not determined [85].

The group of Marco-Contelles also used the structure of tacrine for the design of novel multi-target hybrid compounds, but in contrast with most approaches where tacrine was conjugated to a second pharmacophoric moiety through different linkers, they merged the structure of tacrine with that of the calcium channel blocker dihydropyridine drug nimodipine, which resulted in the design of the so-called tacripyrines, which keep the pharmacological activities of the parent compounds, i.e. AChE inhibitory activity and calcium channel blocking activity, and show neuroprotective and antioxidant properties [86,87]. Recently, this group prepared the methoxytacripyrine (*S*)-**19** (Fig. **5**) and its enantiomer by chromatographic resolution of the racemic compound [88]. According to kinetic studies, both enantiomers are mixed-type inhibitors of AChE, as suggested also by molecular docking studies, which

predicted different energetically favorable binding modes for both enantiomers of **19** either at the CAS of AChE or at the PAS. The (*S*)-enantiomer turned out to be more potent inhibitor of eeAChE ($K_I$ 16 nM vs 125 nM for the (*R*)-enantiomer) but both enantiomers were roughly equipotent for inhibiton of the AChE-induced aggregation of Aβ40 (29% and 25% inhibition at 100 μM, for (*S*)-**19** and (*R*)-**19**, respectively) and self-induced aggregation of Aβ42 (89% and 84% inhibition at 50 μM, and with a ratio [I]:[Aβ42] 1:1, for (*S*)-**19** and (*R*)-**19**, respectively, $IC_{50}$ 33 and 30 μM, respectively). Worthy of note, the ability of these compounds to inhibit amyloid fibril formation was confirmed by transmission electron microscopy (TEM). Curiously, despite the very similar *in vitro* biological profile of (*S*)-**19** and (*R*)-**19**, only the (*S*)-enantiomer was found to be able to protect neuroblastoma SH-SY5Y cells against the cytotoxicity induced by a short Aβ fragment (Aβ25-35) [88].




methoxytacripyrine (*S*)-**19**

**Fig. (5).** Chemical structure of the tacrine-dihydropyridine hybrid (*S*)-**19**.

**Memoquin derivatives**

Other dual inhibitors of AChE and Aβ aggregation have been recently developed from scaffolds other than tacrine. Memoquin (**20**, Fig. **6**) is a very promising multi-target anti-Alzheimer compound which was designed some years ago by the group of Bolognesi by inserting the antioxidant benzoquinone moiety of ubiquinone into a polyamine scaffold that displayed AChE inhibitory activity [89,90]. A comprehensive *in vitro* biological characterization has shown that memoquin is able to potently interact with a number of important targets for AD treatment, namely, AChE and oxidative processes, as the parent compounds from which it was designed, but also BACE-1, and Aβ aggregation. More interestingly, a proof-of-concept in a mouse model of AD showed that memoquin can positively affect the mechanisms behind neurodegeneration, thereby leading to cognitive enhancement [89,90]. In the light of the great potential of

memoquin as a disease-modifying anti-Alzheimer drug candidate, further studies have been carried out by the same group around the structure of memoquin to shed more light on the structure-activity relationships of this structural family and, eventually, to perform a lead optimization process. Thus, because the PAS and CAS of AChE are rich in hydrophobic amino acid residues, the group of Bolognesi studied the effect of the insertion of a methyl substituent at position α of the terminal benzylamino groups of memoquin on its multi-target profile [91], mainly with the hope that the additional methyl groups would lead to more tight interactions at both the CAS and PAS of AChE, thereby increasing the inhibitory activities against the catalytic and the Aβ pro-aggregating action of AChE. Indeed, the (*R*,*R*)-stereoisomer (**21**, Fig. **6**) exhibited a 3-fold increased inhibitory activity toward both processes (Table **2**), while retaining the inhibitory activity of memoquin against Aβ42 self-aggregation and hBChE (memoquin: $IC_{50}$ 1440 nM; **21**: 1480 nM) [91]. Worthy of note, the *meso* (*R*,*S*)-stereoisomer exhibited similar potencies than **21** against the different tested targets.

As previously mentioned, the high hindrance of some dimeric or hybrid AChEIs enable a better fit into the large substrate binding site of BACE-1, and as a result, some of these compounds exhibit significant BACE-1 inhibitory activities. However, the high molecular weight of such compounds might compromise membrane penetration, including BBB penetration. Even if it was not the case for memoquin, for which oral bioavailability and brain penetration had been confirmed [92], a series of memoquin analogs with lower molecular weights, and potentially better pharmacokinetic profile, were designed from memoquin through a disjunctive approach, i.e. by simplification of the structure of memoquin essentially by removing one of its 2-methoxybenzylaminoalkylamino chains [93]. The most interesting monomeric derivative of memoquin, **22** (Fig. **6**), seems to keep the multi-target profile of memoquin, albeit with lower potencies. Molecular docking studies predicted a dual site binding of **22** to AChE, like the parent memoquin, which was in agreement with the high potency for its hAChE inhibition (Table **2**), although 6-fold lower than that of the dimeric memoquin. Also, lower, but still significant, potencies for inhibition of AChE-induced and self-induced Aβ aggregation and for BACE-1 inhibition (memoquin: >80% inhibition at 3 μM; **22**: 60% inhibition at 3 μM) were found for **22** relative to memoquin [93]. Worthy of note, compound **22**, which was less toxic than memoquin to primary chicken telencephalon neurons at a concentration up to 50 μM, inhibited the secretion of Aβ38, Aβ40, and Aβ42 in these cells with $IC_{50}$ values of 19, 21, and 46 μM, respectively [93].

