From Antioxidant Chelators to Site-Activated Multi-Target Chelators Targeting Hypoxia Inducing Factor, Beta-Amyloid, Acetylcholinesterase and Monoamine Oxidase A/B

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Abstract: chelators hold great promise as disease-modifying drugs for Alzheimer's therapy, and recent research efforts have focused on designing multi-target chelators with increased targeting and efficacy through rational drug design. In this review, we discuss our research studies on the rational design of new multi-target chelators with the potential not only to simultaneously modulate several disease-related targets, but also contain features designed to improve the BBB permeability, increase the brain targeting, and minimize potential side effects. These new chelators include neuroprotective chelators with brain selective monoamine oxidase (MAO) A/B inhibitory activity, acetylcholinesterase (AChE) inhibitors with site-activated chelating and neurogenesis activity, and AChE-MAO A/B inhibitors with site-activated chelating and neurogenesis activity.

Keywords: AChE-MAO A/B inhibitors, HLA20, HLA20A, M30, M30D, multi-target chelators, site-activated, Alzheimer's disease.

INTRODUCTION

The anti-Aβ (amyloid beta) agents are now the most investigated drugs for Alzheimer's disease (AD) because they may have disease-modifying effects by preventing Aβ aggregation and plaques. However, these drugs have not proven effective in the clinic, and moreover, disappointed results are coming continuously from large-scale clinical trials on these anti-Aβ drugs [1]. In fact, none of the currently pathogenetic hypotheses can explain the overall abnormalities in AD. Effective treatments of AD may need comprehensive approaches, either multifunctional approach or combination therapy [2]. Indeed, comprehensive approaches have proven more effective than single target-based approaches in treating multi- factorial diseases such as depression and cancer, and have become a common practice in the clinic [3]. In Alzheimer's field, encouraging news come from recent studies that patients treated with acetylcholinesterase (AChE) inhibitors and memantine have shown greater efficacy over monotherapy and significantly slowed the rate of disease progression [4]. In addition, in contrast to traditional views that interaction with several targets increases side effects, studies indicated that simultaneous moderate inhibition of both β and γ-secretase was found effective and safe in AD mice, with no evidence of toxicity, while completely knocking out β or γ-secretase led to serious side effects [5]. Studies have also shown that synergistic combination therapy tends to improve therapeutically relevant selectivity, and can achieve desired therapeutic efficacy with decreased doses of each drug by overcoming compensatory mechanisms, thereby minimizing toxicity and other side effects related to high doses of single drugs [6].

Recent years, numerous compounds have been specifically designed to possess multi-target profiles as potential drugs for AD/PD [2]. Multi-target drugs may have fewer side effects since they are prodrugs by shedding their specific moieties to receptor sites. Furthermore unlike polyphatmacology, there would not be drug-drug interaction, or metabolite-metabolite or drug-metabolite interactions. One example is mirtazapine (Remeron, Avanza, Zispin), one of the most effective multi-target drugs for depression in the clinic [7]. It acts by antagonizing the adrenergic α_2-auto-receptors and α_2-heteroreceptors as well as by blocking 5-HT_2 and 5-HT_3 receptors in the central nervous system (CNS). Mirtazapine has a lower risk to cause many of the side effects encountered with other antidepressants, such as decreased appetite, insomnia, nausea and vomiting [7]. It is superior to all of the selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) reboxetine, bupropion, and mianserin in terms of antidepressant efficacy [7]. The success of multi-target drugs such as mirtazapine indicates the clinical feasibility of designing multi-target ligands to treat CNS disorders such as AD and PD. Examples of the multi-target drugs under

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development for AD/PD include AChE inhibitors with additional activity against Aβ aggregation, β-secretase, monoamine oxidase (MAO), serotonin transporter (SERT), oxidative stress, or metal (Fe, Cu, and Zn) dyshomeostasis [2]. This review will mainly focus on selected examples from our research to demonstrate the path from antioxidant chelators to multi-target site-activated chelators targeting Aβ aggregation, AChE and MAO A/B. For comparison, a few examples of the multi-target chelators developed from others are also presented in Table **1**. Some of these new compounds have emerged as innovative drug candidates with the potential not only to treat AD symptom, but also to address the disease behaviors and slow down its progression as well.

