Multi-target design strategies for the improved treatment of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a multifactorial syndrome resulting in profound misery and poses a substantial burden on human health, economy, and society throughout the world. Based on the numerous AD-related targets in the disease network, multi-target design strategy is a crucial direction to seek for enhanced therapy, and multi-target drugs have the ability to regulate more targets than single-target drugs, affecting the disease network with more potency. Herein, we highlight nine major targets associated with AD, which are acetylcholine esterase (AChE), beta-site amyloid precursor protein cleaving enzyme 1 (β-secretase, BACE-1), glycogen synthase kinase 3 beta (GSK-3β), monoamine oxidases (MAOs), metal ions in the brain, *N*-methyl-D-aspartate (NMDA) receptor, 5-hydroxytryptamine (5-HT) receptors, the third subtype of histamine receptor (H_3 receptor), and phosphodiesterases (PDEs), and their respective relationship to the disease network. Furthermore, eleven multi-target design strategies classified by the involvement of AChE and related promising compounds for improved therapy of AD in recent years are described based on the nine major targets.

Keywords: Alzheimer's disease, multi-target strategy, acetylcholine esterase, donepezil, tacrine

Contents:

Abbreviations:

ACh	Acetylcholine
AChE	Acetylcholine esterase
AChEI	AChE inhibitor
AD	Alzheimer's disease
APP	Amyloid precursor protein
Aβ	beta-amyloid
BACE-1	beta-site amyloid precursor protein cleaving enzyme 1

BBB	Blood-brain barrier
BChE	Butyrylcholinesterase
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
ChE	Cholinesterase
CNS	Central nervous system
COX	Cyclooxygenase
CREB	cAMP response element-binding protein
EC_{50}	Concentration for 50% of maximal effect
ED	Erectile dysfunction
Gly	Glycine
GSK-3β	Glycogen synthase kinase 3 beta
HT	Hydroxytryptamine
IC_{50}	Half maximal inhibitory concentration
K_i	Inhibition constant
LOX	Lipoxygenase
MAO	Monoamine oxidase
MCI	Mild cognitive impairment
MD	Myotonic dystrophy
MMSE	Mini-Mental State Examination
MTDL	Multi-target-directed ligand
NCE	New chemical entity
NFT	Neurofibrillary tangle
NMDA	*N*-methyl-D-aspartate
NO/sGC/cGMP	The nitric oxide/soluble guanylyl cyclase/cGMP
NQO1	NAD(P)H:quinone oxidoreductase 1
ORAC	Oxygen radical absorbance capacity
PAMPA	The parallel artificial membrane permeation assay
PD	Parkinson disease
PDE	Phosphodiesterase
ROS	Reactive oxygen species
sAPP-α	Soluble amyloid precursor protein α
SGZ	Subgranular zone
SSRI	Selective serotonin reuptake inhibitor

1. Introduction

AD is an progressive neurodegenerative disorder associated with the appearance of extracellular senile or neuritic plaques and intracellular neurofibrillary tangles in the brain [1] that induce memory loss and cognition impairment, causing enormous suffering to individuals, families, and society [2]. AD was first reported by Alois Alzheimer in 1907 [3] and is now deemed by World Health Organization as the most common cause of dementia, with approximately 47 million

patients worldwide in 2018, and is estimated to affect 75 million people by 2030 and 140 million people by 2050 globally [4]. The nebulous and complicated pathogenesis of AD makes it difficult to develop new drugs, and also leads to failure of many promising drug candidates when tested in clinical trials with the result of unpredictable clinical manifestations. Despite these challenges, research of AD is still a hot topic, and the efforts and attempts of scientific groups worldwide are continuing, with the aim of finding an effective treatment [5].

Currently, exploration of AD has led to the discovery of many primary targets that influence generation and exacerbation of the disease, such as AChE, BACE-1, GSK-3β, MAOs, metal ions in the brain, NMDA receptor, 5-HT receptors, H_3 receptor, and PDEs (Figure 1). And lots of potent and effective compounds for the treatment of AD are discovered based on these targets, among which donepezil (**1**), galantamine (**2**), rivastigmine (**3**), and memantine (**14**) (Table 1) (Figure 2) are the only four drugs approved by the FDA for the treatment of AD. And all of them are single-target drugs that can notably improve the conditions of patients in cognition deterioration and memory loss, but these drugs cannot completely cure the disease. It is a unique and intricate feature of AD that relevant targets in different cell signaling pathways can interact with each other and form a disease network, and this results in a poor curative effect of single-

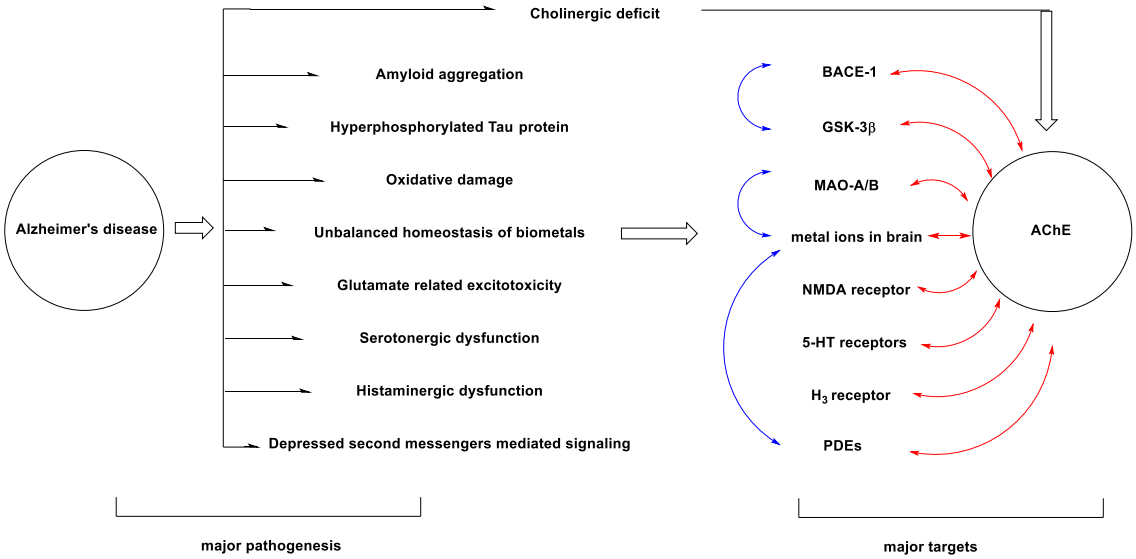

Figure 1. Concise relationships between AD and nine major targets, and eleven multi-target design strategies based on the targets. Red lines: multi-target strategies involving AChE. Blue lines: multi-target strategies without AChE involvement.

target drugs. Based on the complicated pathogenesis of AD and the imperfect single-target drugs, multi-target strategies are becoming more and more popular for potential treatment of AD because several targets associated with AD could be affected by one multi-target drug, which can have a synergistic effect on the disease network, leading to better improvement on memory and cognition. Therefore, multi-target design strategy for AD therapy is an important direction in current research compared with single-target drugs, and multi-target drugs will likely be more pivotal and effective in regulating disease progression.

2. Multi-target strategies involving AChE

Nine related targets in AD are presented in this section, which are AChE, BACE-1, GSK-3β, MAOs, metal ions in the brain, NMDA receptor, 5-HT receptors, H_3 receptor, and PDEs. These nine targets and their respective signaling pathways could influence the disease progression by themselves and interactions among them, also resulting in the complicated and unclear disease network of AD. Among them, AChE is the most pivotal target of the well-known AD network, and multi-target drugs are designed to involve this target to increase the availability and decrease the failure risk of new chemical entities (NCEs). In short, AChE is the most popular target in AD-related targets and multi-target design strategy involving AChE is the most popular strategy in AD-related multi-target strategies. In this section, eight multi-target strategies involving AChE and related targets are reviewed (Figure 1).

2.1.1. AChE

The cholinergic hypothesis, a significant guidance orientation of AD, was first reported by Davies and Maloney in 1976 [6], states that the reduction of acetylcholine (ACh) in presynaptic cholinergic terminals of the hippocampus and the neocortex regions caused by degeneration of cholinergic neurons of basal forebrain nuclei plays a key role in AD pathology, and the conclusive treatment is to up-regulate the ACh concentration in the synaptic cleft [7]. ACh in the synaptic cleft is secreted by synaptic vesicles to activate muscarinic and nicotinic receptors and is immediately decomposed to choline and acetate [8] by AChE under normal physiological conditions. AChE is a highly kinetically efficient enzyme, and each AChE molecule can hydrolyze 5000 ACh molecules per second [9]. Butyrylcholinesterase (BChE) can also hydrolyze ACh, and its levels progressively rise in advancing AD, while AChE activity remains unchanged or declines

[10]. BChE is becoming an interesting target because selective BChE inhibitors have displayed amyloid-lowering activity which is a useful effect to improve dementia condition [11]. Highly selective BChE inhibitors or dual AChE and BChE inhibitors represent another new potential approach for AD treatment; however, this multi-target strategy is not discussed in this article

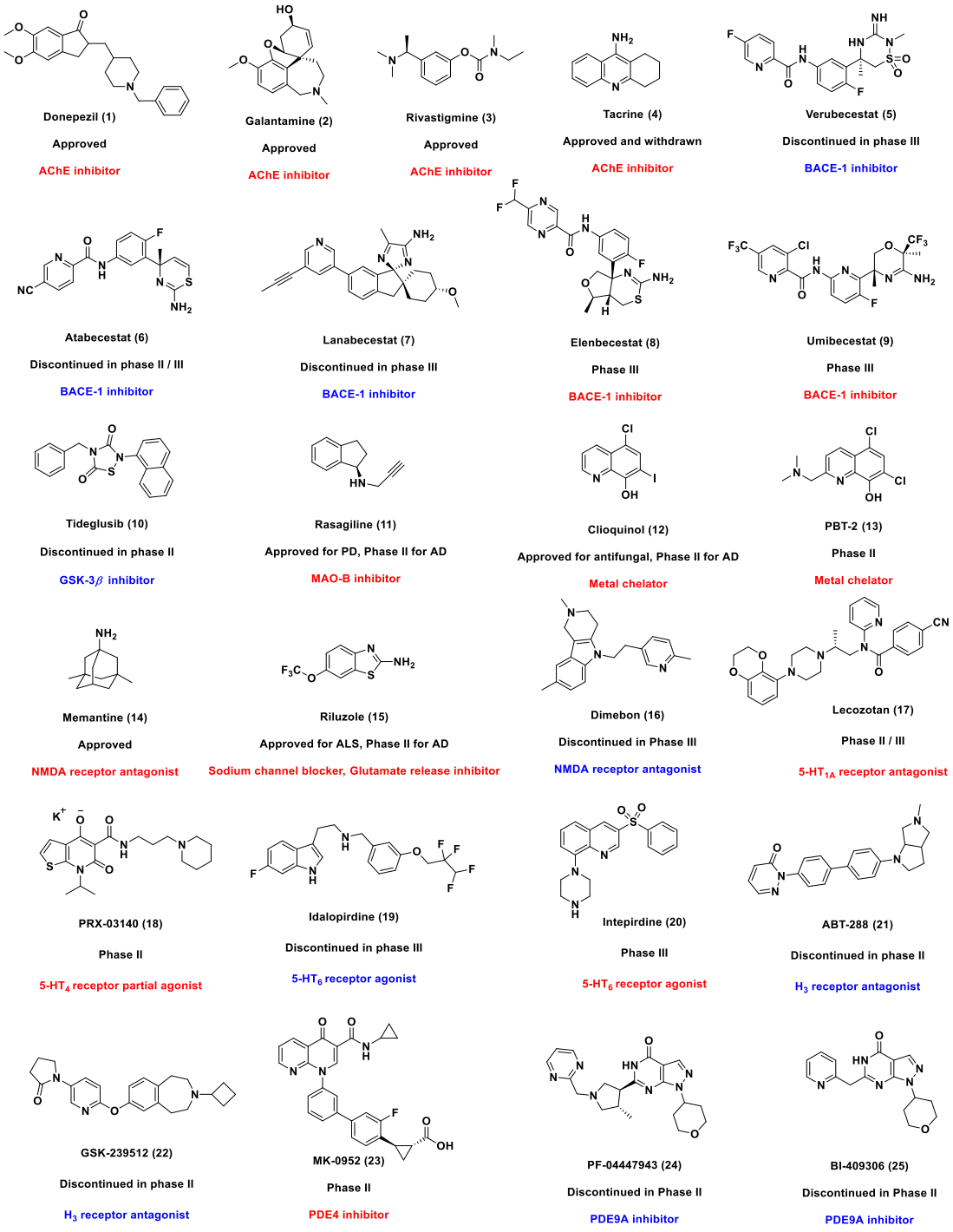

Figure 2. Related single-target drugs that are approved or in clinical trials for the treatment of AD. Red refers to the approved drugs or candidates in clinical trials, and blue refers to the discontinued candidates in clinical trials.

because of the unclear theoretical basis of BChE and the close relationship between the two targets [12].