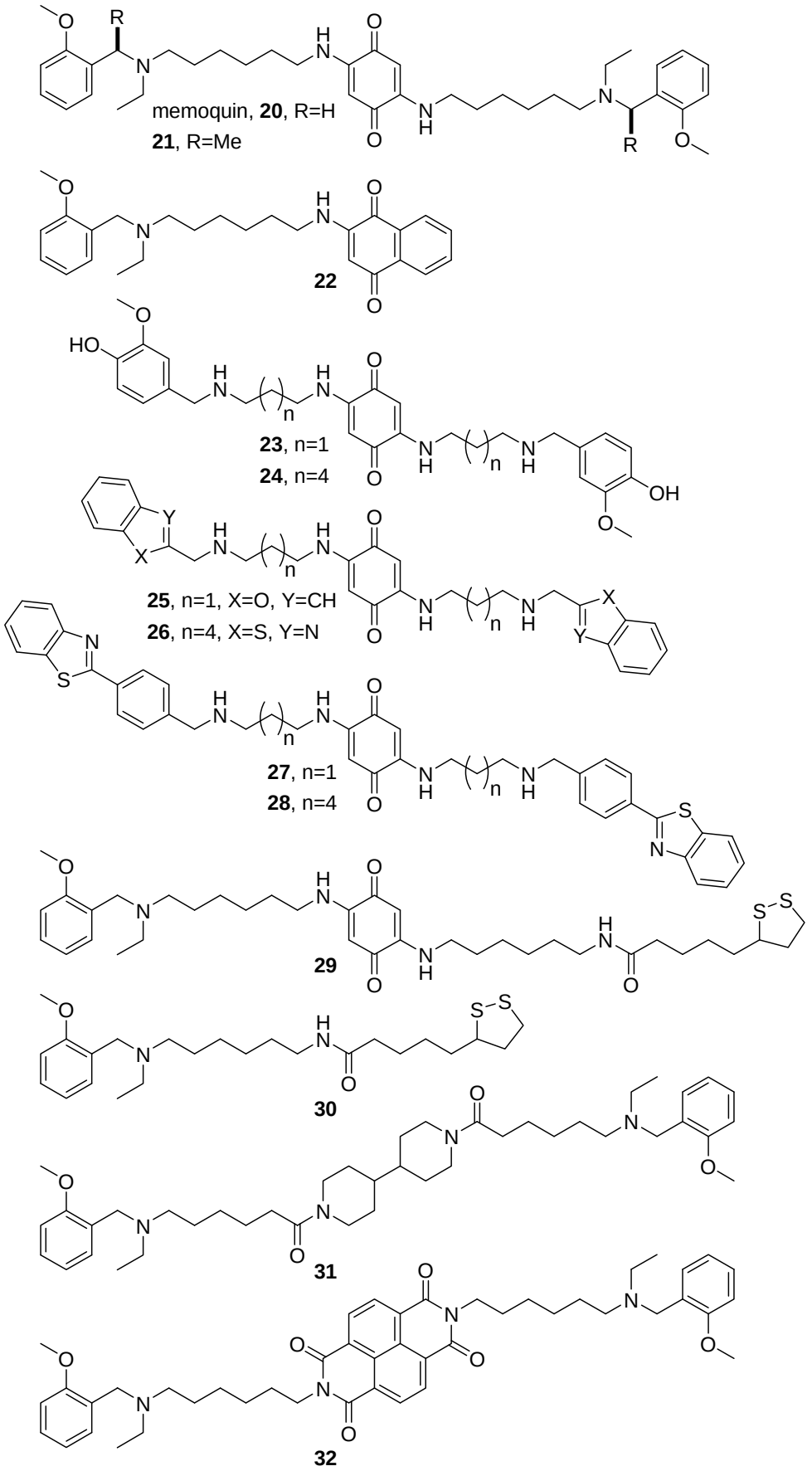



**Fig. (6).** Chemical structures of memoquin and derivatives thereof.

**Table 2. *In Vitro* Dual Inhibitory Activities of Memoquin Derivatives Against AChE and Aβ Aggregation**

| Compound | hAChE ($IC_{50}$ nM) | AChE-induced Aβ40 aggregation (% inhibition at 100 μM[a] and/or $IC_{50}$ μM) | self-induced Aβ42 aggregation (% inhibition at 10 μM[b] and/or $IC_{50}$ μM) |
|---|---|---|---|
| memoquin, **20** | 1.55 | 87 / $IC_{50}$ 28.3 μM | 67 |
| **21** | 0.50 | $IC_{50}$ 9.34 μM | 64 |
| **22** | 9.73 | 69 / $IC_{50}$ 45 μM | 27 |
| **23** | 198 | | 22 |
| **24** | 102 | | 50 |
| **25** | 21800 | | 27 |
| **26** | 22000 | | 19 |
| **27** | 31400 | | 41 |
| **28** | 305 | | 26 |
| **29** | 100 | | 45 |
| **30** | 256 | 17 | |
| **31** | 3.32 | 41 | 14 |
| **32** | 0.37 | >90 / $IC_{50}$ 8.13 μM | 55 / $IC_{50}$ 9.69 μM |

[a] [I]=100 μM, [Aβ40]=230 μM, [AChE]=2.30 μM, Aβ40:AChE ratio 100:1. [b] [I]=10 μM, [I]:[Aβ42] ratio 1:5.

Notwithstanding the promising multi-target profile of memoquin, its activities against AChE (low nanomolar range) and Aβ (micromolar range) are not well balanced. In order to discover memoquin analogs with more balanced multi-target profile, and with the assumption that the planar 2,5-diaminobenzoquinone moiety of memoquin might play a pivotal role in the modulation of protein-protein interactions, the terminal 2-methoxybenzyl groups of memoquin were replaced with structural moieties from compounds with known amyloid binding abitity, such as the vanillic ring (as in compounds **23** and **24**, Fig. **6**), benzofuran moieties (as in compound **25**), benzothiazole system (as in compound **26**) or 2-

phenylbenzothiazole fragment (as in compounds **27** and **28**) [94]. These structural changes resulted in a very important drop of hAChE inhibitory activity from the nanomolar to the micromolar or submicromolar range and in a slightly decreased Aβ42 anti-aggregating activity (Table **2**), thereby affording weaker but more balanced multi-target lead compounds [94]. Interestingly, compound **23** at 10 μM elicited a protective effect against the neurotoxicity induced by Aβ42 in neuroblastoma SH-SY5Y cells similar to that of memoquin (approx. 80% inhibition of neurotoxicity).

The 2-methoxybenzyl group of memoquin has also been replaced by a unit of the antioxidant lipoic acid, with the expectation of deriving compounds with multiple antioxidant mechanisms, of interest for the treatment of AD [95]. The memoquin-lipoic acid hybrid **29** (Fig. **6**) at 10 μM significantly decreased the ROS production in bovine heart submitochondrial particles and in human glioma cell line T67, and retained the dual inhibitory effect of memoquin against hAChE and Aβ42 aggregation, albeit with lower potencies, thereby resulting again in a more balanced multi-target profile (Table **2**) [95]. Further removal of the aminohexylaminobenzoquinone fragment of memoquin and direct linkage of lipoic acid to the remaining *N*-ethyl-*N*-(2-methoxybenzyl)-1,6-hexanediamine moiety led to compound **30** (Fig. **6**), which significantly decreased ROS production in SH-SY5Y cells, from 82% of increased intracellular ROS induced by *tert*-butyl hydroperoxide to 70% at 10 μM or to 38% at 50 μM [96]. Compound **30** also potently inhibits hAChE (Table **2**) but again with clearly lower potency than memoquin, and had a very weak inhibitory activity against the AChE-induced Aβ40 aggregation [96].

Based on previous studies by Tumiatti *et al.* on dimeric polyamine based AChEIs containing *N*-ethyl-*N*-(2-methoxybenzyl)aminoalkyl moieties similar to those of memoquin, which suggested that increasing rigidity within the linker, as in compound **31** (Fig. **6**), increased Aβ anti-aggregating activity relative to flexible oligomethylene-linked counterparts [97], and taking into account that aromatic systems may enable additional interactions with aromatic amino acid residues at mid-gorge of AChE as well as inhibition of Aβ self-aggregation, the dipiperidino moiety of **31** was replaced by still more constrained cyclic systems as the 1,4,5,8-naphthalenetetracarboxylic diimide moiety of the novel analog **32** (Fig. **6**) [98]. Indeed, as suggested by kinetic and docking simulations, compound **32** was able to simultaneously interact with CAS, PAS and mid-gorge aromatic residues, this three-site binding mode accounting for its higher hAChE and AChE-induced Aβ40 aggregation inhibitory activities relative to the potent AChEI **31** (Table **2**). Also, the insertion of the aromatic system in the linker of **32**, as expected, endowed the compound with a significant inhibitory activity toward Aβ42 self-aggregation, in contrast with **31**, which

was essentially inactive for this activity [98]. Worthy of note, the Aβ anti-aggregating activity of **32** was in the same range than that of propidium and only slightly lower than that of Congo red [98].

**Natural products and natural-based compounds**

A number of dual inhibitors of AChE and Aβ aggregation are natural products or natural-based compounds. The structure of the isoquinoline alkaloid berberine, **33** (Fig. **7**), with known AChE inhibitory activity [99], has been linked to different (hetero)aromatic systems. Thus, the group of He and Li has synthesized series of berberine-based hybrids substituted at position 9 of the berberine scaffold with a linker that connects it to a phenylbenzoheterocyclic system, as in compounds **34** and **35** (Fig. **7**) [85], to a triazole ring, as in compounds **36** and **37** [100], to a benzenediol ring, as in compounds **38** and **39** [101], to a melatonin moiety, as in compound **40** [101], or to a ferulic acid unit, as in compound **41** [101]. Most berberine-based hybrids were less potent eeAChEIs than berberine, with $IC_{50}$ values in the micromolar or submicromolar range, with the exceptions of compounds **36**-**38** (Table **3**). Precisely, a dual site binding to AChE has been predicted by molecular docking experiments for compound **38**, which seems to position its berberine moiety at the PAS and the triazole ring at the CAS of AChE [100], which might account for its higher AChE inhibitory activity relative to berberine. Conversely, all of these hybrids turned out to be more potent inhibitors of Aβ42 self-aggregation than berberine and another reference compound such as curcumin (52% inhibition of Aβ42 aggregation at 20 μM, $IC_{50}$ 18.7 μM). Worthy of note, in the case of the berberine-based hybrids bearing a polyphenol moiety (**38**, **39**, **41**) or a melatonin unit (**40**), the antioxidant activity of these scaffolds was preserved in the hybrids, which exhibited oxygen radical absorbance capacity (ORAC) values ranging from 3.4 to 9.5 Trolox equivalents, clearly higher than that of the parent berberine (0.4 Trolox equivalents) [101].