FIRST GENERATION CHELATORS

Oxidative stress is one of the earliest, if not the earliest, change in AD pathogenesis, as extensive oxidative damage was observed in the brains of persons with MCI (mild cognitive impairment), a condition that often precedes AD [8]. Oxidative stress can increases Aβ production resulting in its aggregation. Conversely, Aβ aggregation also induces oxidative stress both *in vivo* and *in vitro* [9]. In addition, both oxidative stress and Aβ aggregation are closely associated with metal dyshomeostasis in the brain. First, metal (Fe, Cu, and Zn) ions are increased from 3 to 5-fold in the AD patient brains compared to those in age-matched controls, with significant accumulation in and around Aβ plaques, and/or neurofibrillary tangles (NFTs). Second, these metal ions favor protein misfolding and aggregation (formation of Aβ plaques and NFTs), for example, Zn^{2+} induces Aβ aggregation rapidly even at low physiological concentrations (submicromolar); Cu^{2+} and Fe^{3+} promote Aβ aggregation at mildly acidic condition. Third, copper interacts with Aβ promoting tau hyperphosphorylation, and subsequent NFT formation. Copper, like iron, also catalyzes the production of reactive oxygen species (ROS), inducing oxidative damage associated with neuronal death [10].

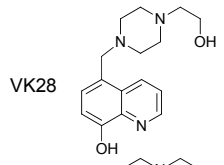
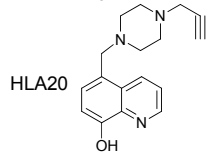
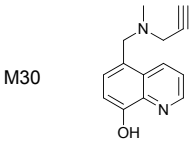
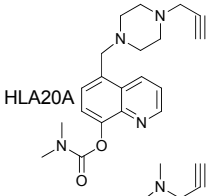
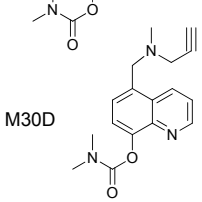
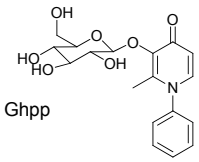
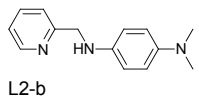
The direct link between iron metabolism and AD/PD pathogenesis is the presence of an iron responsive element (IRE) in the 5' untranslated region (UTR) of APP mRNA or in the Parkinsons's alpha synuclein (ASYN) transcript [11, 12]. Iron was found to closely regulate APP / ASYN expression, affecting APP production / ASYN translation, *via* IRP-1(iron regulatory protein-1) binding to the APP mRNA IRE or the ASYN mRNA IRE. Iron chelation with DFO selectively increased the binding of IRP1 to the APP IRE sequences and reduced APP protein expression (> 3-fold). Mycophenolic acid (iron chelator) was found to suppress ASYN levels in an iron-dependent manner and decrease ASYN 5' UTR directed translation in neural cell lines [13]. Besides, ASYN can also function as a cellular ferrireductase, binding to both copper and iron and reducing Fe^{3+} to Fe^{2+} that potentially catalyzes the Fenton reaction generating free radicals [14]. By contrast, APP possesses ferroxidase activity with a potential to oxidize Fe^{2+} to Fe^{3+} preventing the Fenton reaction. In AD cortex, zinc trapped by accumulated Aβ was found to inhibit APP ferroxidase activity; in HEK293T cells and human cortical tissue, APP exhibited a major interaction with ferroportin, which facilitates iron export from neurons. In addition, dietary iron exposure to APP−/− mice was shown to cause Fe^{2+} accumulation and oxidative stress in the cortical neurons [15].