Inhibition of AChE activity to prevent degradation of ACh in synapses is the most primary method in medicinal chemistry, which makes AChE a vital target and leads the inhibition activity of AChE to be a fundamental evaluation criterion. The present clinical drugs approved by the FDA are principally AChE inhibitors (AChEIs), such as donepezil (**1**), galantamine (**2**), and rivastigmine (**3**) [13] (Table 1) (Figure 2), which can notably slow down memory loss and improve the cognition function of patients, while dementia cannot be prevented or completely cured by them [14]. Tacrine (**4**) (Figure 2) was approved in 1993 as the first AChEI for AD treatment and was withdrawn shortly after due to liver toxicity [2], but its structure is still important for the new drug design. Donepezil (**1**) (Figure 2) of Eisai and Pfizer, the top-selling AChEI drug worldwide among AD-related clinical drugs, was approved in 1997 for the treatment of mild-to-moderate AD, and was approved in 2014 for additional treatment of Lewy body dementia. Donepezil (**1**) is available as disintegrating tablets or oral solutions to be administered at a dose of 5-10 mg once a day due to its long half-life, with a starting dose of 5 mg/day. Donepezil (**1**) is a safe and well tolerated drug based on current clinical evidence, it can improve cognition and shows clinical amelioration in overall functioning even for patients with severe AD [14]. Tacrine (**4**) and donepezil (**1**) are still subjected to various modifications by research groups [15], and are often used as positive controls in enzyme activity tests *in vitro* and in pharmacological experiments with AD-related animal models *in vivo* to test the AChE inhibitory potency and safety of NCEs. The cholinergic hypothesis and approved drugs make AChE and AChEIs a high profile in AD field, lead single-target strategy aiming at AChE and multi-target strategy involving AChE to become common thoughts in new drug design for the potential treatment of AD.

2.1.2. BACE-1

The amyloid hypothesis, a currently popular theoretical foundation first outlined by Hardy and Higgins in 1992 [16], states that the production, oligomerization and self-aggregation of beta-amyloid (Aβ) cause synaptic dysfunction and senile plaques which is the typical hallmark of AD [17]. The degradation of major amyloid precursor protein (APP) occurs through a two-step proteolysis reaction catalyzed by α-secretase and γ-secretase with no physical damage [18], while

a small proportion of APP is degraded by β-secretase (BACE-1) and γ-secretase to generate Aβ which is composed of 37-49 amino acid residues and mainly shown as Aβ_{40} and Aβ_{42} peptides [19]. Aβ_{40} is the more common metabolite and may actually be anti-amyloidogenic, whereas Aβ_{42} and longer peptides are highly self-aggregating and lead to profound Aβ deposition [20].

Table1. Fundamental information regarding four drugs which have been approved by the FDA for the treatment of AD

Drug	Donepezil (**1**)	Galantamine (**2**)	Rivastigmine (**3**)	Memantine (**14**)
Salt type	Hydrochloride	Hydrobromide	Tartrate	Hydrochloride
Launch year for AD	1997	1995	1997	2002
Brand name	Aricept	Reminyl	Exelon	Ebixa
Indication of AD	Mild to moderate	Mild to moderate	Mild to moderate	Moderate to severe
Mode of action	Selective AChE inhibition	Selective AChE inhibition	Slowly reversible AChE and BChE inhibition	Non-competitive NMDA-receptor antagonist
Half-life [1]	70 h	7-8 h	1 h	60-100 h
Metabolism [14]	CYP2D6 and CYP3A4 in liver	CYP2D6 and CYP3A4 in liver	AChE	Kidney
Given with food	Irrelevant	Recommended	Yes	Irrelevant
Clinically efficient dose	5-10 mg/day	16-24 mg/day	6-12 mg/day	20 mg/day

Despite continuing arguments for the amyloid hypothesis [18], the growing consensus is that anti-amyloid drugs will be most effective in the early AD process [21]. Obviously, BACE-1 is the most suitable target to decrease Aβ production in the APP metabolic pathway by inhibiting its activity, and mice with BACE-1 deficiency showed a healthy and fertile clinical phenotype with abolished Aβ production in the brain [22], making BACE-1 a popular target and its inhibitors an attractive therapeutic strategy. The clinical situation of BACE-1 inhibitors is cruel (Figure 2). The drug verubecestat (**5**) of Merck in Phase III was discontinued in February 2017 due to lack of

efficacy [23, 24]. The Phase II/III drug atabecestat (**6**) of Johnson & Johnson was discontinued in May 2018 for the increase of hepatic enzyme, and the Phase III drug lanabecestat (**7**) of Eli Lilly and AstraZeneca was discontinued in June 2018 because no curative endpoint was achieved. The optimistic cases of BACE-1 inhibitors are that the drug LY3202626 (structure not disclosed) from Eli Lilly is in Phase II clinical trials, the drug elenbecestat (**8**) from Biogen and Eisai is in Phase III clinical trials [25], and the drug umibecestat (**9**) from Novartis and Amgen is in Phase III clinical trials.

2.1.3. AChE and BACE-1 multi-target strategy

Design of multi-target NCEs based on AChEI drugs, such as donepezil (**1**) and tacrine (**4**), is popular because the scaffolds of approved drugs with evident AChE inhibition capacity can hopefully guarantee the efficacy of new compounds, at least for targeting AChE, reducing the failure risk of developing new drugs. Combining the pharmacophores of AChEI drugs and BACE-1 inhibitors with a suitable linker is a common approach to design novel dual inhibitors targeting both enzymes.

Zhu and co-workers [26] reported a series of hybrid compounds with AChE and BACE-1 dual inhibitions, and the scaffold was fused through three types of linkers with AChEI donepezil (**1**) and isophthalamide (**26**), a widely used pharmacophore in BACE-1 inhibitors [27]. Among these compounds, hybrid **27** (Figure 3) was considered as the most potential multi-target agent for therapeutic application of AD, and showed excellent dual inhibition in enzyme inhibitory potency assay (IC_{50} = 1.83 μM for AChE, IC_{50} = 0.567 μM for BACE-1), good inhibitory effects on Aβ production in APP-transfected HEK293 cells (IC_{50} = 98.7 nM), and a mild antioxidative effect against H_2O_2-induced PC12 cell injury at 10 μM. Further *in vivo* experiments in APP transgenic mice showed that compound **27** caused a 29% reduction in A$\beta_{1\text{-}40}$ production through intracerebroventricular administration. Another compound, hybrid **28** (Figure 3), reported by the Praveen group [28], was fused with donepezil (**1**) and 2,4-disubstituted pyrimidine, a template with an Aβ-aggregation inhibition profile [29], and showed dual inhibitory activities in the enzyme assay (IC_{50} = 9.9 μM for AChE, 34% inhibition at 10 μM for BACE-1), 17.4% self-induced A$\beta_{1\text{-}40}$ aggregation inhibition at 100 μM, and 81.0% neuroblastoma cell viability at 40 μM.

Fernandez-Bachiller and co-workers [30] reported a new family of AChEI tacrine (**4**) and 4-oxo-4*H*-chromene (**29**) hybrids, in which the tacrine (**4**) scaffold was selected for AChE

inhibition and a flavonoid fragment derived from 4-oxo-4*H*-chromene (**29**) was chosen for BACE-1 inhibition. The most promising compound, hybrid **30** (Figure 3), showed potent dual inhibition against human AChE and BACE-1 (IC_{50} = 8.0 nM for AChE, IC_{50} = 2.8 μM for BACE-1), 1.3-fold more potent antioxidant activity than trolox (a vitamin E analogue), and good central nervous system (CNS) permeability in the parallel artificial membrane permeation assay for blood-brain barrier (PAMPA-BBB). A promising compound, hybrid **34** (Figure 3), reported by the Munoz-Torrero group [31], was fused through a long alkylamine linker with rhein (**33**) and huprine Y (**32**), another hybrid from tacrine (**4**) and huperzine A (**31**) [32], and showed potent inhibitory activities against human AChE and BACE-1 (IC_{50} = 3.6 nM for AChE, IC_{50} = 120 nM for BACE-1) and 47.9% Aβ_{42} anti-aggregating activity at 10 μM. Further *in vivo* experiments in APP-PS1 transgenic mice showed a central soluble Aβ-lowering effect four weeks later after intraperitoneal administration with compound **34**. Replacement of the chlorobenzene ring of the huprine Y (**32**) moiety of **34** with pyridine led to lower AChE and BACE-1 inhibitory activities but potent antioxidant activity [33]. Another potent tacrine (**4**)-benzofuran (**35**) hybrid **36** (Figure 3), reported by the Bartolini group [34], showed an interesting profile as a dual inhibitor against human AChE and BACE-1 (IC_{50} = 0.86 nM for AChE, IC_{50} = 1.35 μM for BACE-1) and 61.3% inhibition toward self-induced Aβ aggregation at 10 μM. Further *in vivo* studies confirmed that **36** caused cognitive improvement in scopolamine-treated ICR mice and exhibited no significant hepatotoxicity.

Computer-aided structure-based design is a practical and efficient method to find new active hits. Dominguez and co-workers [35] reported that after two cycles of design and screening of candidates based on pharmacophores and required interactions with the targets, compound **37** (Figure 3) was discovered, synthesized, and showed evident dual inhibition activities against AChE and BACE-1 (IC_{50} = 9.1 μM for AChE, IC_{50} = 2.5 μM for BACE-1). Innovating new compound scaffolds through the self-accumulated effort and knowledge of scientific groups is another classical drug discovery approach. Belluti and co-workers [36] reported that compound **38** (Figure 3) with a benzophenone core, showed dual-target inhibitory potency against human AChE and BACE-1 (IC_{50} = 1.57 μM for AChE, 10.72% inhibition at 3.38 μM for BACE-1). Recently, a new compound **39** (Figure 3) with a benzophenone core was reported by the Gabr group [37], showing potent human AChE and BACE-1 inhibition (IC_{50} = 4.11 nM for AChE, IC_{50} = 18.3 nM

for BACE-1), potential metal chelating capability, low toxicity toward SH-SY5Y neuroblastoma cells, and the ability to permeate the BBB tested by PAMPA.