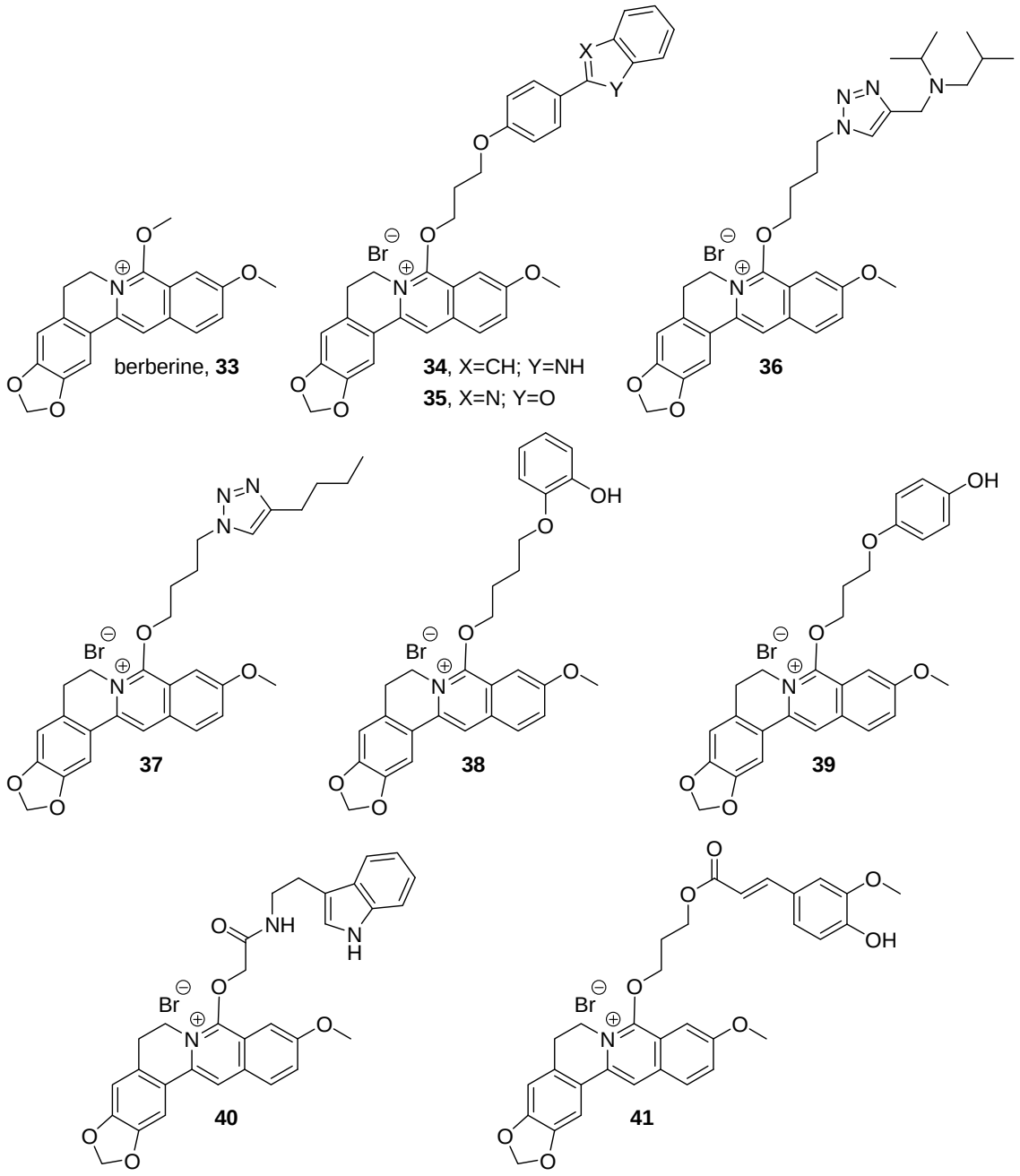



**Fig. (7).** Chemical structures of berberine and derivatives thereof.

**Table 3. *In Vitro* Dual Inhibitory Activities of Berberine Derivatives Against AChE and Aβ Aggregation**

| Compound | eeAChE ($IC_{50}$ nM) | self-induced Aβ42 aggregation (% inhibition at 20 μM[a] and/or $IC_{50}$ μM) |
|---|---|---|
| Berberine, **33** | 374 | 36 |
| **34** | 774 | $IC_{50}$ 4.69 μM |
| **35** | 2120 | $IC_{50}$ 3.75 μM |
| **36** | 44 | 53 |
| **37** | 201 | 78 |
| **38** | 123 | 85 |
| **39** | 460 | 92 |
| **40** | 1110 | 83 |
| **41** | 3210 | 76 |

[a] [I]=20 μM, [I]:[Aβ42] ratio 2:5.

The scaffold of oxoisoaporphine, which is present in the hybrid **14** (Fig. **4**), has been used by the same group in the synthesis of a series of oxoisoaporphine derivatives substituted at position 10 with different basic side chains [102]. Among these derivatives, compounds **42** and **43** (Fig. **8**) emerged as potent inhibitors of eeAChE ($IC_{50}$ 720 nM and 210 nM, respectively) and AChE-induced aggregation of Aβ40 (**42**: 88% and 61% of inhibition at 100 μM and 10 μM, respectively; **43**: 94% and 74% of inhibition at 100 μM and 10 μM, respectively), and also moderately potent inhibitors of equine BChE ($IC_{50}$ 1.13 μM and 2.81 μM, respectively). These compounds were less potent cholinesterase inhibitors than the reference compound tacrine ($IC_{50}$ eeAChE 130 nM, $IC_{50}$ equine BChE 21 nM, in the same assay conditions) and more potent inhibitors of the AChE-induced aggregation of Aβ40 than the PAS inhibitor propidium (85% and 40% inhibition at 100 μM and 10 μM, respectively), but less potent than Congo red (97% inhibition at 100 μM and 10 μM). As in the case of propidium, the potent inhibitory activity of these oxoisoaporphine derivatives seems to arise from interaction with the PAS of AChE, as suggested by kinetic studies. Despite the close structural similarity between compounds **42** and **43**, the former was

predicted by the PAMPA-BBB assay to be able to cross the BBB, whereas surprisingly a low BBB permeation was predicted for its upper homolog **43** [102].

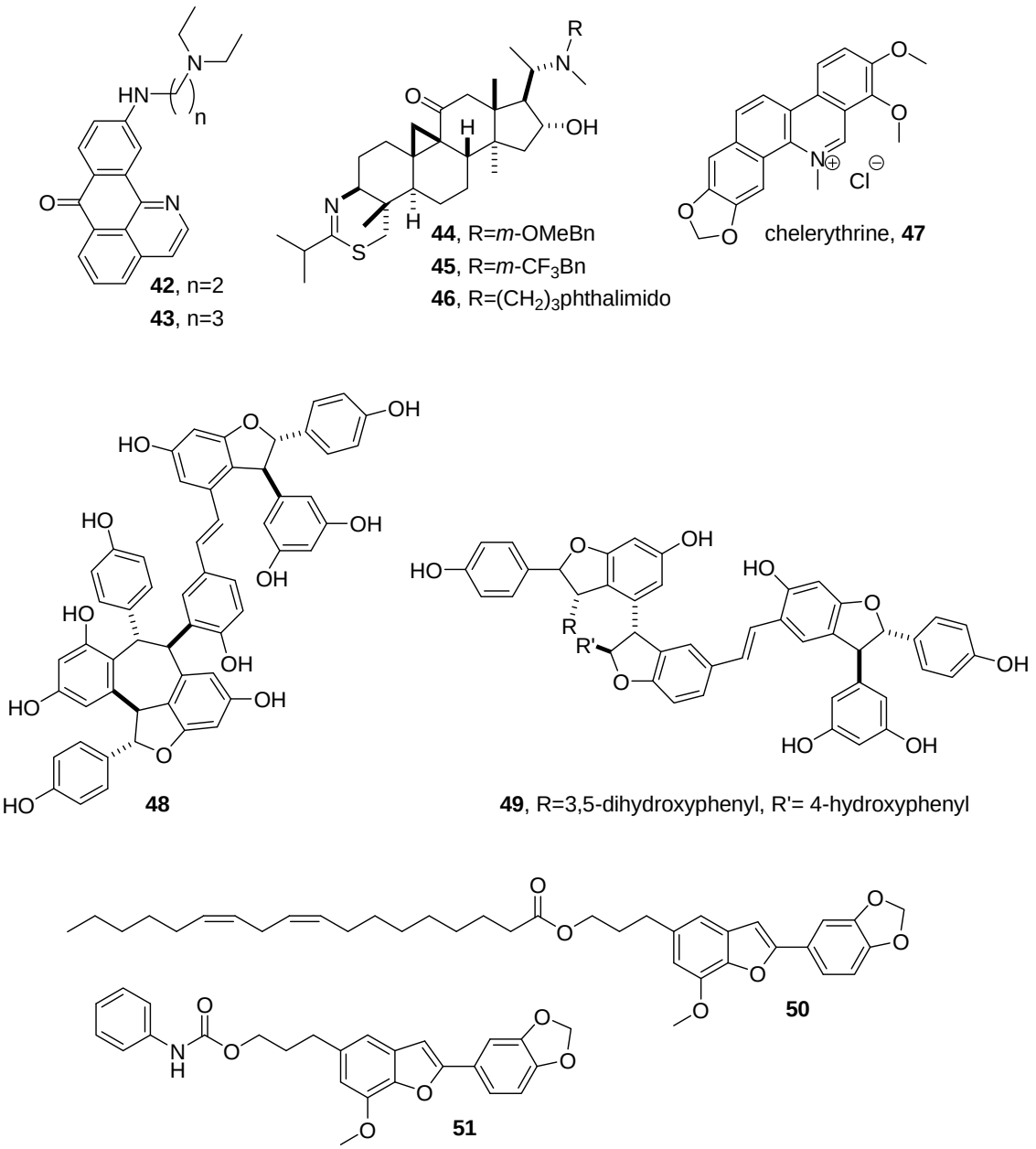



**Fig. (8).** Chemical structures of other natural products and natural-based compounds with dual Aβ aggregation and AChE inhibitory activities.