The identification of IREs in APP mRNA / in ASYN transcript opens a new approach to the reduction of amyloisis/ ASYN overproduction and aggregation by targeting the IREs. The IRE-targeted drugs under investigation include DFO (Fe^{3+} chelator), dimercaptopropanol (Pb^{2+} and Hg^{2+} chelator), and tetrathiomolybdate (Cu^{2+} chelator); all were found to suppress APP holo-protein expression and lower Aβ secretion [11, 16]. VK28 is the lead of our first generation antioxidant chelators developed as potential IRE-targeted drugs for AD/PD (Table **1**) [17, 18]. Studies found that VK28 suppressed translation of a luciferase reporter mRNA *via* the APP 5'UTR, which includes the APP IRE. In PC12 cells, VK28 protected against 6-OHDA induced apoptosis, reducing the cell death by 23% at 1 μM, with slightly more potent than anti-PD drug rasagiline in the same conditions. When injected to 6-OHDA lesioned rats, either intraventricularly or intraperitoneally, VK28 was able to protect against 6-OHDA induced striatal dopami-nergic lesion in rats [22]. In a recent study, given intraperitoneally 7 days before or after lactacystin (proteasome inhibitor) injection, VK28 significantly improved behavioral performances and attenuated lactacystin-induced DA neuron loss, proteasomal inhibition, iron accumulation, and microglial activation in SN [23].

SECOND GENERATION CHELATORS

Propargylamine phamacophore plays a crucial role in the activities of propargylamine-containing compounds, including the anti-PD drug rasagiline, and the multi-target drug ladostigil. This phamacophore confers not only MAO-A/B inhibitory activity (such as MAO B inhibitor rasagiline), but also a wide range of neuroprotective and neurorestorative activities. These activities include regulation of APP promoting Aβ reduction, protection against a variety of insults in cell culture and *in vivo*, promotion of neuronal survival, and elevation of the brain-derived nerve factor (BDNF) mRNA expression [24]. Based on these new findings, new antioxidant chelators with potentially neuroprotective and neurogenic activities were designed and synthesized by rationally amalgamating propargylamine phamacophore (red part) into our first generation antioxidant chelators (Table **1**) [18]. HLA20, the lead of this class, showed metal (Fe, Cu and Zn) chelating activity, and anti-lipid peroxidation effects *in vitro*, with the potency similar to VK28 [17, 25]. In addition to its neuroprotection against PC12 cell death induced by serum deprivation and 6-OHDA, HLA20 also displayed a wide range of activities in mouse NSC-34 motor neuron cells. Examples includes neuroprotection against hydrogen peroxide and 3-morpholinosydnonimine induced neurotoxicity, induction of differentiation, and up-regulation of hypoxia-inducible factor (HIF)-1 and HIF-target genes such as enolase1 and vascular endothelial growth factor (VEGF) [26]. More importantly, HLA20 exhibited neurogenesis activity, demonstrated by inducing the characteristics of neuronal differentiation including cell body elongation, stimulation of neurite outgrowth in different neuron cells [27].

Table 1. Examples of Multi-Target Chelators/Prochelators as Potential Drugs for AD/PD