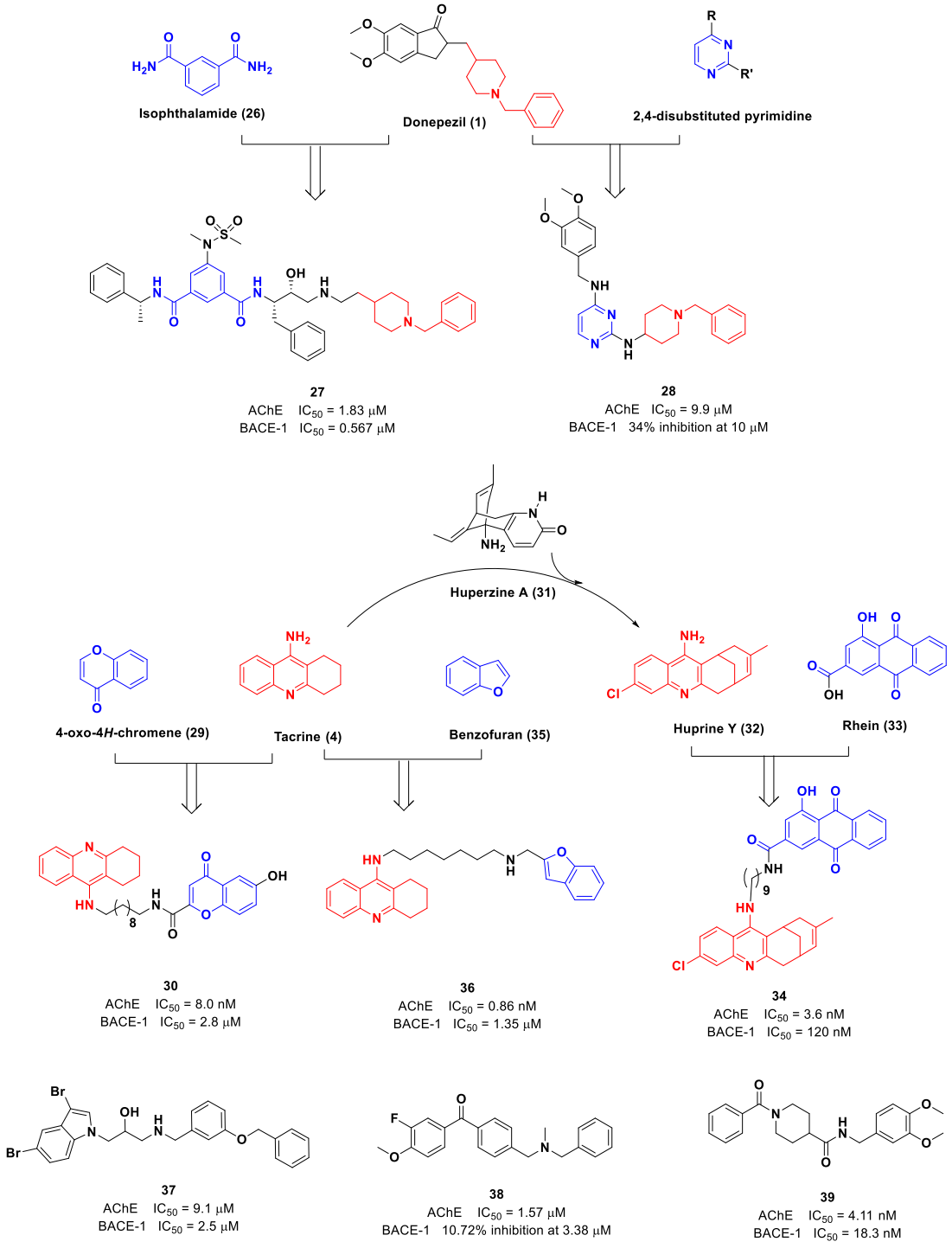

Figure 3. AChE and BACE-1 multi-target strategy

2.2.1. GSK-3*β*

The tau protein hypothesis is based on intracellular neurofibrillary tangles (NFTs) in the brain

which is another histopathological hallmark of AD just as senile plaques. NFTs are composed of paired helical filaments and straight filaments which are mainly caused by hyperphosphorylated tau protein [38]. The primary physiological function of tau protein, the most abundant microtubule-associated protein expressed in neurons, is to form microtubules with microtubule proteins, maintain microtubules stabilization, and promote microtubules to concentrate in clusters. When tau protein is hyperphosphorylated by the highly conserved threonine-serine kinase GSK-3β, primarily at Ser396, Ser199, and Ser413 [39], from the original 2-3 phosphate groups to 5-9 phosphate groups, it is separated from microtubules and subsequently aggregated into insoluble NFTs which ultimately cause cell death [40]. Not only can GSK-3β phosphorylate tau protein, increased GSK-3β levels could regulate γ-secretase to induce Aβ formation in a unique way, resulting in toxicity to cultured neurons [41]. The three aspects of therapies based on the tau protein hypothesis include inhibiting the phosphorylation, preventing the aggregation of tau protein, and stabilizing microtubules, and GSK-3β is the major and specific target in the upstream tau pathway, making it the most popular target for small molecule compounds. The GSK-3β inhibitor tideglusib (**10**) (Figure 2) from AMO-pharma belongs to the thiadiazolidinone family and is currently in Phase II clinical trials for the treatment of myotonic dystrophy (MD). Tideglusib (**10**) was in Phase II clinical trials for the treatment of AD, but the trial was discontinued in 2012 due to a lack of drug efficacy, even though it showed positive trends in four out of five clinical scales and induced a significantly benign response on the Mini-Mental State Examination (MMSE) [42].

2.2.2. AChE and GSK-3β multi-target strategy

AChE and GSK-3β, each of which has been frequently adopted in multi-target strategies, are two vital AD-related targets in ACh concentration modulation and tau protein phosphorylation, respectively. However multi-target-directed ligands (MTDLs) possessing inhibitory potency for both enzymes are rarely reported.

Hui and co-workers [43] reported that hybrid **41** (Figure 4), fused with the AChEI tacrine (**4**) and phenothiazine which is the key pharmacophore of the tau aggregation inhibitor methylene blue (**40**) (Figure 4), showed AChE inhibitory potency (IC_{50} = 89 nM). And tau hyperphosphorylation induced by okadaic acid in N2α cells was markedly prevented by hybrid **41** with 39.5% down-regulation of the tau protein level when **41** was tested at 100 μM. After initial

ADME properties screening using PreADMET (v2.0), molecular docking studies were undertaken using Molegro Virtual Docker 2009 to test the inhibition potency of AChE and GSK-3β among 26 hybrids, and **41** had significant affinity toward AChE, with a MolDock score of -183.585 kJ/mol, and toward GSK-3β, with a MolDock score of -148.821 kJ/mol. However, enzyme inhibitory activity of compound **41** against GSK-3β was not reported.

Jiang and co-workers [44] reported a novel series of hybrids, fused with tacrine (**4**) and compound **42** (GSK-3β inhibitor, IC_{50} = 1.1 nM) which was reported by the Sivaprakasam group [45]. The most promising hybrid **43** (Figure 3) showed important human AChE and GSK-3β dual-target inhibitory activity (IC_{50} = 6.5 nM for AChE, IC_{50} = 66 nM for GSK-3β), good performance on Aβ self-aggregation with a 46% inhibitory rate at 20 μM, inhibition of tau protein hyperphosphorylation in mouse neuroblastoma N2α-Tau cells, less hepatotoxicity, and significant *in vivo* cognitive improvement in scopolamine-treated ICR mice.

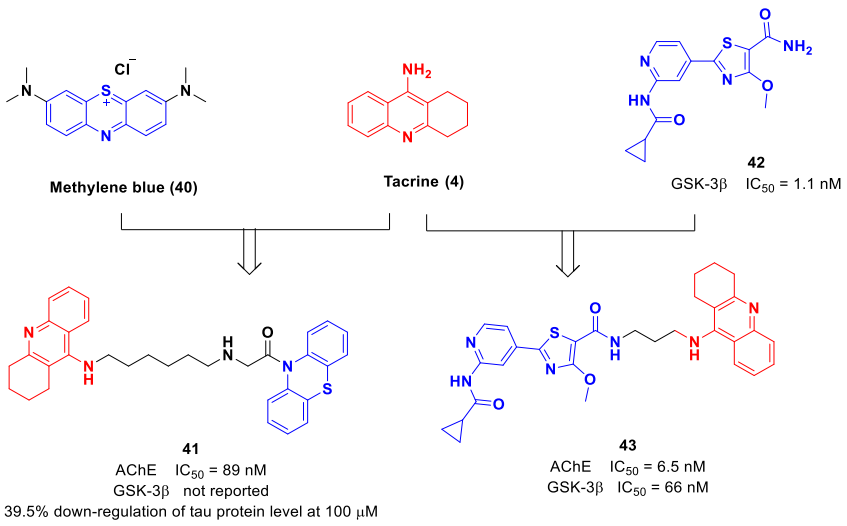

Figure 4. AChE and GSK-3β multi-target strategy

2.3.1. MAOs

MAOs are metabolic enzymes that exist in two isoforms, MAO-A and MAO-B, distinguished by their difference on specificity of substrates and inhibitors [46]. MAOs can catalyze the oxidative deamination reactions of various biogenic and xenobiotic amines and play an important role in neurodegenerative diseases, such as AD and Parkinson disease (PD). The bioactivity of MAO-B is significantly increased in the cerebral cortex and the hippocampus regions of AD

patients, representing more than 80% of the total MAOs activity in the brain [47], and the activity and gene expression of MAO-A are also up-regulated in different brain areas [48]. High levels of MAOs catalyze oxidative deamination and increase the production of hydrogen peroxide and reactive oxygen species (ROS) which are responsible for oxidative injuries and the toxic environment characteristics of neurodegeneration [49], and the increased MAO-B levels can enhance astrogliosis in the brain [50]. MAO-A inhibitors are used in the clinic to treat anxiety and depression, which are also common symptoms considered to be risk factors in AD progression [51], while MAO-B inhibitors are used in the treatment of PD [52]. MAOs inhibitors can increase monoaminergic neurotransmission, decrease ROS formation and oxidative stress, and exert pharmacological effects including antioxidation, neuroprotection and cognitive improvement [53], which are potentially valuable for the treatment of AD. Rasagiline (**11**) from Teva pharma (Figure 2) is a potent and irreversible selective MAO-B inhibitor approved in 2005 for the treatment of PD and has been in Phase II clinical trials for the treatment of AD.

2.3.2. AChE and MAOs multi-target strategy

Ladostigil (**44**) from Avraham pharma (Figure 5) is currently in Phase II clinical trials for the treatment of mild cognitive impairment (MCI) and Alzheimer's type dementia [54]. This drug shows inhibitory activities against AChE, BChE, MAO-A, and MAO-B in the brain [55], which is combined with the carbamate moiety of the cholinesterases (ChEs) inhibitor rivastigmine (**3**) and the indolamine moiety of the MAO-B inhibitor rasagiline (**11**).

