Multifunctional Neuroprotective Derivatives of Rasagiline as Anti-Alzheimer's Disease Drugs

Orly Weinreb, Silvia Mandel, Orit Bar-Am, Merav Yogev-Falach, Yael Avramovich-Tirosh, Tamar Amit, and Moussa B. H. Youdim

Eve Topf and USA National Parkinson Foundation Centers of Excellence for Neurodegenerative Diseases Research and Department of Pharmacology, Rappaport Family Research Institute, Technion-Faculty of Medicine, Haifa, 31096, Israel

Summary: The recent therapeutic approach in which drug candidates are designed to possess diverse pharmacological properties and act on multiple targets has stimulated the development of the multimodal drugs, ladostigil (TV3326) [(N-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate] and the newly designed multifunctional antioxidant iron chelator, M-30 (5-[N-methyl-N-propargylaminomethyl]-8-hydroxyquinoline). Ladostigil combines, in a single molecule, the neuroprotective/neurorestorative effects of the novel anti-Parkinsonian drug and selective monoamine oxidase (MAO)-B inhibitor, rasagiline (Azilect, Teva Pharmaceutical Co.) with the cholinesterase (ChE) inhibitory activity of rivastigmine. A second derivative of rasagiline, M-30 was developed by amalgamating the propargyl moiety of rasagiline into the skeleton of our novel brain permeable neuroprotective iron chelator, VK-28. Preclinical experiments showed that both compounds have anti-Alzheimer's disease activities and thus, the clinical development is oriented toward treatment of this type of dementia. This review discusses the multimodal effects of two rasagiline-containing hybrid molecules, namely ladostigil and M-30, concerning their neuroprotective molecular mechanisms *in vivo* and *in vitro,* including regulation of amyloid precursor protein processing, activation of protein kinase C, and mitogen-activated protein kinase signaling pathways, inhibition of cell death markers and upregulation of neurotrophic factors. Altogether, these scientific findings make these multifunctional compounds potentially valuable drugs for the treatment of Alzheimer's disease. **Key Words:** Alzheimer's disease, amyloid precursor protein, multifunctional drugs, propargyl moiety, cholinesterase inhibitor, iron chelator.

INTRODUCTION

The novel anti-Parkinsonian drug, rasagiline (N-propargyl-1-(R)-aminoindan) (Azilect, Teva Pharmaceutical Co.), is a second generation, irreversible selective inhibitor of monoamine oxidase (MAO)-B.[1] In light of the reported benefits in patients with early and late illness,[2–5] and the results of the recent large clinical study, phase III Attenuation of Disease Progression with Azilect Given Once Daily (ADAGIO) (presented during the 12th Congress of European Federation of Neurological Societies, 2008 in Madrid, Spain), it has been suggested that rasagiline could be the first Parkinson's disease (PD) treatment to receive the label "disease-modifying" from the the U.S. Food and Drug Administration. In preclinical studies, rasagiline has been shown to have a broad neuroprotective activity against a variety of neurotoxins in neuronal cell cultures and in animal models of neurodegenerative diseases. This includes attenuation of cell death in partially differentiated rat pheochromocytoma (PC12) cells deprived of serum and nerve growth factor (NGF)[6] and neuroprotection against the endogenous neurotoxin N-methyl-(R)-salsolinol (N-M-(R)-Sal),[7–9] 6-hydroxydopamine (6-OHDA),[10] 3-morpholinosydnonimine (SIN-1) (a peroxynitrite donor, NO),[11,12] and glutamate toxicity[13] in human SH-SY5Y neuroblastoma cells. *In vivo* studies have described the protective effect of rasagiline in N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model in mice and monkeys[14] by preventing its conversion to 1-methyl-4-phenylpyridinium (MPP^+), in a focal ischemia model in rats[15] and in a neurotrauma model of head injury in mice.[16] In addition, rasagiline suppresses the cell death cascade initiated by Bcl-2 family pro-apoptotic mitochondrial proteins and caspase-3, preventing the decline in mitochondrial membrane po-