Following a high-throughput screening campaign of an in-house library of natural products that led to the discovery of a tetracyclic triperpene hit with AChE inhibitory activity [103], the group of Guillou

developed a novel class of analogs incorporating structural modifications to enable an additional interaction with the PAS of AChE, apart from interactions at the CAS, thereby potentially leading to increased AChE inhibitory activity and also to inhibitory activity against the AChE-induced aggregation of Aβ [104]. Indeed, molecular docking and molecular dynamics simulations suggested that one of the novel analogs, compound **44** (Fig. **8**), was able to simultaneously reach the CAS and the PAS of AChE, establishing mainly hydrophobic interactions. This might account for the very potent inhibitory activity of **44** and other analogs such as **45** and **46** against hAChE ($IC_{50}$ 3 nM, 3 nM, and 14 nM, respectively) and for their significant inhibitory activity against the AChE-induced aggregation of Aβ40 (69%, 56%, and 42% inhibition at 100 μM, respectively) [104]. These compounds were clearly more potent hAChEIs than the reference compound tacrine and the initial tetracyclic triterpene hit ($IC_{50}$ 484 nM and 299 nM, respectively, in the same assay conditions) and only slightly less potent inhibitors of the AChE-induced Aβ40 aggregation that the prototypic inhibitor of AChE PAS and of the AChE-induced Aβ aggregation propidium (85% inhibition at 100 μM).

Screening of a commercial library of natural and natural-based compounds for AChE and BChE inhibitory activities allowed the identification by the group of Vuorela of chelerythrine, **47** (Fig. **8**), as a hit compound for hAChE and hBChE inhibition ($IC_{50}$ 1.54 μM and 10.3 μM, respectively) [105]. Kinetic and docking simulations suggested that chelerythrine might bind to the PAS of AChE. Further biological profiling of this compound, showed that chelerythrine exhibits an interesting multiple Aβ anti-aggregating action, as it is able to inhibit the self-aggregation of Aβ40 ($IC_{50}$ 4.20 μM) and the AChE-induced aggregation of Aβ40 (49% at 5 μM, 65% at 10 μM, and 88% at 100 μM), and very interestingly, it is also able to disaggregate preformed Aβ40 aggregates ($IC_{50}$ 13 μM). Thus, chelerythrine emerges as a very promising anti-Alzheimer lead with multiple well balanced activities (all of them in the low micromolar range) [105].

Analogously, in a screening campaign for AChE and BChE inhibitors from natural products, the extract of the roots of *Vitis amurensis* was selected, and two resveratrol oligomers, compounds **48** and **49** (Fig. **8**), were isolated from this active extract [106]. Biological characterization of these compounds confirmed their moderately potent inhibitory activity against hAChE ($IC_{50}$ 1.04 μM and 1.66 μM, respectively) and hBChE ($IC_{50}$ 4.41 μM and 1.75 μM, respectively), they being roughly equipotent to the anti-Alzheimer AChEI drug galantamine ($IC_{50}$ AChE 0.93 μM; $IC_{50}$ BChE 9.24 μM, in the same assay conditions). Interestingly, both **48** and **49** also inhibited the self-aggregation of Aβ42 (61% and 39% at 10 μM,

respectively, with a ratio [I]:[Aβ42] 1:2.5) [106]. Thus, as in the case of chelerythrine, the multiple activities of these compounds, all of them in the low micromolar range, are well balanced, which is an important feature in multi-target drugs.

Also, following a high-throughput screening of a plant extract library for AChE inhibition, an ethyl acetate extract of the fruits of *Styrax agrestis* was found to be able to inhibit AChE [107]. Subsequent bioassay-guided fractionation of this extract led to the isolation of the new egonol-type 2-arylbenzofuran **50** (Fig. **8**), which can inhibit both hAChE ($IC_{50}$ 1.7 μM) and the AChE-induced aggregation of Aβ40 (78% at 100 μM). To expand the structure-activity relationships in this structural family, new egonol derivatives were prepared by semisynthesis from egonol, among which compound **51** (Fig. **8**) displayed the most interesting profile ($IC_{50}$ hAChE 4.5 μM; 87% inhibition of AChE-induced aggregation of Aβ40 at 100 μM). Interestingly, both egonol derivatives were more potent inhibitors of the AChE-induced aggregation of Aβ40 than propidium (54% inhibition at 100 μM, in the same assay conditions), and only slightly less potent hAChEIs than tacrine ($IC_{50}$ 1.03 μM, in the same assay conditions) [107]. Molecular docking and molecular dynamics simulations suggested a simultaneous interaction of this kind of derivatives with both the CAS and PAS of AChE, which might account for their significant dual inhibitory activities.

**2-Arylbenzofuran derivatives**

Other synthetic 2-arylbenzofurans have been also developed for dual inhibition of AChE and Aβ aggregation. The group of Rampa combined the 7-(*N*-methyl-*N*-benzylamino)heptyloxy moiety of some previously developed cholinesterase inhibitors [108] and the 4-(2-furyl)phenoxy fragment of SKF-64346, an inhibitor of Aβ fibril formation [109], in the search for novel dual inhibitors of cholinesterases and Aβ aggregation [110,111]. Among the resulting benzofuran-based hybrids, compounds **52**-**55** (Fig. **9**) emerged as the most interesting derivatives. Compounds **52**-**54** display not very strong but well balanced inhibitory activities against hAChE and the self-induced aggregation of Aβ25-35, a short Aβ peptide that retains the aggregation and neurotoxicity properties, exhibiting $IC_{50}$ values in the low micromolar range, whereas compound **55** is more potent against hAChE and less potent for the Aβ25-35 anti-aggregating effect (Table **4**). Worthy of note, all of these compounds also display a similar micromolar or submicromolar activity against hBChE ($IC_{50}$ 0.28 μM, 38.1 μM, 1.82 μM, and 2.88 μM, respectively) [110,111]. Interestingly, compounds **53** and **55** significantly protected neuroblastoma SH-SY5Y cells

against the neurotoxicity induced by Aβ25-35 (58% and 48% inhibition at 30 μM), this activity likely resulting from the hydrophobic properties of these compounds together with their putative ability to block the interaction of the Aβ peptide with the lipid bilayer of neuronal membranes [110,111]. Thus, it has been hypothesized that the benzoyl group of hybrids **53** and **55** is essential for the interaction with hydrophobic residues of Aβ25-35, particularly Ile31, Ile32, and Met35, that seem to be responsible for the aggregation and neurotoxic properties [112]. Worthy of note, at 30 μM hybrids **53** and **55** were found to be able to reduce the binding of Aβ25-35 to the cell membrane surface of SH-SY5Y cells and to inhibit the ROS formation induced by Aβ25-35, which would seem to be the mechanisms behind the neuroprotective effect of these compounds.

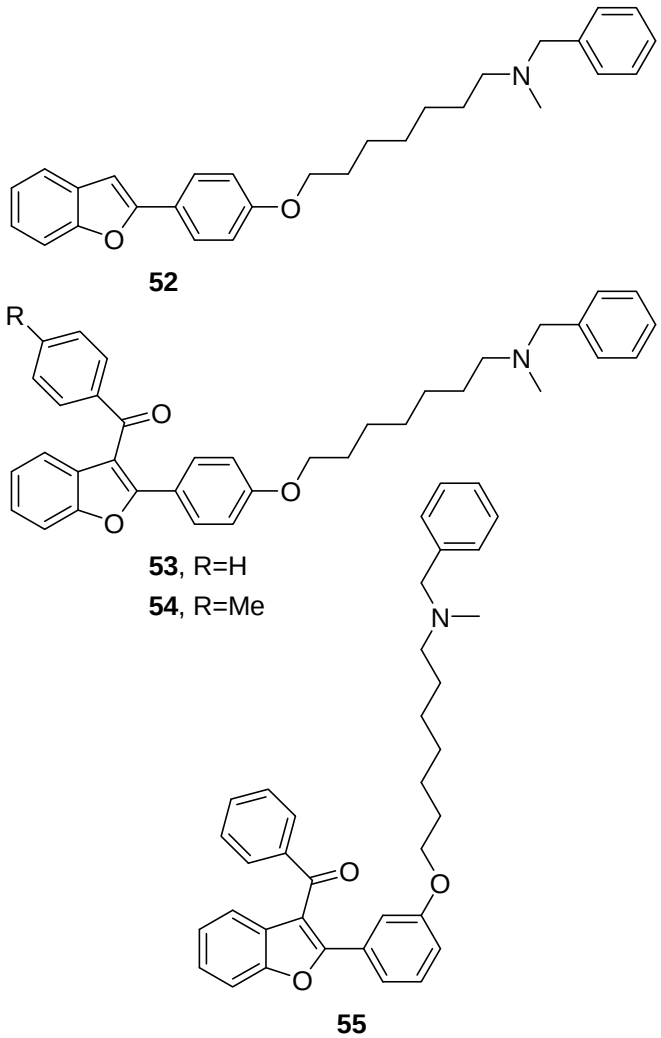



**Fig. (9).** Chemical structures of synthetic 2-arylbenzofuran derivatives.

**Table 4. *In Vitro* Dual Inhibitory Activities of Synthetic Benzofuran-based Hybrid Compounds Against AChE and Aβ Aggregation**

| Compound | hAChE ($IC_{50}$ μM) | self-induced Aβ25-35 aggregation (% inhibition at 10 μM[a] and/or $IC_{50}$ μM) |
|---|---|---|
| **52** | 32.6 | $IC_{50}$ 7.0 μM |
| **53** | 40.7 | 47 / $IC_{50}$ 12.5 μM |
| **54** | 10.5 | $IC_{50}$ 13.0 μM |
| **55** | 0.24 | 35 |

[a] [I]=10 μM, [I]:[Aβ42] ratio 1:10.