In multi-target strategies involving targets AChE and MAOs for AD therapies, donepezil (**1**)-related derivatives and tacrine (**4**)-related derivatives as MTDLs are still popular. The Unzeta group [56] reported a promising compound, hybrid **46** (Figure 5), which was fused with the benzylpiperidine moiety of donepezil (**1**) and the indolyl propargylamino moiety of the MAOs inhibitor **45** [57] (IC_{50} = 100 nM for MAO-A, IC_{50} = 63 nM for MAO-B) (Figure 5), that showed multi-target inhibitory potency against AChE, BChE, MAO-A, and MAO-B (IC_{50} = 0.35 μM for AChE, IC_{50} = 0.46 μM for BChE, IC_{50} = 5.2 nM for MAO-A, IC_{50} = 43 nM for MAO-B) and presented significant profiles with 47.8% self-induced Aβ aggregation inhibition at 10 μM and 32.4% AChE-induced Aβ aggregation inhibition at 100 μM. Afterwards, the same group [58] reported that hybrid **48** (Figure 5), with the juxtaposition structure of donepezil (**1**) and M30 (**47**) which is potent brain-selective MAOs inhibitor and neuroprotective biometal-chelator [59] (IC_{50} =

57 nM for MAO-A, IC_{50} = 1.5 μM for MAO-B) (Figure 5), was a mixed-type ChEs inhibitor and an irreversible MAOs inhibitor with metal-chelating properties (IC_{50} = 1.8 μM for AChE, IC_{50} = 1.6 μM for BChE, IC_{50} = 6.2 μM for MAO-A, IC_{50} = 10.2 μM for MAO-B), and was capable of significantly decreasing scopolamine-induced learning deficits in healthy adult mice. Later, the same group [60] reported another compound, hybrid **49** (Figure 5), which was an improved analogue of **48** and exhibited excellent ChEs inhibition potency, selective MAO-A inhibition (IC_{50} = 29 nM for AChE, IC_{50} = 39 nM for BChE, IC_{50} = 10.1 μM for MAO-A), strong metal chelating capacity to Cu^{2+} and Zn^{2+} ions, and moderate antioxidant properties. Estrada and co-workers [61] reported that hybrid **51** (Figure 5), fused with donepezil (**1**) and the natural antioxidant cinnamic acid (**50**) (Figure 5), was ChEs and MAOs dual inhibitors (IC_{50} = 1.75 μM for AChE, IC_{50} = 0.69 μM for BChE, IC_{50} = 3.5 μM for MAO-A, IC_{50} = 6.0 μM for MAO-B) and was able to improve the differentiation of adult subgranular zone (SGZ)-derived neural stem cells into a neuronal phenotype *in vitro*, thus showing antioxidant, cholinergic, neuroprotective and neurogenic properties. Hybrid **53** (Figure 5) reported by the Sang group [52] was fused with donepezil (**1**) and the notable MAO-B inhibitor **52** (IC_{50} = 2.9 nM) [62], and showed the potent and balanced inhibitory activities against ChEs and MAOs (IC_{50} = 0.56 μM for AChE, IC_{50} = 2.3 μM for BChE, IC_{50} = 0.3 μM for MAO-A, IC_{50} = 1.4 μM for MAO-B). Further investigation confirmed that **53** could cross the BBB *in vitro* and abided by Lipinski's rule of five.

The Li group [63] reported that hybrid **55** (Figure 5), fused with the AChEI tacrine (**4**) and selegiline (**54**) which is an MAO-B inhibitor approved in 1981 for the treatment of PD, provided a good balance of activities toward ChEs and MAOs (IC_{50} = 22.6 nM for AChE, IC_{50} = 9.37 nM for BChE, IC_{50} = 0.37 μM for MAO-A, IC_{50} = 0.18 μM for MAO-B). The same group [64] reported that hybrid **57** (Figure 5), fused with tacrine (**4**) and the homoisoflavonoid compound **56** which is known as an efficient MAO-B selective inhibitor [65] (IC_{50} = 1.06 μM for MAO-B) (Figure 5), showed potent inhibitions against ChEs and MAO-B (IC_{50} = 67.9 nM for AChE, IC_{50} = 33.0 nM for BChE, IC_{50} = 0.401 μM for MAO-B). Later, the same group [66] reported another homoisoflavonoid derivative **58** (Figure 5), which showed the desired balance of AChE and MAO-B inhibitory activities (IC_{50} = 3.94 μM for AChE, IC_{50} = 3.44 μM for MAO-B).

Indolotacrine analogue **59** (Figure 5) reported by the Musilek group [67], was a potent inhibitor of ChEs and MAO-A and a weak inhibitor of MAO-B (IC_{50} = 1.5 μM for AChE, IC_{50} = 2.4 μM for

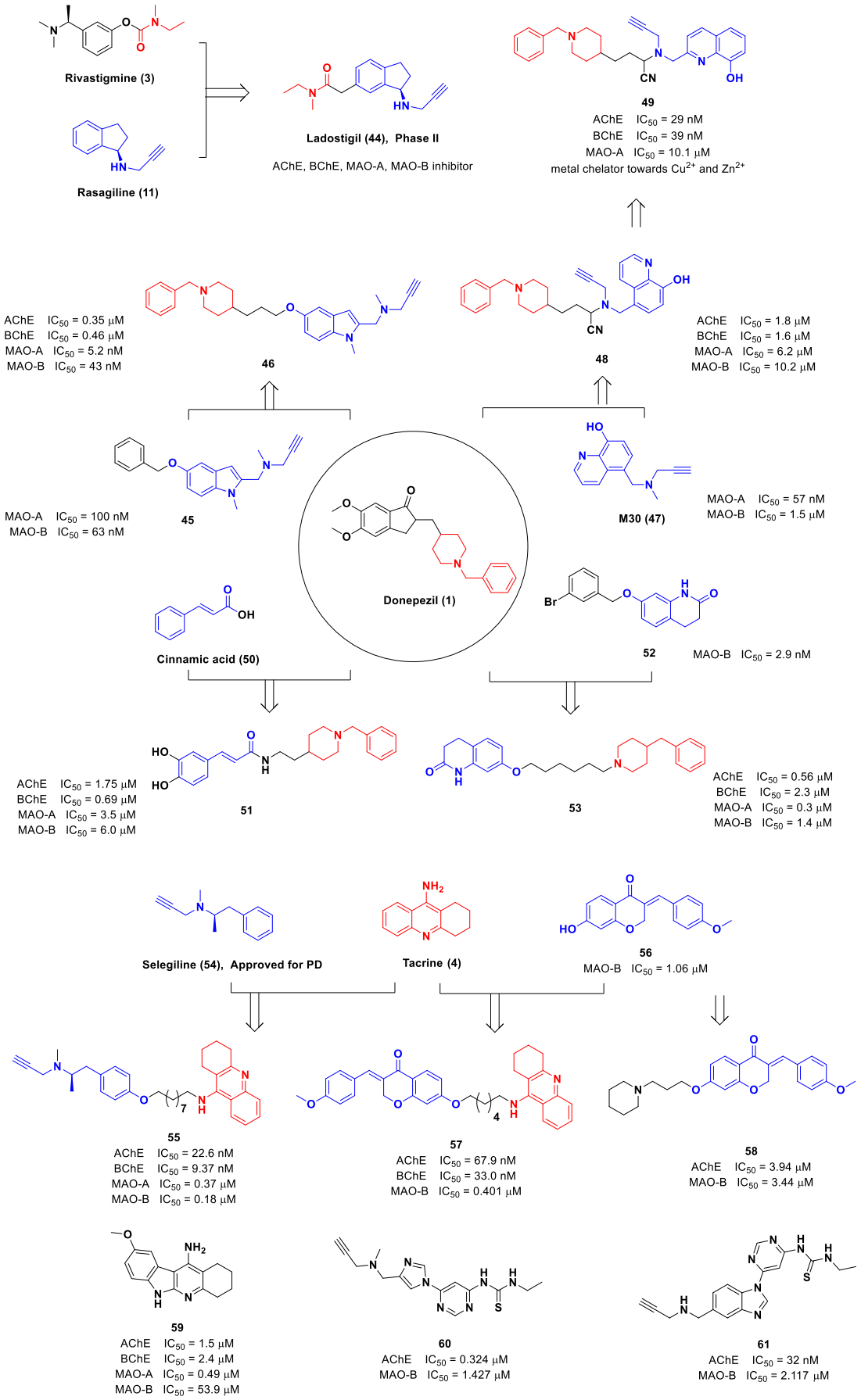

Figure 5. AChE and MAOs multi-target strategy

BChE, IC_{50} = 0.49 μM for MAO-A, IC_{50} = 53.9 μM for MAO-B), while its profiles of cytotoxicity in the CHO-K1 cell line (IC_{50} = 5.5 μM) and hepatotoxicity in the HepG2 cell line (IC_{50} = 1.22 μM) were not sufficiently good, which was tested by MTT assay, respectively. The Li group [53, 68] reported two series of propargylamine-modified pyrimidinylthiourea derivatives, among which compound **60** (Figure 5) was the best compound in the first series with good selective inhibitory activity against AChE and MAO-B (IC_{50} = 0.324 μM for AChE, IC_{50} = 1.427 μM for MAO-B), and demonstrated mild antioxidant ability, good copper chelating ability, and the ability to ameliorate scopolamine-induced cognitive impairment in mice. While compound **61** (Figure 5) was the most promising compound in the second series with a dual functional profile of targeting AChE and MAO-B (IC_{50} = 32 nM for AChE, IC_{50} = 2.117 μM for MAO-B), and showed antioxidant ability, good copper chelating capacity *in vitro*, and the ability to alleviate scopolamine-induced cognitive impairment in mice.

2.4.1. Metal ions in the brain

Bioinorganic chemistry is a significant concept in therapeutic and diagnostic medicine, and the antitumor drug cisplatin (*cis*-[$Pt(NH_3)_2Cl_2$]), a profound prototype in the bioinorganic field, is still a widely used chemotherapeutic agent for cancer and improves the survival rates of patients worldwide [69]. Potential bioinorganic chemical agents are metal ions, active metal complexes, and biometal chelators, which are most often used to limit the adverse effects of metal ions overload, inhibit selected metalloenzymes, or facilitate metal ions redistribution [69]. Excess metal levels in the brain are associated with peptide aggregation and oxidative stress, ultimately leading to cell death, and are responsible for neurodegenerative disorders, such as AD, PD, Creutzfeldt-Jakob disease, and amyotrophic lateral sclerosis [70]. High levels and dysregulation of Cu^{2+}, Fe^{2+}, Zn^{2+}, and Ca^{2+}, which are the four most important biometal ions [71], are closely implicated in the pathogenesis of AD. Cu^{2+} and Zn^{2+} have been shown to induce the generation of toxic Aβ oligomers by binding to Aβ peptides and influencing the Aβ aggregation pathway [72], while the redox-active metals, Cu(I/II) and Fe(II/III), have been demonstrated to generate cytotoxic ROS and cause neuronal damage [73]. Thus, biometal chelators which can down-regulate the high levels of biometals might be a potential therapeutic strategy for the treatment of AD. The approved antifungal drug clioquinol (**12**) (Figure 2), a moderate metal chelator with 8-hydroxyquinoline as a scaffold, was shown to extract metals from extracellular Aβ

aggregates and shift them to copper-carrier proteins in parenchymal cells without perturbing the overall essential metal biochemistry in the brain in recent Phase II clinical trials [74]. PBT-2 (**13**) from Prana Biotechnology (Figure 2) is a second-generation derivative of clioquinol (**12**), another metal chelator with an 8-hydroxyquinoline scaffold, and has been in Phase II clinical trials for the treatment of AD [75].

2.4.2. AChE and metal ions multi-target strategy

Fernandez-Bachiller and co-workers [76] reported that hybrid **62** (Figure 6), fused with the AChEI tacrine (**4**) and the metal chelator drug clioquinol (**12**), showed potent human AChE inhibitory activity (IC_{50} = 5.5 nM) and Cu^{2+} ion chelating ability based on UV-vis spectrometry. The maximum absorption at 242 nm of **62** in Tris buffer suffered a redshift to 248 nm with the addition of $CuSO_4$, indicating the formation of complex **62**-Cu(II). Hybrid **64** (Figure 6) reported by the Kong group [77] is fused with tacrine (**4**) and a naturally occurring compound flavone (**63**), and exhibited a significant ability to inhibit AChE (IC_{50} = 0.13 μM), along with Cu^{2+} and Fe^{2+} ions chelating ability tested by UV-vis spectrometry with wavelength ranging from 200 to 500 nm, 79.1% self-induced Aβ aggregation inhibition at 20 μM, and low toxicity in SH-SY5Y neuroblastoma cells. Xie and co-workers [78] reported that a novel hybrid **66** (Figure 6), fused with tacrine (**4**) and coumarin (**65**) which is a natural product possessing AChE inhibitory activity [79], displayed the ability to inhibit AChE (IC_{50} = 92 nM), chelate Cu^{2+} and Fe^{2+} ions, and inhibit 67.8% self-induced Aβ aggregation at 20 μM. UV-vis spectrometry with wavelength ranging from 200 to 500 nm was used to test the metal chelating effect of **66**, and the increased absorbance of **66** along with increasing Cu^{2+} or Fe^{2+} concentrations indicated the occuring interactions between **66** and these metals.