Address correspondence and reprint requests to: Prof. M. B. H. Youdim, Department of Pharmacology, Technion-Faculty of Medicine, P.O.B. 9697, Haifa, 31096, Israel. E-mail: youdim@tx.technion.ac.il.

tential ($\Delta\Psi m$), and the nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and DNA fragmentation.[12,17,18]

Structure-activity studies provide evidence that the propargyl moiety (propargylamine) of rasagiline promotes neuronal survival via similar neuroprotective/neurorescue pathways, thus enlightening the importance of this moiety for the novel activities of rasagiline.[19–20] Rasagiline and propargylamine significantly attenuated cell death induced by serum deprivation in neuronal cells.[6,12,20–23] The role for protein kinase C (PKC) activation in the neuroprotective mechanism of rasagiline and propargylamine was supported by results that both compounds can increase phosphorylated-PKC (p-PKC) levels and upregulate two essential PKC isoforms involved in cell survival pathways, PKCα and PKCε, in mice hippocampus[19] and PC12 cells.[24] The inhibition of PKC activity blocked the neuroprotective action of rasagiline and its propargyl moiety in serum-deprived PC12 cells. The specific-broad spectrum PKC inhibitor, GF109203X, which exhibits high affinity for conventional PKCs (α, β, γ), and novel isoenzyme PKCε,[25,26] markedly blunted rasagiline and propargylamine-suppressive effects on the expression of the pro-apoptotic regulator, *Bad*, and the cleavage and activation of procaspase-3 and poly (ADP- ribose) polymerase (PARP), in serum withdrawal-induced programmed cell death.[18,20] Similarly, GF109203X, and the extracellular signal-regulated kinase (ERK)1/ERK2 inhibitor (PD98059) prevented rasagiline activation/phosphorylation of p42 and p44 mitogen-activated protein kinase (MAPK), thus indicating that rasagiline directly activates PKC-MAPK pathway.[22] The importance of PKC pathway in rasagiline- and propargylamine-induced neuroprotective activity is supported also by previous data demonstrating that these compounds[20,22] induced the release of the neuroprotective–neurotrophic nonamyloidogenic soluble amyloid precursor protein α (sAPPα) by MAPK-and PKC-dependent mechanisms *in vitro*.[18–21]

Current therapeutic approaches suggest that drugs acting at a single target may be insufficient for the treatment of multifactorial neurodegenerative diseases such as PD, Alzheimer's disease (AD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS), all characterized by the coexistence of multiple etiopathologies. These include among others, oxidative stress (OS) and reactive oxygen species (ROS) formation, protein misfolding and aggregation, mitochondrial dysfunction, inflammation, metal dyshomeostasis and accumulation at the sites of neurodegeneration.[27–29] Noticeably, drug therapy of neurodegenerative diseases with multifunctional compounds embracing diverse biological properties with single bioavailability and pharmacokinetic metabolism, will have pronounced advantage over individual-target drug or cocktail of drugs.