**Pyrimidine and triazine derivatives**

The group of Rao has developed a series of pyrimidine derivatives substituted at positions 2 and 4 with groups of different steric and electronic properties as dual inhibitors of AChE and Aβ aggregation [113,114]. Some representative examples of these 2,4-disubstituted pyrimidines are compounds **56-59** (Fig. **10**), which exhibit moderate to weak potencies against hAChE and AChE-induced aggregation of Aβ40 (Table **5**) that might arise from a dual site binding to AChE as suggested by docking simulations with **58** [114]. Also, compound **59** was found to be weakly active as inhibitor of Aβ40 self-aggregation (28% inhibition at 100 μM), and, together with **58**, also active against equine BChE (**58**: $IC_{50}$ 3.9 μM; **59**: $IC_{50}$ 7.6 μM).

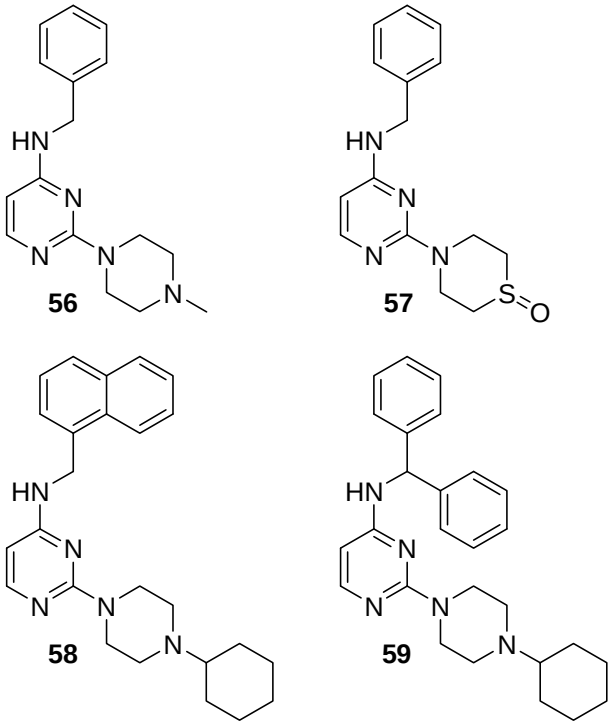



**Fig. (10).** Chemical structures of 2,4-disubstituted pyrimidine derivatives.

**Table 5. *In Vitro* Dual Inhibitory Activities of 2,4-Disubstituted Pyrimidine Derivatives Against AChE and Aβ Aggregation**

| Compound | hAChE ($IC_{50}$ μM) | AChE-induced Aβ40 aggregation (% inhibition at 100 μM) |
|---|---|---|
| **56** | 24.9 | 59 |
| **57** | 12.6 | 56 |
| **58** | 8.0 | 31 |
| **59** | 10.0 | 32 |

Other nitrogen containing heterocycles such as triazines have been also used as the core of dual inhibitors of AChE and Aβ aggregation. The group of Carotti has synthesized series of benzo[*e*][1,2,4]triazin-7(1*H*)-ones, such as **60** (Fig. **11**), and [1,2,4]-triazino[5,6,1-*jk*]carbazol-6-ones, such as **61**, whose quinone/quinonimine and extended planar azaheterocyclic systems were expected to enable π-π stacking,

hydrophobic, and electrostatic interactions with Aβ, thereby leading to an interference with the aggregation process [115]. Indeed, both compounds exhibit a potent inhibitory activity against Aβ40 self-aggregation ($IC_{50}$ 1.4 μM in both cases). Compound **60** also displayed eeAChE and equine BChE inhibitory activities in the same range than its Aβ40 anti-aggregating activity ($IC_{50}$ 1.5 μM and 1.9 μM, respectively), whereas in the case of **61** these additional activities were not so well balanced (55% inhibition of eeAChE at 10 μM and $IC_{50}$ BChE 25 nM) [115]. Worthy of note, further studies on the Aβ anti-aggregating profile of **60** confirmed its ability to inhibit also the self-aggregation of Aβ42 and suggested the stabilization of random coil arranged unstructured peptide as the mechanism underlying its inhibitory activity [115].

Incorporation into a s*ym*-triazine core of acetylcholine substrate analogs and multiple hydrophobic phenyl rings, which might improve β-sheet intercalation into amyloid aggregates, has been used by the group of Kerman as strategies to confer both cholinesterases and Aβ inhibitory activities [116]. Indeed, triazine derivatives **62**-**64** (Fig. **11**) were found to be able to inhibit hAChE ($IC_{50}$ 9.7 μM, 0.3 μM, and 2.8 μM, respectively) and Aβ40 self-aggregation (66%, 51%, and 72% at 100 μM, with a ratio [I]:[Aβ40] 1:1). Also, the most hindered compounds **63** and **64** turned out to be moderately potent inhibitors of hBChE ($IC_{50}$ 3.9 μM and 15.3 μM, respectively). TEM studies confirmed the Aβ anti-aggregating activity of triazines **62**-**64**, which seemed to lead to the formation of globular aggregates instead of fibrillar structures [116]. Moreover, compounds **63** and **64** were nontoxic to neuroblastoma SH-SY5Y cells at a concentration as high as 400 μM.

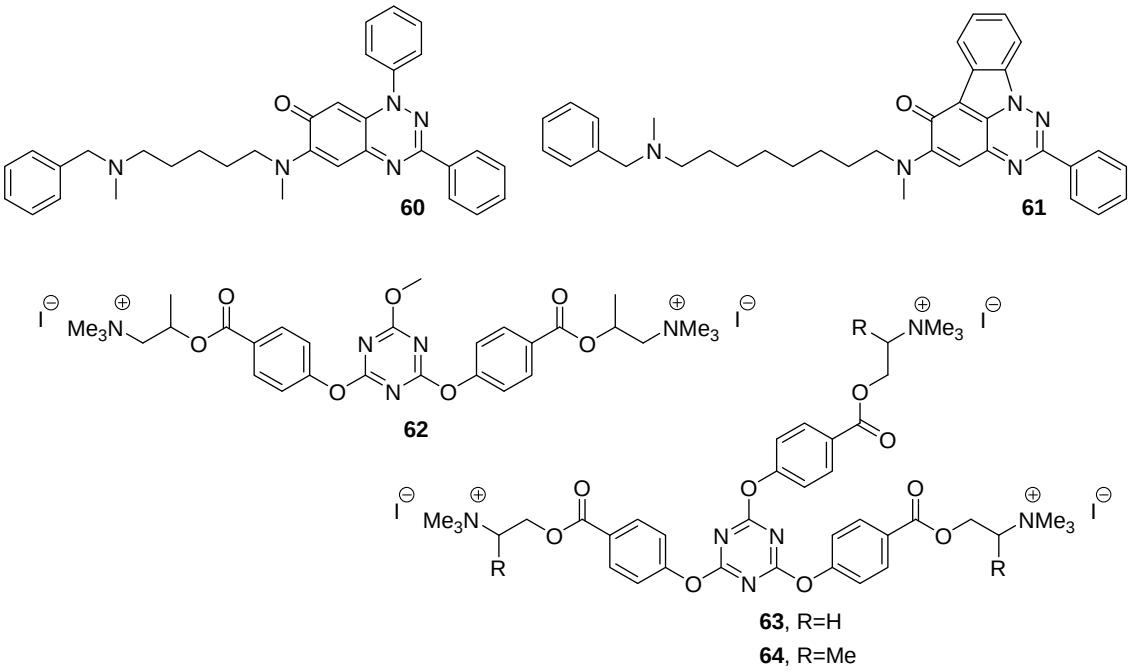



**Fig. (11).** Chemical structures of triazine derivatives.

**Donepezil-related compounds**

Donepezil, **3**, is the sole marketed dual binding site AChEI, with a proven, albeit weak, inhibitory activity against the AChE-induced Aβ aggregation [35,55]. Several series of dual inhibitors of AChE and Aβ aggregation have been designed from the structure of donepezil. The group of Muñoz-Torrero developed some donepezil-huprine hybrids bearing a donepezil-like 5,6-dimethoxy-2-[(4-piperidinyl)methyl]indane moiety [117], in which the indanone of donepezil had been replaced by an indane system on the basis of previous studies on a family of donepezil-tacrine hybrids where this replacement resulted in increased inhibitory activity against the AChE-induced aggregation of Aβ40 [118]. A better dual site binding to AChE of these compounds relative to donepezil, as suggested by molecular dynamics simulations and kinetic studies, accounted for their improved dual inhibitory potencies. The most interesting compound of the series was the hybrid (–)-(7*S*,11*S*)-**65** (Fig. **12**, Table **6**), which additionally was found to be a moderately potent inhibitor of hBChE ($IC_{50}$ 349 nM) and BACE-1 ($IC_{50}$ 11.0 μM) [117]. Moreover, this compound was predicted to be able to cross BBB in a PAMPA-BBB assay, and more importantly, it was confirmed to penetrate into the brain of OF1 mice (unpublished results). Despite the multi-target profile of compound **65**, a proof-of-concept study in a transgenic mouse model of AD was not completely satisfactory, as this compound was able to improve cognition, likely by a purely cholinergic effect, but