Name and structure	Mechanisms of action/activities	Status
 VK28	Free radical scavenger; metal chelator; protected against 6-OHDA induced striatal dopaminergic lesion in rats.	Preclinical
 HLA20	Free radical scavenger; metal chelator; inhibitor of cyclin D1 resulting in cell differentiation; neuroprotective/neurotrophic activities in cellular and animal models of AD/PD.	Preclinical
 M30	Free radical scavenger, brain-selective MAO A/B inhibitor; metal chelator, inhibitor of cyclin D1 resulting in cell differentiation; neuroprotecive/ neurotrophic activities in cellular and animal models of AD/PD; increased dopamine, serotonin, and adrenaline levels; decreased Aβ levels in the brain.	Preclinical
 HLA20A	Free radical scavenger; elective AChE inhibitor; Prochelator of HLA20; activation by inhibition of AChE releaseing an active chelator HLA20.	Preclinical
 M30D	Free radical scavenger; MAO A inhibitor; selective AChE inhibitor; prochelator of M30; activation by inhibition of AChE releaseing an active chelator M30.	Preclinical
 Ghpp	Prochelator of hydroxypyridinone, with a pendant glucosyl moiety for improving blood–brain barrier targeting; Glycosidase removal of the glucosyl moiety producing an active chelator that passivates excess metal ions, traps radicals and dissolves Aβ plaque [19].	Preclinical
 L2-b	Amyloid-binding metal chelator; MAO B inhibitor; Interaction with both metal ions and Aβ aggregates; disassembly of Aβ aggregates; regulation of ROS production and metal-Aβ neurotoxicity [20].	Preclinical
PBT2 (8-hydroxyquinoline analog, structure not disclosed to date)	Metal chelator; copper/zinc ionophore; in APP/PS1 Tg mice, decrease in soluble interstitial Aβ, insoluble Aβ load and the phosphorylation of tau, and improvement in cognitive performance; in an earlier Phase IIa trial, significant improvement in cognitive tests in Alzheimer's patients [21].	Phase IIa

NEW RASAGILINE ANALOGS

MAO-B activity increases in the aging brain and in brains burdened with neurodegenerative diseases including PD and AD, particularly high around the senile plaques (SP) in AD patients [28]. This increase leads to exacerbated oxidative stress as a consequence of enhanced production of hydrogen peroxide. Besides, the increased activity of MAO can lead to increased metabolism of neurotransmitters such as DA (dopamine), NA (noradrenaline), and ST (serotonin) in the CNS. These neurotransmitters are decreased in PD/AD patients compared to age-matched control [29]. Thus, MAO inhibitors have the potential not only to suppress oxidative stress to interfere with Aβ formation and tau hyperphosphorylation, but also to restore the levels of neurotransmitters. MAO B inhibitor rasagiline, in addition to its symptomatic benefits in treating PD patients, also shows an apparent disease-modifying effect at a dose of 1 mg per day [30]. This effect is likely a result of this drug's interaction with an array of neuroprotective/neurorescue pathways, which are mediated by its key propargylamine moiety [24]. M30 is one example of the new rasagiline analogs developed in an attempt to incorporate the