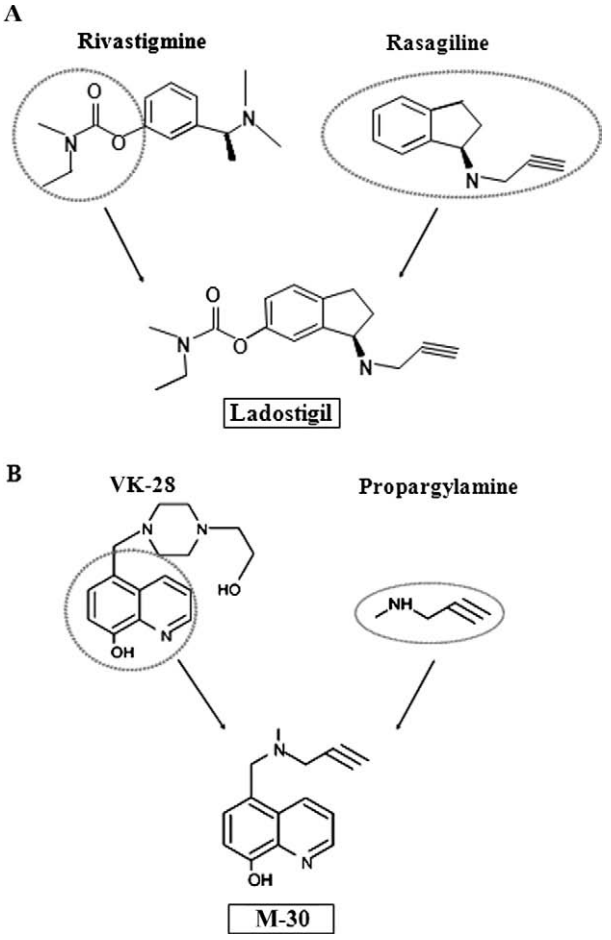

FIG. 1. The chemical structures of the multifunctional-anti-Alzheimer compounds: (**A**) The R-enantiomer, ladostigil (TV3326) [(N-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate] with a carbamate cholinesterase inhibitory moiety of rivastigmine in the aminoindan structure of the selective MAO-B inhibitor, rasagiline. (**B**) The antioxidant iron chelator, M-30 (5-[N-methyl-N-propargylaminomethyl]-8-hydroxyquinoline) which has been designed by introducing the propargyl moiety of rasagiline into the pharmacophore of the iron chelator, VK-28.

Based on this reasoning, our group has designed and synthesized several chimeric compounds by amalgamating the neuroprotective/neurorestorative drug, rasagiline, or its propargyl moiety, either into a carbamate cholinesterase inhibitory (ChEI) moiety or into the antioxidant-iron chelating skeleton of a 8-hydroxyquinoline derivative of VK-28[30] to produce the multimodal compounds (FIG. 1), ladostigil (TV3326) [(N-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate], or M-30, (5-[N-methyl-N-propargylaminomethyl]-8-hydroxyquinoline), respectively, as powerful new drugs for combating AD.[31,32] This review discusses the scientific evidence for the potential use of the novel multifunctional compounds, ladostigil, and M-30, containing the propargyl moiety of rasagiline for AD.

SELECTED STRATEGIES IN THE DEVELOPMENT OF MULTIFUNCTIONAL DRUGS FOR AD

AD is the most prevalent neurodegenerative disease in the elderly population and it has been estimated that approximately 5% of adults greater than 65 years of age is affected by this disease.[33] Its predominant clinical manifestation is the progressive memory deterioration and other changes in brain function, including disordered behavior and impairment in language, comprehension, and visual-spatial skills.[34] The neuropathology of AD is characterized by several features, including extracellular deposition of amyloid β (Aβ) peptide-containing plaques in the cerebral cortical regions, accompanied by the presence of intracellular neurofibrillary tangles and a progressive loss of basal forebrain cholinergic neurons leading to reductions in cholinergic markers, such as acetylcholine levels, choline acetyltransferase (ChAT), and muscarinic and nicotinic acetylcholine receptor binding.[35,36] In addition, there is accumulating evidence that many cytotoxic signals in the AD brain can initiate apoptotic processes, including OS, inflammation, and accumulation of iron at the sites of neurodegeneration.[27–29] Significant reduction also occurs in serotoninergic and noradrenergic transmission, which might explain the relatively high incidence of depression found in AD patients.[37,38] Thus, it seems likely reasonable to conclude that AD therapy will require multiple drug therapy to address the varied pathological aspects of this disease.