not amyloid pathology (unpublished results), which likely reflects the significant difference in its potencies against cholinesterases (nanomolar range) and Aβ (micromolar range), thereby highlighting the importance for multi-target compounds of having well balanced activities against the different targets.

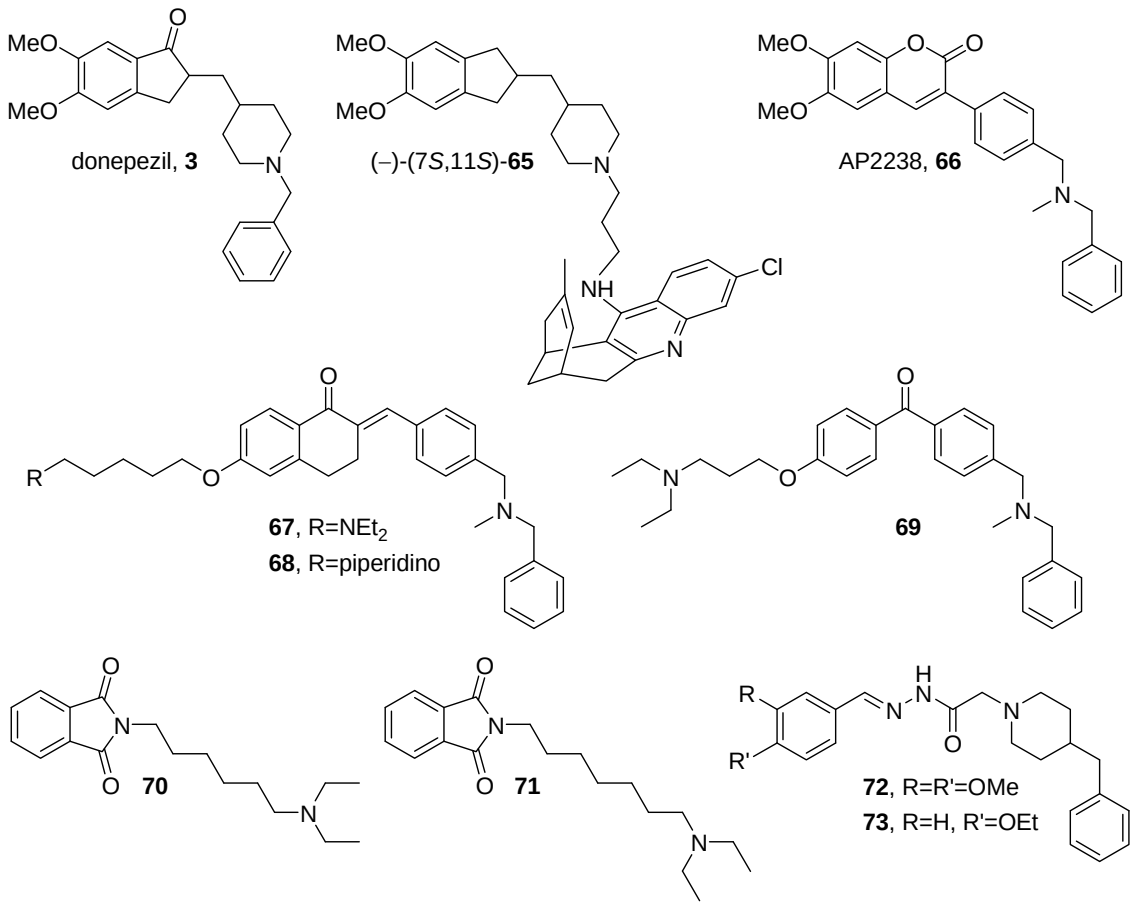



**Fig. (12).** Chemical structures of donepezil-related compounds.

**Table 6. *In Vitro* Dual Inhibitory Activities of Donepezil-Related Compounds Against AChE and Aβ Aggregation**

| Compound | hAChE ($IC_{50}$ nM) | AChE-induced Aβ40 aggregation (% inhibition at 100 μM[a] and/or $IC_{50}$ μM) | self-induced Aβ42 aggregation (% inhibition at 10 μM[b] and/or $IC_{50}$ μM) |
|---|---|---|---|
| Donepezil, **3** | 21.4 | 22 | <5 |
| **65** | 2.61 | 42 | 29 |
| AP2238, **66** | 44 | 35 | <5 |
| **67** | 56 | 43 | 26 |
| **68** | 52 | 48 | 27 |
| **69** | 25 | 34 | |

[a] [I]=100 μM, [Aβ40]=230 μM, [AChE]=2.30 μM, [Aβ40]:[AChE] ratio 100:1. [b] [I]=10 μM, [I]:[Aβ42] ratio 1:5.

The group of Rampa has developed a series of hybrid compounds that conjugate the (*N*-benzyl-*N*-methylamino)benzyl moiety of AP2238, **66** (Fig. **12**), the first dual binding site AChEI purposely designed to hit both AChE and AChE-induced Aβ aggregation [119], and a tetralone moiety, structurally related to the indanone system of donepezil [120]. The most interesting hybrids, **67** and **68**, which seemed to be also dual binding site AChEIs according to kinetic studies, exhibited slightly lower AChE inhibitory potencies but increased Aβ anti-aggregating effects than the parent compounds (Table **6**), as well as significant hBChE inhibitory activities ($IC_{50}$ 8.05 μM and 5.01 μM, respectively). The same group developed some hybrid compounds that merged the (*N*-benzyl-*N*-methylamino)benzyl moiety of AP2238 with another moiety structurally related to the indanone system of donepezil, namely a dimethoxybenzophenone scaffold [121]. Because the initial lead compound of this series was devoid of inhibitory activity against the AChE-induced aggregation of Aβ, further modifications were carried out to increase this activity, which consisted of introducing at the benzophenone moiety aminoalkyl chains that might mimic the role of the diethylmethylammonium alkyl chain of the prototypic inhibitor of AChE PAS and, hence, of AChE-induced Aβ aggregation propidium [122]. Indeed, the most interesting compound of the series, **69** (Fig. **12**), exhibited improved hAChE and AChE-induced Aβ40 aggregation

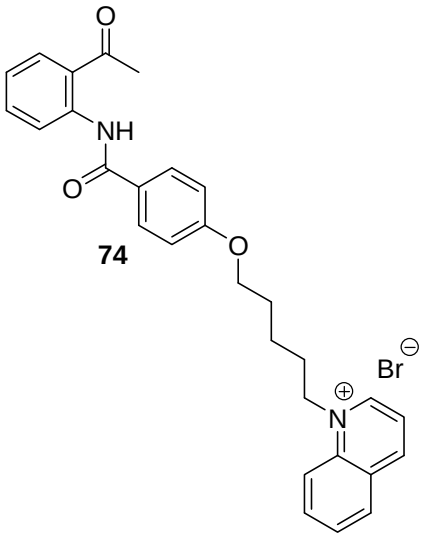
inhibitory potencies than the initial lead, it being equipotent to donepezil for hAChE inhibition but clearly less potent than propidium for inhibition of AChE-induced Aβ aggregation (Table **6**) [122], as the other previously mentioned donepezil-related derivatives.

The group of Malawska has used another system structurally related to the indanone ring of donepezil, namely an isoindoline-1,3-dione scaffold, as the core of a series of dual inhibitors of AChE and Aβ aggregation [123]. The most interesting compounds, **70** and **71** (Fig. **12**), exhibit a significant inhibitory activity against eeAChE ($IC_{50}$ 1.2 μM and 1.1 μM, respectively) and against the self-aggregation of a short Aβ peptide (34% and 39% inhibition at 80 μM, respectively, with a ratio [I]:[Aβ] 1:1.25) [123].