Compound **67** (Figure 6) with a chromone scaffold reported by the Deng group [80] showed excellent inhibitory potency against rat AChE (IC_{50} = 70 nM), selective biometal chelating ability with Cu^{2+} via UV-vis spectrometry, moderate antioxidative activity, 59.2% self-induced Aβ aggregation inhibition at 25 μM, and 48.3% Cu^{2+}-induced Aβ aggregation inhibition at 25 μM. The electronic spectra of compound **67** were recorded when the salts $CuCl_2$, $FeSO_4$, $ZnCl_2$, or $AlCl_3$ were added, and the curve had a redshift (the peak at 340 nm shifted to 420 nm) with the addition of $CuCl_2$, whereas no significant shift was observed with the other salts, suggesting the formation of complex **67**-Cu(II). The Li group [72] reported a series of non-fused and non-assembly

pyrimidinylthiourea derivatives by screening hits from 630 compounds. Compound **68** (Figure 6) was the most promising compound which exhibited potent AChE inhibition (IC_{50} = 0.204 μM), specific Cu^{2+}-chelating ability with the maximum absorption dramatically decreased at 290 nm in the UV-vis spectroscopy assay, antioxidant effects, regulatory function toward Cu^{2+}-induced Aβ aggregation, low cytotoxicity, and moderate neuroprotection to human neuroblastoma SH-SY5Y

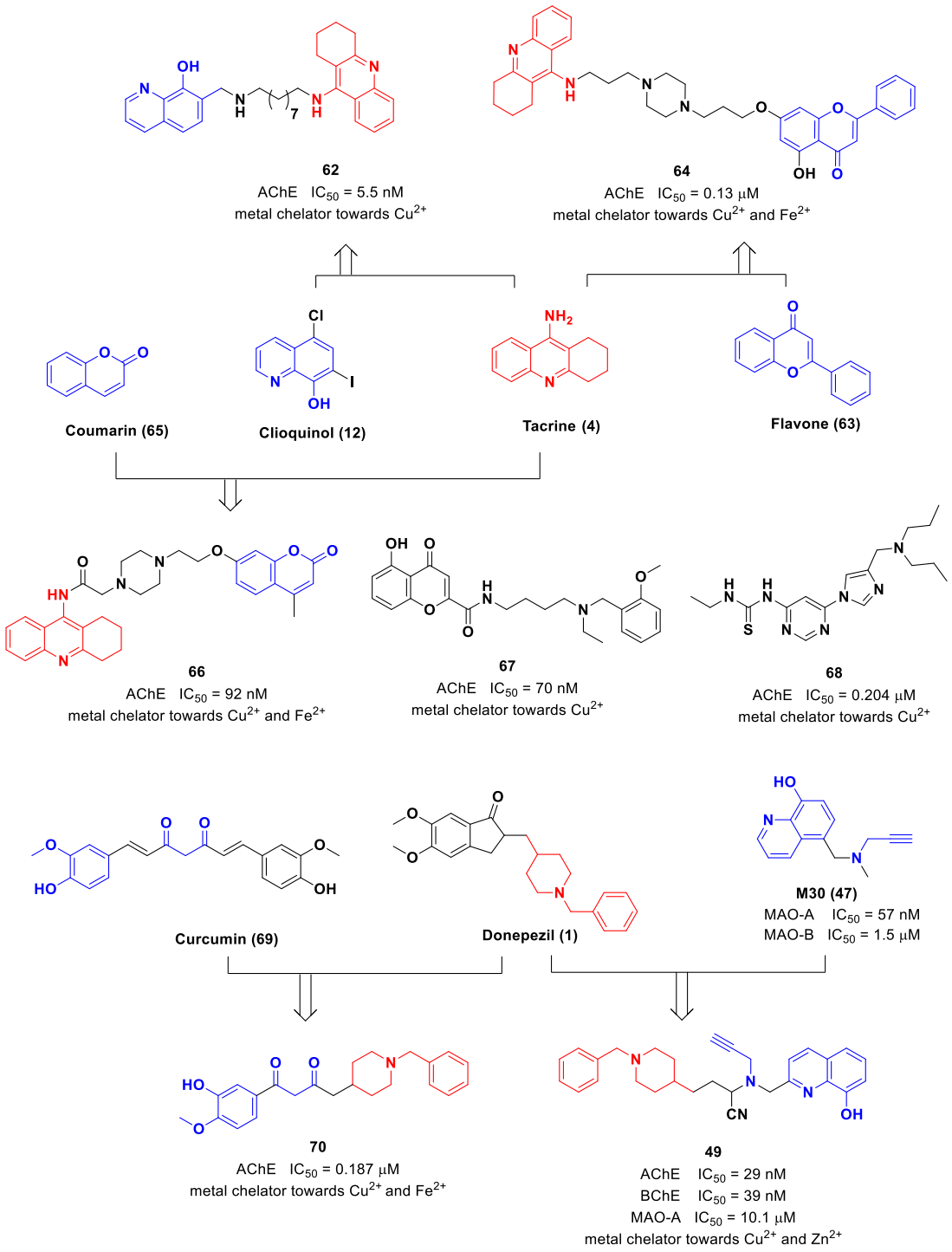

Figure 6. AChE and metal ions multi-target strategy

cells. Further experiments showed appropriate BBB permeability of **68** both *in vitro* and *in vivo*, and memory and cognitive function improvement in scopolamine-induced amnesia mice after the administration of compound **68**. Yan and co-workers [81] reported that a novel compound, hybrid **70** (Figure 6), fused with the AChEI donepezil (**1**) and curcumin (**69**) which is extracted from the rhizome of *Curcuma longa L.*, showed potent AChE inhibition (IC_{50} = 0.187 μM), metal-chelating ability with Cu^{2+} and Fe^{2+} via UV-vis spectrometry, 45.3% Aβ self-aggregation inhibition at 20 μM, and a remarkable antioxidant effect.

2.5.1. NMDA receptor

The glutamatergic hypothesis of AD is based on the NMDA receptor which plays a crucial role in modifying major forms of synaptic plasticity, certain types of learning and memory formation, and consolidation of short-term memory into long-term memory under physiological conditions [82], and the states that appropriate inhibition toward NMDA receptor would ameliorate overall condition of AD patients. The NMDA receptor is a type of ionotropic glutamate receptor composed of two NR1 subunits and two NR2 (NR2A-D) subunits or occasionally NR3 subunit, and can be activated when bound with the excitatory neurotransmitter glutamate or the modulatory agent glycine (Gly). The NMDA receptor exerts important bio-functions in normal synaptic transmission, meaning it cannot be totally antagonized, whereas glutamate-related excitotoxicity and cell death could be caused when NMDA receptors are overstimulated by excess glutamate [83]. An optimal balance of NMDA receptors between glutamate stimulation and glutamate-related excitotoxicity is crucial to achieve the ideal treatment of AD.

Memantine (**14**) (Table 1) (Figure 2) from Merz Pharma is a noncompetitive antagonist with a relatively low to moderate affinity toward NMDA receptor. Memantine (**14**) was approved in 2002 and is the only NMDA receptor antagonist drug used in the clinic for the treatment of AD, while it still cannot prevent neuronal loss, stop deterioration of dementia, or reverse the disease progression of AD. Memantine (**14**) was formulated as tablets with four dose forms (5, 10, 15, 20 mg) and an oral solution (10 mg/mL) in the European Union, whereas lower doses of tablet forms (5, 10 mg) and a more dilute oral solution (2 mg/mL) in the USA were licensed a year later. It is well absorbed by oral administration with nearly 100% bioavailability, it undergoes partial hepatic metabolism with a half-life of 60-100 h, and 48% of the administered drug is excreted as the original with unchanged structure in the urine [14]. Riluzole (**15**) (Figure 2) from Covis Pharma is

a sodium channel blocker approved in 1996 for the treatment of amyotrophic lateral sclerosis (ALS), is currently in Phase II clinical trials for mild AD and has exhibited a cognitive improvement effect. Riluzole (**15**) can lower extracellular glutamate levels, inhibit presynaptic glutamate release, and enhance glutamate transporter activity [2], suggesting a new clinically applicable therapeutic approach for AD. The withdrawn antihistamine drug dimebon (**16**) (Figure 2), an NMDA receptor antagonist with the ability to bind AChE and NMDA receptor, was evaluated by Pfizer in Phase III clinical trials for the treatment of moderate to severe AD, but negative results were announced, and the development of dimebon (**16**) was discontinued [55].

2.5.2. AChE and NMDA receptor multi-target strategy

Cholinergic and glutamatergic neuronal systems influence each other through their joint dysfunction, and cholinergic deficits and glutamate-related excitotoxicity are central to AD pathology [84]. Excessive activation of NMDA receptor is implicated in the degenerative process of cholinergic neurons in AD, with the instance that neuronal decline caused by direct injection of NMDA into the rat basal forebrain generates reduced activity of choline acetyltransferase in the cortex [85]. Thus, the AChE and NMDA receptor multi-target strategy can affect the cholinergic and glutamatergic systems and is becoming an important idea because NMDA receptor antagonists can confront neurodegeneration and AChEIs can recover memory and cognition. Namzaric from Allergan approved in 2015 for the treatment of AD is a once-daily fixed-dose combination drug and comprised of memantine (**14**) hydrochloride and donepezil (**1**) hydrochloride, under the consideration of reducing pill burden and alleviating administration. In addition, caregivers can sprinkle the drug into food for patients who have difficulty in swallowing.

The Rosini group [86] has done much work to develop novel compounds with dual AChE and NMDA receptor inhibitory potency for potential treatment of AD. Hybrid **72** (Figure 7), named carbacrine, was fused with AChEI tacrine (**4**) and carbazole (**71**), and showed dual inhibitory activity against AChE and NMDA receptor (IC_{50} = 2.15 nM for AChE, IC_{50} = 0.74 μM for NR1/NR2A), 36.0% self-induced Aβ aggregation inhibition, 57.7% AChE-induced Aβ aggregation inhibition, and the ability to reduce oxidative stress [87]. Later, compound **73** (Figure 7), named memagal, was reported as a novel hybrid formed by linking the AChEI galantamine (**2**) and the NMDA receptor antagonist drug memantine (**14**) [88]. Memagal (**73**) showed remarkably inhibitory potency against AChE (IC_{50} = 1.16 nM), and NMDA receptor inhibition which was

tested by a [^{3}H] MK-801 binding assay (K_i = 4.6 μM, the value was derived from an iterative curve-fitting procedure). Further investigation revealed that hybrid **73** possessed NR2B-containing NMDA receptor inhibition based on a [^{3}H] ifenprodil binding assay (K_i = 4.6 μM, the value was derived from an iterative curve-fitting procedure) and could inhibit NMDA (500 μM) mediated