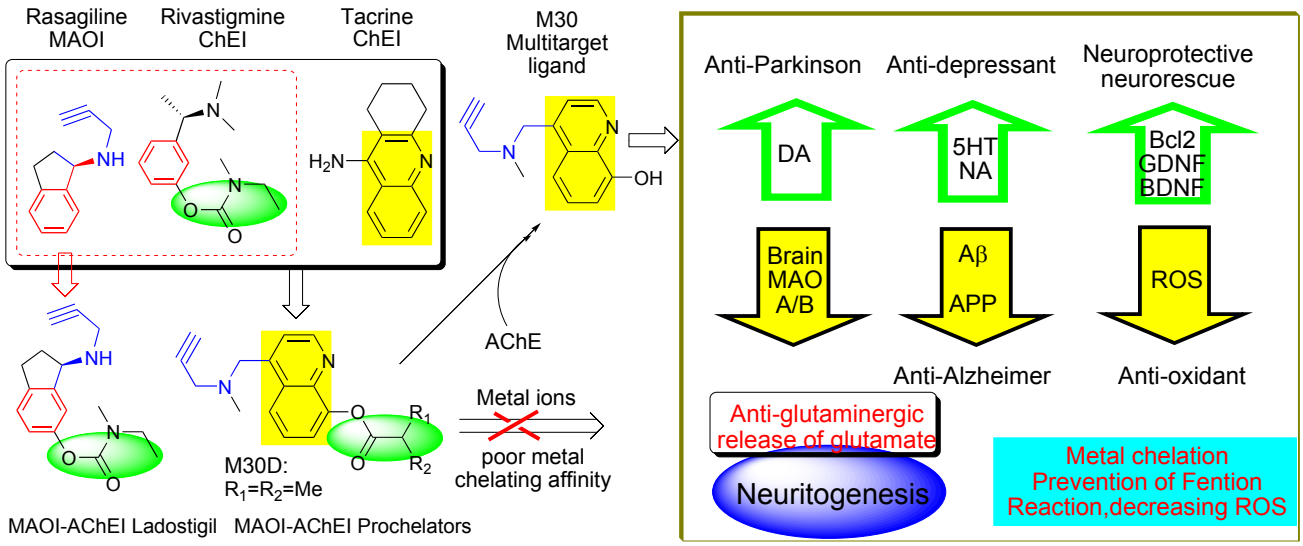


Fig. (1). Design strategy leading to multi-target ligands ladostigil and M30D. M30D has poor metal chelating capacity but can be activated to liberate the active chelator M30 upon binding to and inhibition of AChE. M30 exhibits multiple activities including: (1) brain selective inhibition of MAO A/B; (2) induction of antiapoptotic protein, Bcl-2, and neurogenesis; (3) elevation of dopamine (DA), noradrenaline (NA), and serotonin (ST) in the brain; (4) reduction of APP formation, Aβ aggregation, and glutamate release; (5) elevation of the brain-derived nerve factor (BDNF) mRNA expression, glial cell line-derived neurotrophic factor (GDNF); (6) metal (Fe, Cu, and Zn) chelation and prevention of Fention Reaction decreasing ROS (reactive oxygen species) production.

neuroprotective/neurorescue and MAO inhibitory activities of rasagiline into our antioxidant chelators (Table **1**) [18]. M30 inhibited both MAO-A and MAO-B *in vitro* with IC_{50} values of 0.037 ± 0.02 and 0.057 ± 0.01 μM, respectively [25]. *In vivo* with the rat and mouse models, M30 possessed brain selectivity for MAO A and B with little inhibition of these enzymes in the liver and small intestine [31]. This suggests that M30 may also have anti-depressant activity by increasing brain levels of DA, NA, and ST with limited potentiation of tyramine pressor effect. Indeed, studies have shown that rats treated with M30 following oral administration of tyramine showed limited potentiation of blood pressure, similar to that of moclobemide, a selective reversible MAO-A inhibitor used as an antidepressant in the clinic [32]. Fig. (**1**) summarizes the main activities of M30.

NEW AChE INHIBITORS WITH SITE-ACTIVATED CHELATING ACTIVITY

AChE inhibitors are the first FDA-approved and still the primary drugs for AD treatment, with proved safety and efficacy in improving cognition and global functions in AD patients. Moreover, studies also indicate AChE colocalizes with Aβ in senile plaques and serves to increase Aβ neurotoxicity and to accelerate Aβ aggregation into amyloid fibrils [33]. Therefore a new class of AChE inhibitors were developed to explore the potential synergistic effects by simultaneously targeting AChE, metal toxicity and oxidative stress [34]. Fig. (**2**) shows the design strategy to the lead HLA20A, which involves rationally merging the important pharmacophores from three FDA-approved drugs (rasagiline, rivastigmine and donepezil) into single molecules. Studies revealed that HLA20A inhibited AChE activity with slightly more potency than rivastigmine *in vitro*. In contrast to rivastigmine, which is a dual inhibitor of AChE and BuChE with selectivity toward BuChE, HLA20A is a selective AChE inhibitor with weak inhibition of BuChE (IC_{50} 0.50 ± 0.06 μM for AChE versus 42.58 ± 6.67 μM for BuChE). It acted as a pseudo-irreversible inhibitor and inhibited AChE activity in a time-dependent manner. More importantly, HLA20A was found to act as a prochelator with poor metal chelating activity until it was activated by inhibition of AChE to liberate the active chelator HLA20. As AChE is