The structure of donepezil has also inspired the design by the group of Ozadali of a series of acylhydrazones which incorporated the dimethoxybenzene and benzylpiperidine moieties of donepezil or related systems [124]. Compounds **72** and **73** (Fig. **12**), as representative examples of this series, are rather weak inhibitors of hAChE ($IC_{50}$ 63 μM and 53 μM, respectively) and equine BChE ($IC_{50}$ 55 μM and 67 μM, respectively) but exhibit balanced inhibitory activity toward self-induced aggregation of both Aβ40 and Aβ42 (70-80% inhibition at 100 μM) [124].

**Benzamide derivatives**

The group of Yang and Yang has reported the synthesis of a series of benzamides linked to a quinoline and to an acetophenone moieties, rationally designed as dual binding site AChEIs [125]. Compound **74** (Fig. **13**) was indeed predicted by docking simulations to be able to simultaneously reach both CAS and PAS of AChE. This compound exhibit potent inhibitory activities against hAChE ($K_i$ 6.50 nM) and hBChE ($K_i$ 55 nM) but lower inhibitory activities toward Aβ42 self-aggregation ($IC_{50}$ 79 μM) and BACE-1 ($IC_{50}$ 85 μM) [125].




**74**

**Fig. (13).** Chemical structure of the benzamide derivative **74**.

**Carbamate derivatives**

Most research endeavors on the discovery of novel anti-cholinesterase agents pursue reversible inhibitors. The group of Rampa has been involved in the development of pseudoirreversible AChEIs with additional activity against Aβ aggregation [126]. Structural modification of an initial lead, xanthostigmine [127], had led to the design of novel phenylcoumarin and flavone derivatives, such as **75** and **76**, respectively (Fig. **14**) [126]. Docking and molecular dynamics simulations suggested a dual site binding to AChE for these compounds, which accordingly exhibited potent inhibitory activity against hAChE ($IC_{50}$ 1.20 nM and 0.73 nM), although unexpectedly quite weak AChE-induced Aβ40 aggregation inhibitory activity (13% and 18% inhibition at 100 μM, respectively).

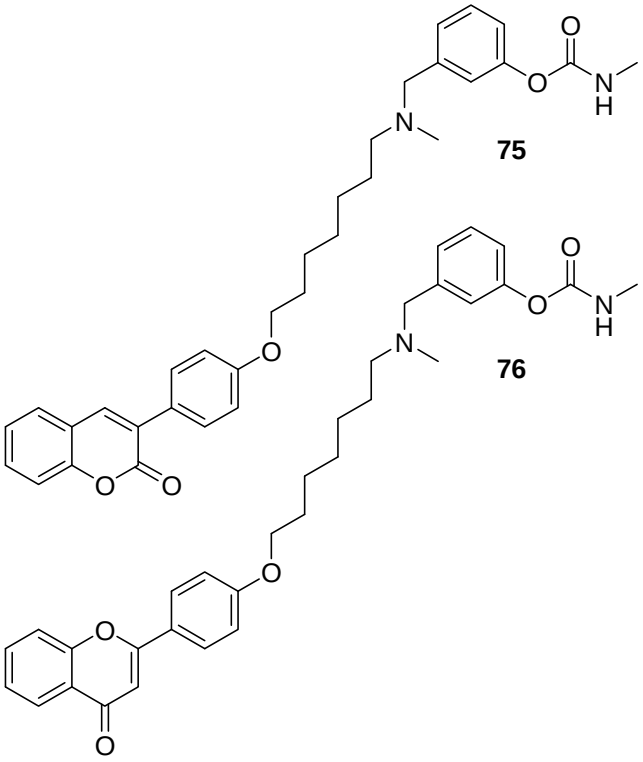



**Fig. (14).** Chemical structures of carbamate derivatives **75** and **76**.

As shown in this section, very intensive research is being carried out by an increasing number of groups toward the development of structurally diverse multi-target anti-Alzheimer agents, and particularly of those having at least a dual inhibitory activity against AChE and Aβ aggregation (either spontaneous or AChE-induced). Much attention is paid to obtaining very high potencies at any target, maybe underestimating the crucial issue of balancing the different potencies at the multiple targets, which will eventually determine the *in vivo* efficacy of multi-target drug candidates. Therefore, it would be worth devoting more efforts to properly balancing the dual AChE and Aβ aggregation inhibitory profile of already existing leads, even at the expense of a little drop of potency.

## SIMPLIFIED *IN VIVO* ASSAYS FOR THE SCREENING OF Aβ ANTI-AGGREGATING COMPOUNDS

The discovery of Aβ anti-aggregating hit and lead compounds as well as the lead optimization process for increasing potency and/or balancing Aβ anti-aggregating activity need robust and reliable methods for the evaluation of this activity. The screening of Aβ anti-aggregating compounds is mostly carried out *in vitro*

through specific amyloid-like dyes as thioflavin-T (Th-T)-based fluorometric assays that use expensive synthetic peptides [35,36,128] and that seem not too easy to be implemented, inasmuch as amyloid aggregation is very sensitive to experimental conditions (purity of peptides, nature of solvents, concentrations of buffer solutions, incubation times, mixing conditions, or even the nature of the vessel surface, etc.) [9,15]. Apart from the high cost and technical difficulties, the sensitiveness of aggregation to the assay preparation conditions may lead to significant differences between assays performed in different conditions or by different researchers. Another important issue that applies for *in vitro* assays performed with isolated protein targets, in general, is the fact that they are increasingly perceived as being too far from the (patho)physiological conditions where these targets are expected to be hit by the drug candidates under evaluation. In living organisms proteins are not isolated but they are prone to interact with other molecules, and, indeed, they take part in complex interaction networks that are not present in *in vitro* tests [39,129,130]. In particular, amyloid aggregation seems to be greatly influenced in physiological conditions by interactions with other molecules such as metal ions, chaperones or proteases. These interactions are stimulated under the molecular crowding caused by the high concentration of macromolecules inside cells that favor processes as aggregation that lead to an increased available volume [131,132]. Because these key aspects of the biological complexity of amyloid aggregation cannot be captured in *in vitro* assays there is an increasing demand of novel phenotypic assays, which recapitulate better the physiological scenario of the amyloid aggregation process and its inhibition, which are less expensive than classical *in vivo* studies with mouse models and *in vitro* tests with synthetic peptides, and which are simple, rapid, and reliable.

In this light, bacteria are emerging as suitable models to monitor protein aggregation [132]. Protein aggregation occurs intracellularly in bacteria, at least during the overexpression of heterologous genes, usually in the frame of the production of recombinant proteins for biotechnological purposes. Indeed, the high translation rate in bacteria results in high intracellular concentrations of the recombinant proteins, and in these conditions, as mentioned above, aggregation is favored over other processes such as protein folding, thereby leading to the formation of insoluble protein deposits called inclusion bodies (IBs) [133-135]. IBs are very dense particles that can be formed in the cytoplasmic or in the periplasmic space of bacteria, which were initially regarded as amorphous aggregates, devoid of any ordered structure and activity and thought to result from molecular mechanisms completely unrelated with those mediating protein deposition in eukaryotic cells. Consequently, not only IBs constituted a serious bottleneck for the

biotechnological production of proteins in bacteria, but also they were considered to be useless for basic or applied research purposes [136]. Contrary to the initial assumptions, it has been recently found that bacterial IBs share common features and common mechanisms of formation and even toxicity with highly ordered amyloid structures, including the ability to seed further amyloid formation [137-141]. Thus, X-ray diffraction, Fourier transform infrared spectroscopy (FTIR), and circular dichroism experiments have demonstrated the presence of amyloid-like structures inside IBs with β-sheet motifs, which render IBs amenable to tinction with specific amyloid-like dyes such as Th-T or Congo red [140].

In the light of the similarities between bacterial IBs and amyloid aggregates and of the easy genetic and biochemical manipulation of bacteria, bacterial IBs are emerging as a very interesting tool for studying protein aggregation processes and for screening protein aggregation inhibitors, as well [135].

Thus, aggregation of a given protein or peptide of interest can be monitored inside bacteria, particularly in *Escherichia coli*, genetically engineered to express a fusion protein where a given protein or peptide is fused to a functional reporter protein that can elicit a readily measurable property that is blocked upon peptide-triggered aggregation of the entire fusion protein or enabled by peptide aggregation inhibitors that block or delay the aggregation of the peptide thereby allowing a correct folding of the reporter protein, which becomes functional. One of such reporter proteins, widely used in these studies, is the green fluorescent protein (GFP) [139,142,143].