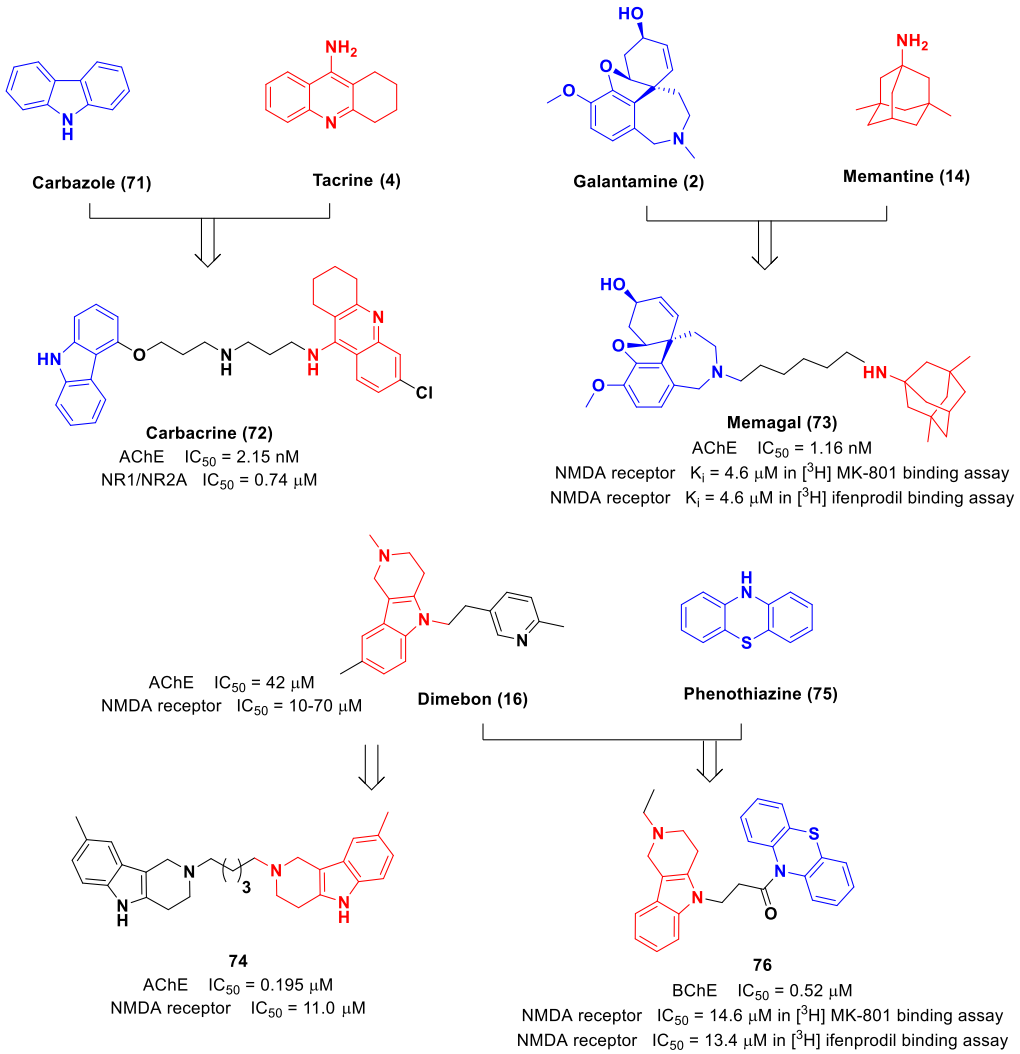

Figure 7. AChE and NMDA receptor multi-target strategy

neurotoxicity in SH-SY5Y cell viability assay (IC_{50} = 0.28 nM), showing a highly potent neuroprotective effect. Dimebon (**16**) (Figure 7) has a multi-target profile for AD, while the activity against AChE and NMDA receptor is weak (IC_{50} = 42 μM for AChE, IC_{50} = 10-70 μM for NMDA receptor). In contrast, the optimized dimebon-derivative **74** (Figure 7) showed a potent multi-target profile against both AChE and NMDA receptor (IC_{50} = 0.195 μM for AChE, IC_{50} = 11.0 μM for NMDA receptor) and 67.0% self-induced Aβ aggregation inhibitory potency [89]. Another interesting compound, hybrid **76** (Figure 7), reported by Makhaeva and co-workers [90],

was fused with dimebon (**16**) and phenothiazine (**75**). And compound **76** showed dual BChE and NMDA receptor inhibition potency with IC_{50} = 0.52 μM for BChE, IC_{50} = 14.6 μM for NMDA receptor in a [^{3}H] MK-801 binding assay, and IC_{50} = 13.4 μM for NMDA receptor in a [^{3}H] ifenprodil binding assay.

2.6.1. 5-HT receptors

The serotonergic neurotransmitter system has crucial physiological functions in emotion and depression, while it is implicated with the cholinergic system in cognitive function of neurodegenerative disease [91]. 5-HT, also named serotonin, is considered as an inhibitory neurotransmitter compared with the excitatory neurotransmitter glutamate, while 5-HT has the bio-function to generate feelings of well-being and happiness, suggesting that the concentration of 5-HT should be increased and the serotonergic system should be enhanced in depressive disorder. Selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine and paroxetine, are primarily approved as antidepressant drugs with the ability to increase 5-HT concentration in the synaptic cleft by inhibiting neuronal reuptake of 5-HT, improving the emotional state of depressive patients. There are seven subtypes of 5-HT receptors (5-HT_1 to 5-HT_7 receptors), which are classified by their structural and functional characteristics. 5-HT_{1A}, 5-HT_4, 5-HT_6, and 5-HT_7 receptors in the brain are associated with learning and memory. 5-HT_{1A} receptor plays a significant role in therapeutics for major depressive disorder [92], and both agonists and antagonists of this receptor could be potential therapies for AD. 5-HT_4 receptor is involved in memory processes, and partial agonists of this receptor could be utilized to treat the cognitive symptoms of AD [93]. 5-HT_6 receptor is expressed primarily in the cortex and the hippocampus areas of the brain and is associated with learning and memory processes, and antagonists of this receptor would be beneficial for ameliorating AD symptoms.

Lecozotan (**17**) (Figure 2) from Pfizer is an orally available, potent and selective 5-HT_{1A} receptor antagonist in Phase II/III clinical trials for the treatment of AD. PRX-03140 (**18**) (Figure 2) from Ology Bioservices is a 5-HT_4 receptor partial agonist in Phase II clinical trials for the treatment of AD. Idalopirdine (**19**) (Figure 2) from Otsuka and Lundbeck is an orally available and selective 5-HT_6 receptor antagonist with promising efficacy and safety data in Phase II trials, but it did not meet its primary efficacy endpoint and was discontinued in Phase III clinical trials [2]. Intepirdine (**20**) (Figure 2) from Axovant Sciences is another 5-HT_6 receptor antagonist in Phase

III clinical trials for the treatment of mild-to-moderate AD, and Phase II data indicated that the treatment of intepirdine (**20**) in combination with AChEI donepezil (**1**) may improve cognitive function in mild-to-moderate AD patients [2].

2.6.2. AChE and 5-HT receptors multi-target strategy

The serotonergic and cholinergic systems have physiological and pathological interactions with each other. Brain functions mediated by 5-HT_4 receptor require a synergistic effect from cholinergic neurotransmission [94], and activation of 5-HT_4 receptor can enhance the release of Ach in the hippocampus [95]. 5-HT_4 receptor agonists could promote the nonamyloidogenic cleavage of APP, forming neurotrophic human soluble amyloid precursor protein α (sAPP-α) fragments and decreasing Aβ secretion in primary neurons [96]. Meanwhile, 5-HT_6 receptor antagonists are thought to have the ability to enhance cholinergic neurotransmission [97]. Thus, AChE and 5-HT receptors could act synergistically on cognitive deficits, Aβ-mediated damage, and unhealthy emotions, meaning a multi-target strategy targeting these two targets could lead to a potential treatment for AD.

RS67333 (**77**) (Figure 8) is a 5-HT_4 receptor partial agonist and well-known for its precognitive effect, and showed a synergistic effect with AChEI donepezil (**1**) on memory performance in mice [98]. RS67333 (**77**) has the ability to down-regulate Aβ level by activating 5-HT_4 receptor, directly inhibit human AChE, and the capacity to induce sAPP-α release, reducing amyloid plaque formation [99] (IC_{50} = 403 nM for AChE, K_i = 13 nM for 5-HT_4 receptor, EC_{50} = 27.2 nM for sAPP-α release). The Dallemagne group [100] reported a further optimized compound **78** (Figure 8), which had a multi-target profile with both AChE inhibitory effect and 5-HT_4 receptor agonist activity (IC_{50} = 95.8 nM for AChE, K_i = 4.2 nM for 5-HT_4 receptor). Interestingly, compound **78** was more antagonistic than agonistic toward 5-HT_4 receptor, and molecular modeling studies failed to explain the antagonist profile of compound **78**. Later, the same group [101] reported that the hybrid **79** (Figure 8), named donecopride, which was fused with RS67333 (**77**) and donepezil (**1**), showed dual AChE inhibition and 5-HT_4 receptor agonist activity (IC_{50} = 16 nM for AChE, K_i = 6.6 nM for 5-HT_4 receptor). Donecopride (**79**) stimulated the nonamyloidogenic 5-HT_4 receptor-mediated cleavage of APP and had greater potency in promoting neurotrophic sAPP-α release (EC_{50} = 11.3 nM) compared with RS67333 (**77**) [99]. Donecopride (**79**), which presented profiles with promising druggability parameters, favorable bioavailability and low toxicity,

exhibited a precognitive effect with an improvement in memory performance observed at 0.3 and 1 mg/kg of donecopride (**79**) administration via intraperitoneal injection in further *in vivo* studies. Hybrid **81** (Figure 8), reported by the Li group [102], was fused with the AChEI tacrine (**4**) and vilazodone (**80**) which is 5-HT_{1A} receptor partial agonist and 5-HT reuptake inhibitor for the treatment of major depressive disorder (Figure 8), and exhibited moderate ChEs inhibitory activities, 5-HT_{1A} receptor agonist activity, and 5-HT reuptake inhibitory activity (IC_{50} = 1.72 μM for AChE, IC_{50} = 0.34 μM for BChE, EC_{50} = 0.36 nM for 5-HT_{1A} receptor, IC_{50} = 20.42 nM for 5-HT reuptake). Further investigation showed that compound **81** also possessed good BBB permeability, making it a promising compound for the treatment of depression with cognitive impairment.

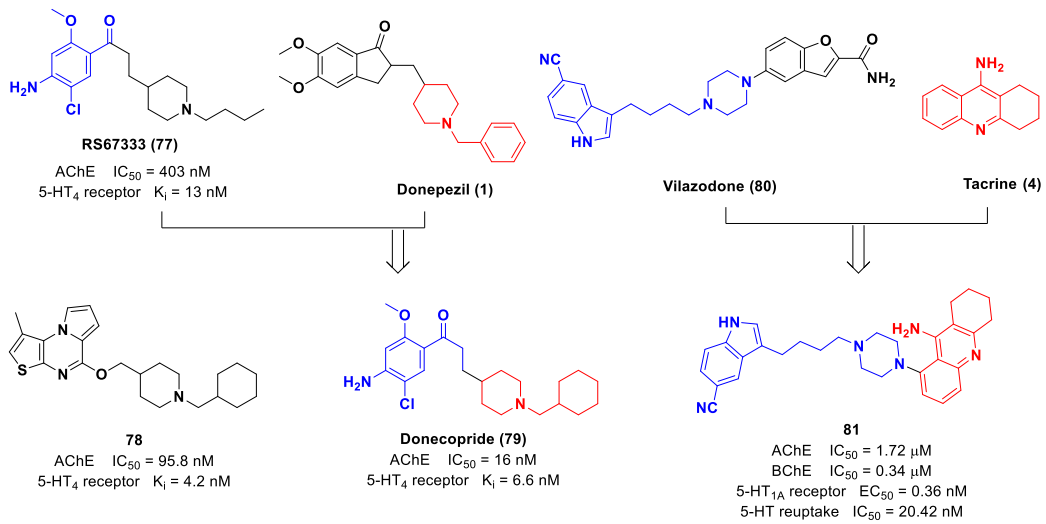

Figure 8. AChE and 5-HT receptors multi-target strategy

2.7.1. Histamine receptors

The histaminergic system is composed of histamine and its receptors, plays an important role in maintaining brain homeostasis function and higher brain functions in the CNS, and participates in smooth muscle contraction, dilatation of capillaries, gastric acid secretion, and inflammation in the periphery. Histamine is an endogenous biogenic amine distributed throughout the body that acts as a neurotransmitter in nervous centralis and a local mediator in the periphery, and has high concentrations in the lung, skin, and gastrointestinal tract, carrying out its physiological functions with histamine receptors [103]. Histamine receptors, which are also distributed throughout the

body, are G protein-coupled receptors with different bio-functions. There are four histamine receptor subtypes, which are H_1 receptor, H_2 receptor, H_3 receptor, and H_4 receptor. H_1 receptor activation mainly promotes allergic symptoms in the periphery and antagonists against this receptor are typically anti-allergic drugs, such as loratadine, while H_2 receptor activation primarily stimulates the secretion of gastric acid and antagonists against this receptor can be antiulcer drugs, such as ranitidine. The interesting H_3 receptor is an antoreceptor and heteroreceptor, providing negative feedback on histaminergic system and inhibiting the release of other neurotransmitters when it is activated, and antagonists against this receptor could augment the release of histamine and other neurotransmitters. H_4 receptor is the most recently discovered histamine receptor, found in immunocompetent cells, and the development of anti-inflammatory drugs based on H_4 receptor is anticipated [103].