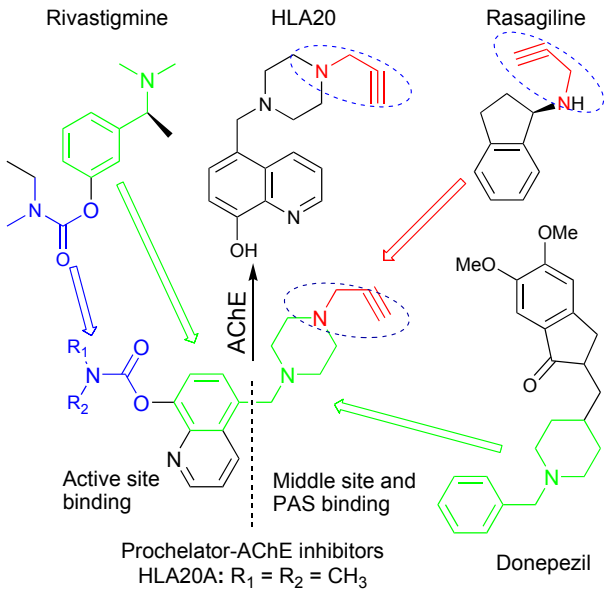


Fig. (2). Design strategy to new AChE inhibitors with site-activated chelating activity.

mainly found in the brain, this unique profile of HLA20A may help minimize the interruption of metal homeostasis in the body and increase its targeting to the brain with potentially reducing side effects related to non-targeted chelators. In fact, studies indicated that HLA20A exhibited lower cytotoxicity than its activated chelator HLA20 when tested in SH-SY5Y neuroblastoma cells [35, 36].

AChE-MAO INHIBITORS WITH SITE-ACTIVATED CHELATING ACTIVITY

Recently a new strategy was developed for designing multi-target ligands M30D series (Fig. (**1**)). This strategy follows the current use of cocktails of drugs for AIDS, but this time with the use of multiple targets on a single molecule. [37]. It involves rationally combining into single molecules the key pharmacophores from three FDA-approved drugs tacrine, rivastigmine (ChE inhibitor) and rasagiline (MAO-B inhibitor), instead of mixing them as drug-cocktails. We selected these drugs as the starting leads for three reasons. First, tacrine and rivastigmine are two AChE inhibitors for treating AD in the clinic. Second, rasagiline (Azilect), a novel MAO-B inhibitor, in addition to its symptomatic benefits in treating PD patients, was found to have an apparent disease-modifying effect at a dose of 1 mg per day, slowing the neurodegeneration. This disease-modifying effect is mediated by the propargylamine moiety in rasagiline. Third, the propargylamine moiety is a neuroprotective and neurorestorative moiety responsible not only for MAO-A/B inhibitory profile but also for APP processing (which help reduce the SP formation) and for increase in nerve growth factor [24]. Using the key pharmacophores from the three drugs as building blocks, we reconstructed our drug candidates by highly merging the underlying pharmacophores (Fig. (**1**)). One of the advantages of this strategy is to give rise to small and simple molecules with better drug-like properties and high probability of crossing the blood brain barrier (BBB). The novelty of our design strategy is to create small molecules acting as prochelators to slowly release the multi-target chelator M30 upon binding to and inhibition of AChE. Studies have found that the lead M30D highly inhibited MAO A (IC_{50} = 0.0077±0.0007 μM) with moderate inhibition of MAO B (IC_{50} = 7.90 ± 1.34 μM) *in vitro*; It was more potent than rivastigmine against AChE with an IC_{50} value of 0.52 ± 0.07 μM. M30D was a weak inhibitor of BuChE (IC_{50} = 44.90 ± 6.10 μM) with high selectivity toward AChE (IC_{50} BuChE / IC_{50} AChE = 86). Studies have also indicated that M30D was a poor metal chelator *in vitro*. However, after incubation with AChE *in vitro*, M30D displayed high metal binding affinity similar to other 8-hydroxyquinol analogs like VK28. It was found that M30D was metabolized into an active chelator M30 following inhibition of AChE [36].