Currently, numerous clinical trials have demonstrated the safety and efficacy of acetyl cholinesterase inhibitors (AChEIs) in the treatment of AD. Yet their benefits in AD as symptomatic drugs are likely to be more complex than simply replacement of lost acetylcholine.[39–42] As recently reviewed, there is growing preclinical evidence that AChEIs have minor symptomatic activity and may block some of the fundamental neurodegenerative processes involved in AD.[43] AChEIs, such as tacrine, donepezil, galantamine, huperazine A, and ganstigmine were reported to protect neurons from death in various cell culture models of neurodegenerative diseases.[43] In addition, there is evidence that several ChEIs also affect various neuropathological markers of AD and modulate the cleavage of the nonamyloidogenic APP processing.[42] Therefore, it has been suggested that AChEIs, possessing properties of neuroprotection and/or APP processing, might be more beneficial than those that only inhibit acetyl cholinesterase (AChE) to treat AD, and in particular to prevent the pathogenesis of AD. However, none of these drugs have been shown to possess clinical neuroprotective or disease-modifying activity.

To date, various AChEIs have been demonstrated as producing significant symptomatic improvement in cognitive performance and behavioral abnormalities that occur in AD and the related disorders, Vascular and Lewy bodies dementias.[44,45] Currently, the available anti-AD medications, which include AChEIs or N-methyl-D-aspartate receptor antagonists for the treatment of moderate to severe Alzheimer dementia cases, are efficient to produce modest symptomatic improvements in some of the patients, but not to cure or stop the disease progression.[46,47] To fill this gap, the multifunctional drug, ladostigil (FIG. 1) was designed to possess the neuroprotective and neurorestorative activities of rasagiline, as well as target various underlying pathogenic mechanisms of AD, and thus it is expected to have a disease-modifying effect. The underlying principle in the design of ladostigil was to amalgamate the neuroprotective propargyl moiety of rasagiline to the carbamate ChEI moiety of rivastigmine[48] (FIG. 1). The resulting molecule is a novel cholinesterase and brain-selective MAO-A/MAO-B inhibitor, intended for the treatment of dementia comorbid with extra pyramidal disorders (Parkinsonism) and depression (presently in Phase IIa studies in man). Ladostigil was shown to produce neuroprotective and antidepressant-like activities in several preclinical models of neurodegeneration,[49–53] improve cognitive deficits in aged monkeys,[54] possess a novel potential antidepressant activity in rat models of anxiety and depression,[48,55–57] and prevent memory deficits induced by scopolamine [58] or streptozotocin (STZ) in rat models of AD.[59]

A supplementary pathology of AD is the accumulation of iron in and around amyloid senile plaques and neurofibrillary tangles, leading to alterations in the pattern of the interaction between iron regulatory proteins and their iron responsive element (IRE) and disruption in the sequestration and storage of iron.[60,61] Also, high levels of iron have been reported in the amyloid plaques of the transgenic mouse model for AD, Tg2576, resembling those seen in the brains of AD patients.[62] In addition to the accumulation of iron in senile plaques, it was demonstrated that the amount of iron present in the AD neuropil is twice that found in the neuropil of nondemented brains.[60] Further studies have suggested that accumulated iron supports the AD pathology as a possible source of OS-dependent reactive oxygen radicals, demonstrating that neurons in AD brains experience high oxidative load.[63] A recent study reported that ribosomal RNA provided a binding site for redox-active iron and serves as a redox center within the cytoplasm of vulnerable neurons in AD brain, in advance of the appearance of morphological change indicating neurodegeneration.[64] In addition, other evidence suggests that the metabolism of iron is disrupted in AD. For example, the location of the iron-transport protein, transferrin, in senile plaques (instead of its regular location in the cytosol of oligodendrocytes) indicated that transferrin becomes