ABT-288 (**21**) (Figure 2) from AbbVie is an H_3 receptor antagonist in Phase II clinical trials for the treatment of mild-to-moderate AD, but its clinical efficacy is not sufficient [104]. GSK-239512 (**22**) (Figure 2) from GlaxoSmithKline is an H_3 receptor antagonist in Phase II clinical trials for the treatment of AD and was discontinued due to lack of improvement in memory test [105], suggesting that single H_3 receptor antagonists are not sufficiently effective in treating cognitive dysfunction in AD patients. SUVN-G3031 (structure not disclosed) from Suven Life Sciences is an H_3 receptor antagonist in Phase I clinical trials for the treatment of cognitive disorders associated with AD patients.

2.7.2. AChE and H_3 receptor multi-target strategy

H_3 receptor is considered as a significant target in AD because its activation decreases the presynaptic release of ACh, and its blockade augments the presynaptic release of ACh and improves cholinergic neurotransmission in the cortex. Thus, a multi-target strategy involving AChE and H_3 receptor may exert a synergistic effect on up-regulating synaptic levels of ACh, which is a potential approach for the treatment of AD, but studies exploring this strategy have rarely been reported.

Huang and co-workers [106] reported a series of compounds with a quinoxaline scaffold that showed related inhibitory activities against AChE, H_3 receptor, and BACE-1 targets. The structure of the compounds was first designed to fuse the BACE-1 inhibitor **82** (IC_{50} = 11 nM) with the AChEI compound **83**, named BYYT-25 (IC_{50} = 50 nM), two compounds reported by the same

group. In addition, the dihydroquinazoline moiety of **82** and benzyl pyrrolidine fragment of **83** were selected to assemble the scaffold of novel H_3 receptor inhibitor. Then, a virtual database consisting of plentiful compounds with novel scaffolds was screened on a pharmacophore model of BACE-1 inhibitor and next filtered by a molecular docking model of AChEI, resulting in the selection of 17 quinoxaline derivatives. Among these compounds, hybrid **84** (Figure 9) showed the most promising profiles, with potent activity toward AChE, H_3 receptor, and BACE-1 targets (IC_{50} = 483 nM for AChE, IC_{50} = 280 nM for H_3 receptor antagonism, IC_{50} = 189 nM for H_3 receptor inverse agonism, 46.64% inhibition for BACE-1 at 20 μM) and high selectivity for H_3 receptor over H_1, H_2 or H_4 receptors. Further receptor-binding studies of hybrid **84** demonstrated that it could achieve several key interactions with AChE and BACE-1, suggesting that quinoxaline derivatives may be a potential therapeutic for AD.

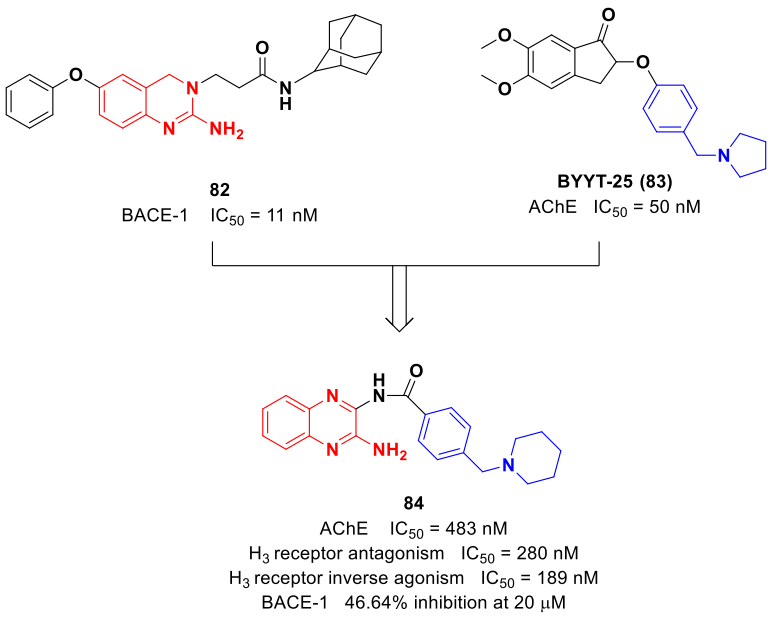

Figure 9. AChE and H_3 receptor multi-target strategy

2.8.1. PDEs

PDEs are enzymes with the ability to break phosphodiester bonds, and usually referred to cyclic nucleotide phosphodiesterases. They are a group of enzymes that can hydrolyze and degrade intracellular second messengers, such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), thus playing a major role in cell signal transduction throughout the brain and the periphery [107]. PDEs are classified into eleven isoenzyme families,

from PDE1 to PDE11, of which most have several subtypes, contributing to in total 100 specific human PDEs according to the current estimation [108]. Among these families, PDE4, 7, and 8 are specific enzymes for cAMP hydrolysis, and PDE5, 6, and 9 are specific enzymes for cGMP hydrolysis. PDE1, 2, 3, 10, and 11 are dual-substrate enzymes that can hydrolyze both cAMP and cGMP [109]. PDEs with the ability to degrade second messengers are important regulators of signal transduction in neuroplasticity and neuroprotection, and thus, PDEs inhibitors with the capacity to up-regulate the concentrations of cAMP and cGMP are receiving increased attention as potential agents to treat cognitive decline in AD. In addition, the nitric oxide/soluble guanylyl cyclase/cGMP (NO/sGC/cGMP) signaling pathway plays a pivotal role in learning and memory by regulating synaptic transmission and synaptic plasticity in the hippocampus and the cerebral cortex [110], and PDEs inhibitors could have a positive effect on that pathway.

Vinpocetine from Gedeon Richter is a PDE1 inhibitor for the treatment of dementia and cognitive disorders that was approved in 1980, but it was reported to be ineffective on cognitive impairment in AD patients [109]. Cilostazol from Otsuka Pharma is an oral PDE3 inhibitor for the improvement of various symptoms of chronic arterial obstruction and was originally launched in 1988. Cilostazol was tested as a co-treatment with the AChEI donepezil (**1**) for patients with mild-to-moderate AD. Rolipram from the National Institute of Mental Health is a PDE4D inhibitor in Phase II clinical trials for the treatment of major depression and had beneficial effects in a hippocampal-dependent memory assay, confirming that PDE4D plays a crucial role in memory consolidation [111]. MK-0952 (**23**) (Figure 2) from Merck is a PDE4 inhibitor in Phase II clinical trials for the treatment of mild-to-moderate AD, but no recent report could be found. PF-04447943 (**24**) (Figure 2) from Pfizer is a PDE9A inhibitor in Phase I clinical trials for the treatment of sickle cell disease and was tested in Phase II clinical trials for the treatment of mild-to-moderate AD, while it was discontinued in 2011 due to the absence of any effects on cognition. BI-409306 (**25**) (Figure 2) from Boehringer Ingelheim, a PDE9A inhibitor in Phase II clinical trials for the treatment of schizophrenia, was evaluated in Phase II clinical trials for the treatment of cognitive impairment in AD patients, and it was discontinued because the efficacy endpoint was not met.

2.8.2. AChE and PDEs multi-target strategy

PDEs have eleven isoenzyme families with extensive physiological functions, meaing PDEs could be drug targets in many signaling pathways. Whereas multi-target strategies involving

targets of AChE and PDEs are rarely reported, indicating that such potential therapies would need to be explored for the treatment of AD. As a special cGMP hydrolytic enzyme, PDE5 has only one hypotype, PDE5A, and is distributed in the hippocampus, cortex and cerebellum of the brain [112]. Tadalafil (**85**) (Figure 10) of Lilly Icos, a selective PDE5 inhibitor drug launched in 2003 for the treatment of erectile dysfunction (ED), was found that it has the ability to reverse cognitive impairment and improve learning and memory in J20 transgenic mouse model of AD [113].

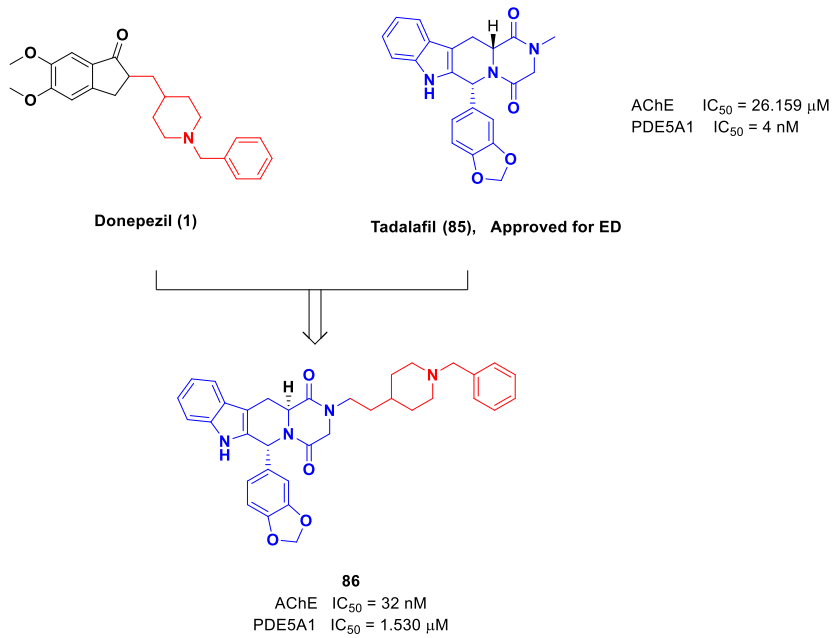

Figure 10. AChE and PDEs multi-target strategy

Repurposing or redeveloping existing drugs for new uses is an important drug discovery strategy with effective and quick characteristics. Through the screening of old drug library, the Li group [114] found that PDE5A inhibitor drug tadalafil (**85**) has certain AChE inhibitory potency (IC_{50} = 26.159 μM for AChE, IC_{50} = 4 nM for PDE5A1). The long half-life and safety of tadalafil (**85**) for the chronic treatments of ED and hypertension make it a good lead compound to discover dual-target inhibitors against AChE and PDE5, being a potential therapy for the treatment of AD. To further explore this synergistic therapeutic route, a series of novel tadalafil (**85**) derivatives, as the first-generation of dual-target inhibitors toward AChE and PDE5, were synthesized and biologically evaluated. The most promising compound **86** (Figure 10), fused with AChEI donepezil (**1**) and tadalafil (**85**), exhibited good inhibitory potency against both targets of AChE and PDE5 (IC_{50} = 32 nM for AChE, IC_{50} = 1.530 μM for PDE5A1), excellent selectivity among

relative enzymes (IC_{50} = 3.880 μM for BChE, IC_{50} > 100 μM for PDE2A1, PDE3A, PDE4D3, PDE6C, PDE7A and PDE9A2), and improved BBB penetrability. Further *in vivo* experiments showed that citrate of **86** could reverse the cognitive dysfunction in scopolamine-induced AD mice with comparable curative effect against donepezil (**1**), and had effect on enhancing cAMP response element-binding protein (CREB) phosphorylation, a crucial factor in memory formation and synaptic plasticity [115], by increasing cGMP levels, which may ameliorate the cognitive impairment and restore synaptic function in AD.