EFFECTS ON HYPOXIA INDUCING FACTOR-1 SIGNALING PATHWAY

HIF-1 regulates the expression of a wide range of genes that affect many biological processes such as glucose/iron metabolism, cell cycle control, and cell proliferation/survival [38]. In AD brain, GLUT-1 and GLUT-3, the two major glucose transporters responsible for glucose uptake into neurons, were significantly reduced, most likely due to the decreased HIF-1 levels in the brain. The reduction of GLUT-1 and GLUT-3 levels contribute to abnormal hyperphosphorylation of tau and AD neurofibrillary degeneration. It was found that activation of HIF-1 by overexpression of a non-degradable HIF-1α prevented Aβ-induced neurotoxicity. Neuronal cells and primary cortical neurons, after exposure to low-dose Aβ or HIF-1α inducers such as iron chelators, survived subsequent lethal dose of Aβ and showed increased levels of HIF-1α, enhanced flux of glucose, and reduced ROS. The iron chelator DFO was found to protect primary cortical neurons against oxidative damage, by activating the hypoxia pathway leading to enhanced HIF-1 activity and increased expression of target genes, such as glycolytic enzymes, p21, and erythropoietin (EPO) [38]. M30 and HLA20 were recently shown to regulate the hypoxia pathway with significantly increasing the levels of both mRNA and protein expression of HIF-1 in primary cortical neurons, leading to enhanced levels of HIF-1-dependent genes, including VEGF, erythropoietin (EPO), p21, enolase-1, and tyrosine hydroxylase (TH) [39]. In addition, systemic chronic administration of M30 to adult mice produced a significant up-regulation of HIF-1α expression in various brain regions (e.g. cortex, striatum and hippo- campus and spinal cord) and induced transcription of HIF-1 target genes. These target genes include VEGF, EPO, enolase-1, transferrin receptor (TfR), heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), and GLUT-1 [40].

CONCLUSION

AD is a multifactorial disease with complex and multiple pathways involving its etiology and neuron death; therefore its effective treatment is unlikely achieved by any drug acting on a single pathway or target. Multi-target ligands that simultaneously modulate several key disease-related targets offer great hope for fighting this devastating disease. As discussed in this paper, chelators can be good candidates for designing multi-target ligands as potential drugs for AD. Chelators can be designed to include antioxidative, neuroprotective, neurogenesis and anti-MAO A/B activities. Chelators can also be designed as prodrugs to increase their permeability and targeting to the brain and to minimize their potential side effects, while possessing anti-MAO A/B and/or anti-AChE activities. In fact, recent researches on chelators as potential anti-AD drugs have moved to design multi-target ligands that combine metal chelation and additional functionality. In addition to the strategies introduced in this paper, chelators have also been designed to carry various moieties, for example, amyloid-binding moieties for targeting to amyloid fibrils, glucose transporters for facilitating the BBB uptake [19, 20]. Although none of the multi-target chelators discussed here or in the literature has prove more efficacious in the clinic than the existing FDA-approved drugs for AD, the strategies discussed here should be considered to open a new direction for rationally designing site-activated multi-target chelators. Besides, the development of site-activated multi-target chelators as potential drugs is not limited to AD, but could be applicable to other diseases including PD, ALS (amyotrophic lateral sclerosis), and cancer, where metal dyshomeostasis is implicated in their pathologies.

CONFLICT OF INTEREST

HZ, MF and MBHY have financial interest in varinel Inc. USA.

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