The group of Hecht and Chang have developed a method for the screening of Aβ42 aggregation inhibitors that is based on the use of *E. coli* cells expressing an Aβ42-GFP fusion protein [144]. Induction of the expression of such fusion protein within *E. coli* in the absence of Aβ42 aggregation inhibitors leads to misfolding and aggregation of the Aβ42 sequence, which in turn triggers the misfolding of the whole fusion protein, thereby preventing the folding of GPF into its correct fluorescent structure. Conversely, addition of inhibitors of Aβ42 aggregation to 96-well plates containing the *E. coli* cells before the induction of the expression of the Aβ42-GFP fusion protein will eventually result in a blockade of the aggregation of the Aβ42 sequence, and hence of the fusion protein, thereby allowing a proper folding of the GFP moiety into its native fluorescent structure, giving rise to a measurable fluorescent emission signal. Indeed, by using this methodology several inhibitors of Aβ42 aggregation have been identified from a library of about one thousand triazine derivatives in only several hours [144]. Interestingly, conventional *in vitro* assays using synthetic Aβ42 confirmed the Aβ42 anti-aggregating activity of one compound, selected among those identified in the *in vivo* test, thus highlighting the reliability of the *in*

*vivo* test for the identification of Aβ42 anti-aggregating hit compounds, apart from its simplicity, cost-effectiveness, reproducibility, and scalability through robotization technologies [144]. Moreover, this *in vivo* test selects compounds that are nontoxic and membrane permeable. Even if these properties are desirable for drugs, some initial hits, either toxic to *E. coli* cells or unable to cross their membranes but otherwise active as inhibitors of Aβ42 aggregation, whose toxicity or membrane permeability profile might be subsequently optimized in a classical hit-to-lead process, will remain undetected, and therefore lost, in the *in vivo* screen [144].

These limitations can be overcome using *in vitro* tests that use purified IBs previously formed in *E. coli*, and consisting of an Aβ42-GFP fusion protein, as reported by the group of Ventura [145]. This method involved the initial induction of expression of the fusion protein, followed by isolation and purification of the formed IBs by centrifugation and resuspension in lysis buffer. The obtained purified IBs were subjected then to an *in vitro* refolding assay which consisted of denaturation of the aggregates contained in the IBs and dissolution in refolding buffer [145]. By adding different compounds to the refolding buffer and measuring the resulting fluorescence signal, pro-aggregating and anti-aggregating compounds can be identified by monitoring the recovery of GFP fluorescence upon folding. Using this method, the group of Ventura confirmed the Aβ42 pro-aggregating action of metal ions, namely $Cu^{2+}$, $Ni^{2+}$, and $Zn^{2+}$, and identified several inhibitors of metal-promoted Aβ42 aggregation [145]. Even if the screening of Aβ42 aggregation inhibitors is carried out under *in vitro* conditions, it avoids the use of expensive synthetic peptides, is rapid, simple and amenable to high-throughput scale.

An analogous *in* vivo approach to that developed by the group of Hecht and Chang, but conceptually more complex, has been reported by the group of DeLisa, using a tripartite fusion protein instead of Aβ42-GFP [146]. The tripartite fusion protein was composed of: i) a signal peptide (ssTorA) that targets an export pathway to the bacterial periplasm, namely the twin-arginine translocation (Tat) system; ii) the enzyme β-lactamase to confer ampicillin resistance to *E. coli* cells; and iii) Aβ42. Upon aggregation of the Aβ42 sequence of the tripartite fusion protein the folding quality control mechanism of Tat precludes the Tat-dependent export of the fusion protein to the periplasm, with which β-lactamase can not localize there, thus resulting in low levels of bacterial resistance to ampicillin. Conversely, incubation of *E. coli* cells with inhibitors of Aβ42 aggregation should allow the Tat-dependent export of the fusion protein to the periplasm, thereby resulting in an increased resistance to ampicillin. For a more easy detection of the periplasmic localization of β-lactamase in living cells, a precursor of a fluorogenic substrate of β-

lactamase (CCF2/AM) was added to the cell cultures. Cleavage of the fluorogenic substrate of β-lactamase (CCF2), which resulted in a measurable strong blue fluorescence, needed a previous hydrolysis of the substrate precursor CCF2/AM by a periplasmic cutinase enzyme that had to be heterologously expressed by the *E. coli* cells. Thus, this complex engineered assay with *E. coli* involved the induction of the coexpression of cutinase and the ssTorA-Aβ42-β-lactamase fusion protein, after having added to the culture medium the potential Aβ42 anti-aggregating compounds, followed by addition of CCF2/AM and measurement of the fluorescence and cell density of each well. A large library of triazine derivatives was screened using this method, and the positive Aβ42 aggregation inhibitor hits were confirmed by a conventional *in vitro* assay using synthetic Aβ42 [146].

Alternatively to the use of protein reporters, Aβ42 aggregation can be also monitored *in vivo* through the use of conformational-sensitive dyes [132]. The group of Sabate and Ventura has recently developed a new method for monitoring Aβ42 aggregation within intact *E. coli* cells that takes advantage of the membrane penetration of the amyloid dye thioflavin-S (Th-S) and of the high-speed high-throughput amenable flow cytometry to detect Th-S fluorescence [147]. Sabate has also developed a simple quantitative method for the *in vivo* screening of Aβ42 aggregation inhibitors based on the direct Th-S staining of IBs within living *E. coli* cells and determination of Th-S relative fluorescence. Using this method, the percentages of inhibition of Aβ42 aggregation *in vivo* of several inhibitors were found to be very similar to those previously determined with a classical *in vitro* assay with synthetic peptide, albeit slightly lower, likely as a result of a noncomplete penetration of the compounds through the bacterial membrane as well as the different expression levels of the recombinant Aβ42 (Pouplana, S. *et al.*, submitted). Very interestingly, the possibility of applying this method in automated technologies, like UV/Vis plate reader assays, might allow a rapid, simple, quantitative, and unexpensive *in vivo* testing of large compound libraries.

**CONCLUSION**

Huge research efforts and financial investement are eagerly pursuing the development of drugs that may efficiently treat AD, the so-called disease-modifying drugs, as they represent an acute unmet medical need. It is increasingly accepted that drugs that can hit simultaneously multiple biological targets involved in the neuropathogenesis of AD, regarded as a pathological networtk of interrelated protein targets instead of as a single-protein induced straightforward process, are the most viable option to efficiently confront

the disease. Among multi-target anti-AD drug candidates, dual inhibitors of Aβ aggregation and AChE are especially sought. A large number of synthetic, natural or semisynthetic compounds are being prepared or isolated and profiled against these two targets, and in many cases also against other biological targets of interest such as BACE-1 or oxidative stress, and some candidates with properly balanced multiple potencies are being discovered. However, to expedite the discovery of such candidates some innovations both at the early and late phases of the drug discovery process are necessary.

At the early phases of hit identification and hit-to-lead processes simple *in vivo* methods for the screening of Aβ aggregation inhibitors are being developed that avoid the use of expensive synthetic Aβ peptides, which become cost-prohibitive in high-throughput screening of large compound libraries, and the artificiality of conventional *in vitro* assays. *In vivo* screening methods using bacterial IBs are emerging, which rely on the detection of the function of a reporter protein fused to Aβ or on the direct detection of a fluorescent signal upon binding to Th-S dye within living *E. coli* cells. These *in vivo* methods allow a rapid, reliable, reproducible, and cost-effective screening of Aβ aggregation inhibitors and are amenable to high-throughput automated technologies.

At the final stages of drug development, the clinical success of dual inhibitors of Aβ aggregation and AChE, as well as of any other putative disease-modifying anti-Alzheimer drug, greatly depends on the possibility of initiating treatment very early in the course of AD, before widespread neurodegeneration and severe brain damage has already occured. For this purpose, the discovery of sensitive and reliable biomarkers and imaging agents that can allow an early diagnosis of AD, at the presymptomatic phases, is essential, inasmuch as they would enable both initiation of an early treatment and assessment of the effectiveness of disease-modifying drug candidates.

**ABBREVIATIONS**

| | | |
|---|---|---|
| Aβ | = | β-Amyloid peptide |
| AChE | = | Acetylcholinesterase |
| AChEI | = | AChE inhibitors |
| AD | = | Alzheimer's disease |
| APP | = | Amyloid precursor protein |
| BBB | = | Blood-brain barrier |
| BChE | = | Butyrylcholinesterase |

| | | |
|---|---|---|
| CAS | = | Catalytic anionic site |
| eeAChE | = | Electric eel acetylcholinesterase |
| GFP | = | Green fluorescent protein |
| hAChE | = | Human acetylcholinesterase |
| hBChE | = | Human butyrylcholinesterase |
| I | = | Inhibitor |
| IBs | = | Inclusion bodies |
| ORAC | = | Oxygen radical absorbance capacity |
| PAS | = | Peripheral anionic site |
| ROS | = | Reactive oxygen species |
| *Tc*AChE | = | *Torpedo californica* acetylcholinesterase |
| TEM | = | Transmission electron microscopy |
| Th-S | = | Thioflavin-S |
| Th-T | = | Thioflavin-T |
| Tat | = | Twin-arginine translocation |

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