3. Multi-target strategies without AChE

Multi-target strategies are not limited to strategies involving AChE, and more diverse structures of hits could be designed based on the specific and multiple protein pockets of AD-associated targets. In the disease network of AD, several targets involving AChE or not, could implicated with others, and their respective signaling pathways could cross with others, contributing together to the development of AD progression. The importance of AChE in disease network is prominent, while the potential possibility among other targets should not be neglected for AD therapy. Thus, seeking the specific relationships in the disease network between targets without AChE is also a significant strategy for the treatment of AD. In this section, three multi-target strategies without AChE are demonstrated (Figure 1).

3.1. BACE-1 and GSK-3β multi-target strategy

The histopathological hallmarks in the brain of AD patients are senile plaques and NFTs, and BACE-1 and GSK-3β are two key targets responsible for each pathological cascade, suggesting that a dual inhibitor against BACE-1 and GSK-3β could produce a downstream synergistic effect [116]. The Cavalli group [117] reported a series of triazinone derivatives as the first generation of dual BACE-1 and GSK-3β inhibitors, which were derived from a cyclic amide group and a guanidine moiety under the guidance of a fragment-based approach. Among these derivatives possessing balanced micromolar affinities for both BACE-1 and GSK-3β, compound **87** (Figure 11) showed the most promising profiles, with the best balanced inhibitory potency against BACE-1 and GSK-3β (IC_{50} = 18.03 μM for BACE-1, IC_{50} = 14.67 μM for GSK-3β), effective neuroprotective and neurogenic activities due to the modulation of GSK-3β and simultaneous inhibition toward both targets, and no sign of neurotoxicity in glial and neuronal cells. Further *in*

vivo investigation in mice indicated that compound **87** displayed good drug-like properties in oral bioavailability (66%) and BBB penetration (a brain concentration of 0.62 μM of compound **87** was observed 30 min later when a very low dose of 3 mg/kg was administered with compound **87**). The Belluti group [118] reported another series of dual inhibitors targeting BACE-1 and GSK-3β that showed good neuroprotective and pharmacokinetic properties. This series of inhibitors was based on the natural product curcumin (**69**) which possesses efficacy and safety for both prevention and treatment of various disorders [119]. In this study, the curcumin derivative compound **88** (Figure 11) had the most balanced concurrent inhibitory activity against BACE-1 and GSK-3β (IC_{50} = 0.97 μM for BACE-1, IC_{50} = 0.90 μM for GSK-3β), exerted neuroprotective activity by inducing the NAD(P)H:quinone oxidoreductase 1 (NQO1) enzyme, possessed a weak antioxidant effect, and showed brain permeability, making it a promising drug candidate.

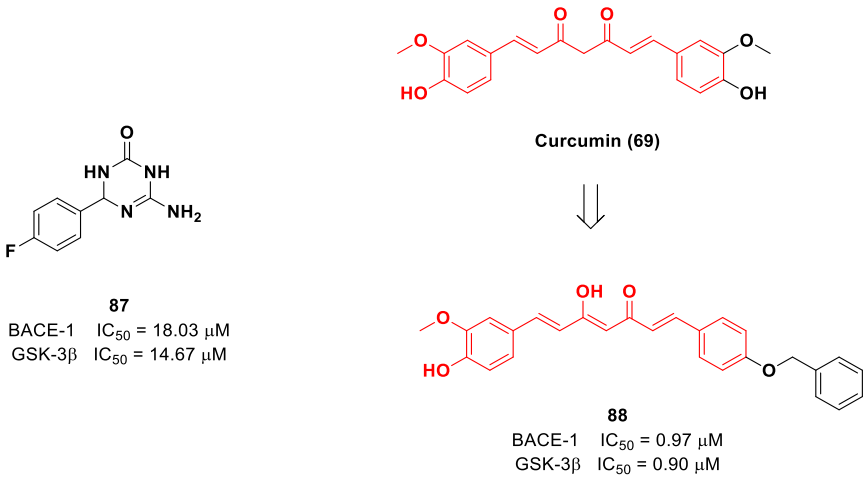

Figure 11. BACE-1 and GSK-3β multi-target strategy

3.2. MAO-B and metal ions multi-target strategy

The Li group [120] reported that a series of novel hybrids fused with the main pharmacophores of selegiline (**54**), an MAO-B inhibitor (IC_{50} = 18.5 nM) approved in 1981 for the treatment of PD, and clioquinol (**12**), a moderate metal chelator approved as an antifungal drug, showed MAO-B inhibitory potency, antioxidant activity, biometal chelating ability, and effective inhibition against Cu(II)-induced Aβ aggregation. The most promising compound **89** (Figure 12) exhibited good inhibitory activity against MAO-B (IC_{50} = 0.21 μM for MAO-B), good antioxidant activity (oxygen radical absorbance capacity (ORAC) = 4.2), and the ability to permeate the BBB in a PAMPA-BBB study. UV-vis spectrometry was used to investigate the metal-chelating ability of

compound **89**, and the results indicated that it could interact with Cu^{2+}, Fe^{2+}, and Zn^{2+} metal ions, especially with Cu^{2+}ion. The maximum absorption peak at 236 nm of the solution of compound **89** was obviously decreased and another absorption peak at 276 nm suffered a redshift to 282 nm after $CuSO_4$ was added to the solution, while the specific absorption peaks at 236 nm and 276 nm shifted slightly after $FeSO_4$ or $ZnCl_2$ was added to the solution, respectively, suggesting that compound **89** possess the biometal chelating ability. These results indicated that compound **89** had the potential profiles to be a good multi-target and multifunctional agent for the treatment of AD.

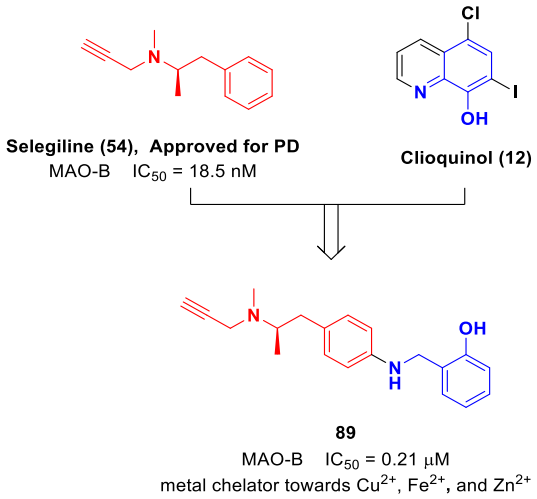

Figure 12. MAO-B and metal ions multi-target strategy

3.3. PDEs and metal ions multi-target strategy

Down-regulation of the NO/sGC/cGMP pathway and impaired homeostasis of biometals play significant and malignant roles in AD, suggesting that PDEs inhibition and metal chelation would have synergistic therapeutic effect and a multi-target strategy involving PDEs and metal chelating is possible.

Su and co-workers [121] reported that a novel hybrid **90** (Figure 13), fused with the PDE9A inhibitor PF-04447943 (**24**) (Figure 2) and clioquinol (**12**) which is a moderate metal chelator approved as an antifungal drug, showed inhibitory potency against PDE9 (IC_{50} = 34 nM) with high selectivity over other PDEs (55-fold), notable Cu^{2+}-induced Aβ aggregation inhibition, and favorable BBB permeability. Hybrid **90** also had remarkable metal-chelating capacity toward metal ions Cu^{2+}, Fe^{2+}, and Zn^{2+}, tested by UV-vis spectrophotometry. The absorption peaks at 259 nm of the solution of compound **90** suffered a redshift to 273 nm after incubation of compound **90** with Cu^{2+}, and similar results were obtained upon incubation of compound **90** with Fe^{2+} and Zn^{2+}.

These results indicated that hybrid **90** could be a promising compound for the treatment of AD. A novel series of hybrids, reported by the Li group [122], were fused with the pharmacophores of the moderate metal chelator clioquinol (**12**) and the PDE4D inhibitor moracin M (**91**) (IC_{50} = 2.91 μM) which is a natural product isolated from the root bark of *Morus alba Linn* with antioxidant and anti-inflammatory activities. The most promising compound, hybrid **92** (Figure 13), had good profiles with excellent PDE4D inhibitory potency (IC_{50} = 0.32 μM), significant antioxidant effects, appropriate biometal chelating functions toward the metal ions Cu^{2+}, Fe^{2+}, and Zn^{2+} according to a UV-vis spectroscopy assay, and the ability to modulate self-induced Aβ aggregation and Cu^{2+}-induced Aβ aggregation.

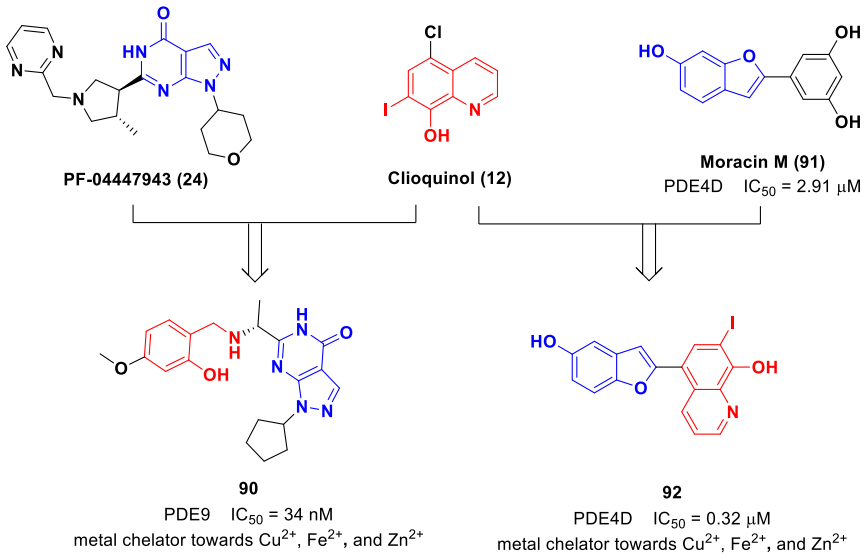

Figure 13. PDEs and metal ions multi-target strategy

4. Conclusion

Currently, AD is an incurable disease with a complicated disease network and brings enormous suffering to patients and their families. Therefore, there is an urgent need to be met for the society. Huge efforts have been made by research groups worldwide to conquer this disease, and lots of AD-related targets have been found with the attempt to solve the puzzle caused by the disease network. Nine major targets were discussed in this overview, among which AChE demonstrates a significant influence on this field, and corresponding single-target drugs or candidates (25 instances) were exhibited. Moreover, the therapies based on other targets, such as cyclooxygenase

(COX) and 5-lipoxygenase (5-LOX) [123] which are not discussed here, can also be considered as potential targets for the possible treatment of AD. There are also some NCEs, with multifunction effects and overall cognitive improvement on AD-related animal models, not discussed here in detail because their corresponding binding-targets have not been clearly figured out. But these NCEs have showed enough and excellent curative effect to be drug candidates for the treatment of AD. For example, mecripyrine (SCR1693) of Yene Pharma, an AChE inhibitor which was developed based on multi-target design strategy, could remarkably attenuate tau hyperphosphorylation and improve memory deficits, and preserve dendrite morphologies as well as spine density [124]. It is in the Phase I clinical trials in China for the potential treatment of AD [125].

Considering the intricate pathogenesis of AD and the shortcomings of single-target drugs, we think the multi-target design strategies are the frontier scientific research for potential treatment of AD because efficient and synergetic modulation toward several targets could be realized by multi-target inhibitors to better affect the disease network and control AD progression than single-target drugs. Eleven multi-target design strategies were discussed in this overview and various promising compounds (42 instances) for the potential treatment of AD were presented. It is deficient for current situation that existing scaffolds are still not sufficient to enrich the compound libraries for AD treatment, and most designed compounds with dual-target activities are hybrids of known structures or drugs. Nevertheless, natural products [126] and computer-aided drug design could be beneficial and promising to explore novel scaffolds and structures with a better curative effect. More efforts are needed to make the multi-target compounds eventually developed into new drugs which could be successfully used in the clinic as ideal AD treatments.

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