trapped within plaques while transporting iron between cells.[65] The mediator of iron uptake by cells, melanotransferrin, and the iron-storage protein ferritin, are altered in AD and are expressed within reactive microglial cells that are present both in and around senile plaques.[66] At the biochemical level, iron was demonstrated to facilitate the aggregation of β-amyloid peptide and increase its toxicity.[67] Indeed, the prototype iron chelator, deferrioxamine (DFO), prevented the formation of β-pleated sheets of Aβ and dissolved preformed β-pleated sheets of plaque-like amyloid.[68] Also, iron-induced aggregation of hyperphosphorylated τ (tau), the major constituent of neurofibrillary tangles.[69] A direct link between iron metabolism and AD pathogenesis was provided recently by Rogers et al.[70] who described the presence of an IRE in the 5' untranslated region (5'UTR) of the APP transcript. Thus, APP 5'UTR is selectively responsive to intracellular iron levels in a pattern that reflects iron-dependent regulation of intracellular APP synthesis. Indeed, iron levels were shown to regulate mRNA translation of holo-APP in astrocytes[27] and neuroblastoma cells[70] by a pathway similar to iron control in the translation of the L- and H-ferritin mRNAs by IREs in their 5'UTRs.

The concept of metal chelators for clinical use in neurological disorders that could remove excess iron in the brain, recently led our group to develop the multifunctional drug, M-30, which is a nontoxic, lipophilic, and brain-permeable iron chelator for neurodegenerative diseases[71] from our prototype brain-permeable iron chelator, VK-28 (Varinel Co.) (5-[4-(2- hydroxyethyl) piperazine-1-ylmethyl]-quinoline-8-ol) (FIG. 1).[71,72] M-30 also possesses the propargyl moiety of rasagiline and thus, inherits some of its neuroprotective properties. Indeed, recent studies have shown a significant neuroprotective action of M-30 against MPTP neurotoxicity[73] or the proteasome inhibitor, lactacystin, in mice.[74] In addition, M-30 is a potent radical scavenger and brain selective irreversible MAO-A and MAO-B inhibitor, with little inhibition of peripheral MAO (liver and small intestine) that limits the potentiation of cardiovascular effect of tyramie ("cheese reaction"),[75] indicating that M-30 may possess antidepressant activity, and thus can be implicated for treatment AD.

NEUROPROTECTIVE MECHANISMS OF ACTION OF LADOSTIGIL

Targeting amyloid precursor protein processing

Much evidence suggests that the accumulation of Aβ in AD may play a pivotal role, thus a bulk of studies has been focused on possible drug intervention along the amyloid pathways in AD.[76] In this context, recent findings demonstrated that ladostigil markedly suppressed holo-APP protein levels and elevated soluble-APPα (sAPPα in different cellular model systems, which can be of clinical value toward accelerating nonamyloidogenic APP processing, thereby reducing the possibility of generation of the toxic β-amyloid peptides.[53,77] Consistent with this, a previous study showed that treatment with ladostigil clearly decreased the levels of cell-associated, holo-APP in the mice hippocampus, which indicates that APP expression can also be regulated by ladostigil under *in vivo* conditions.[19] Moreover, the observation that ladostigil did not alter APP mRNA levels, may suggest that the decrease in APP protein and Aβ levels can be attributed to suppression of APP translation, in analogy to another ChEI, phenserine.[78] A recent study demonstrated that selective butyrylcholinesterase (BuChE) inhibitors reduced APP and Aβ levels *in vitro* and *in vivo*.[79] Thus, the mechanism underlying these effects may involve both cholinergic and noncholinergic actions regulating APP synthesis and processing. Regulation of APP processing by ladostigil was demonstrated to involve PKC- and MAPK-dependent pathways[21] (FIG. 2). However, this neuroprotective effect of ladostigil does not appear to result from its ChE inhibition activity only, since rasagiline or propargylamine were also able to induce PKC and ERK activation and promote sAPPα release.[22] Several ChEIs, such as tacrine,[80] physostigmine,[81] metrifonate,[82] ganstigmine,[83] and donepezil[84] increased sAPPα release in cell culture. However, the observations that phenserine[78] and tacrine, at high concentration,[85] decreased sAPPα release suggests that the regulation of APP processing by ChEIs is not simply associated to AChE inhibition. It is likely that several different mechanisms are in operation. For example, ChEIs increased PKC levels *in vitro*[86] and attenuated the Aβ-induced down-regulation of PKC in rats.[87] Moreover, donepezil promoted the trafficking of α-secretase to the membrane, thus enhancing α-secretase activity.[84]

Similar to ladostigil, its S-isomer, TV3279, which is a ChEI, but lacks MAO inhibitory activity, exerted pronounced neuroprotective properties and APP processing, suggesting that the mode of action is independent of MAO inhibition.[53] These results are consistent with previous data, providing clear evidence that the neuroprotective effect of ladostigil, as well as rasagiline, does not depend on inhibition of MAO, but rather is associated with some intrinsic pharmacological action of the propargyl moiety on the mitochondrial cell survival proteins.[6,10,20,23] Recently, propargylamine was found to inhibit MAO activity significantly less than its abilities to induce neuroprotective, anti-apoptotic activities and regulate APP processing,[18,20] further establishing that MAO inhibition is not a prerequisite for the neuroprotective activity of ladostigil.

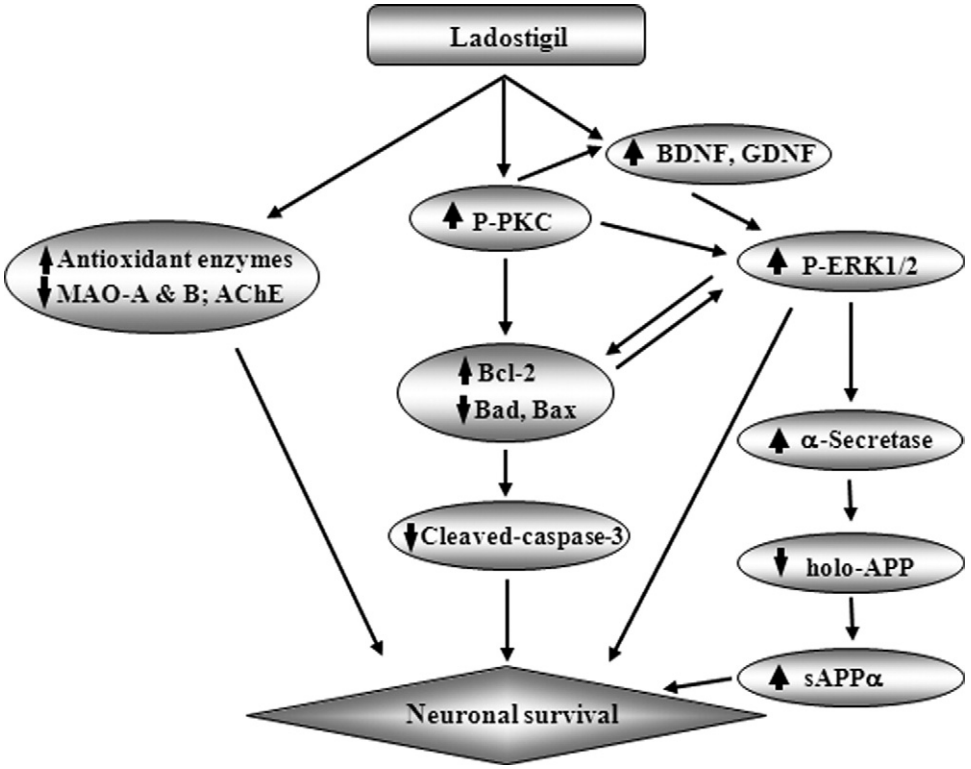

FIG. 2. Schematic overview demonstrating protein and gene targets involved in the neuroprotective activity of ladostigil, with respect to the pathological features described for AD, such as extracellular deposition of Aβ, marked cholinergic cortical afferent dysfunction, lack of trophic factor support and cytotoxic signals that can initiate cell death processes and OS at those neurons and brain areas associated with this disease. AChE = acetyl cholinesterase; APP = amyloid precursor protein; BDNF = brain-derived neurotrophic factor; GDNF = glial cell line-derived neurotrophic factor; MAO = monoamine oxidase; P-PKC = phosphorylated-PKC; P-ERK1/2 = phosphorylated extracellular signal-related kinase 1/2.

Antioxidant activity

Previous studies suggested that in AD, OS is mediated by Aβ-generated ROS.[88,89] Hydrogen peroxide (H_2O_2) is one of the main ROS produced during the redox process and is considered a messenger in intracellular signaling cascades, including cellular metabolism and proliferation.[90,91] It is well acknowledged that H_2O_2 could cause lipid peroxidation and DNA damage, thus inducing apoptosis in many different cell types.[92–94] Several lines of evidence suggested that AChE and BuChE activation might be involved in apoptosis associated with H_2O_2.[95,96] These links between cholinergic signal and OS provide an additional therapeutic target for AChEIs in AD. Indeed, the AChEls, tacrine,[96] and huperzine A[97] were demonstrated to significantly protect rat PC12 cells against H_2O_2 insult. Similarly, ladostgil was found to comprise a significant neuroprotective effect against H_2O_2-induced damage in SH-SY5Y cells.[98] Ladostigil was demonstrated to exert antioxidant activity through both direct scavenging effect on free radicals overproduced in H_2O_2-treated cells, as well as indirect effect by stimulating the expression and activity of cellular antioxidant enzymes, catalase, and glutathione reductase, suggesting their involvement in the cytoprotective effect of ladostigil.[98] In addition, the mRNA expression levels of the antioxidant enzymes, catalase, peroxiredoxin 1, and NADPH, quinone 1 oxidoreductase (NQ01) were elevated by ladostigil in H_2O_2-treated SH-SY5Y cells.[99] In support, results obtained from the high density cytotoxic model of SK-N-SH neuroblastoma cells, widely used in neuronal injury studies as a potential source of ROS,[100] also revealed that ladostigil induced mRNA levels of the same antioxidant enzymes.[98] Indeed, overexpression of NQ01 was demonstrated to induce neuroprotection against various toxins *in vitro* suggesting to play an important role in the central nervous system.[101,102] In addition, NQ01 activity was closely co-localized with AD pathology, indicating that increasing NQ01 activity may provide a neuroprotective avenue for the treatment of neurodegenerative diseases.[103] These findings are in accordance with previous studies showing that ladostigil possesses neuroprotective effects against various *in vivo* and *in vitro* insults, including OS damage induced by either Aβ^{22} SIN-1[52,104] or glucose-oxygen deprivation.[105]

An additional inspection of the activity of ladostigil drug is related to its protective effect against OS *in vivo*. Thus, chronic administration of ladostigil before and after STZ injection significantly reduced the alterations in microglia and astrocytes, and prevented the increase in a

marker of nitrative-oxidative stress, nitrotyrosine, and the development of episodic memory deficits of rats.[59] This report suggests that these actions result from a combination of actions of ladostigil on neuronal and glial cells. The ability to inhibit ChE partially contributes to the effect on episodic memory, since ladostigil inhibits ChE only in the cortex of STZ rat model of AD and no ChE inhibition occurs at the site of recognition, which depends on hippocampal cholinergic activity. Thus, the prevention of memory deficits resulted from distinct activities, such as antioxidative action.[59] Consistent with this, rasagiline (from which ladostigil is derived) was found to increase antioxidant enzyme activities in the brain dopaminergic system in rat.[106] Other propargylamine derivatives were shown to possess antioxidant properties assessed by their ability to scavenge peroxynitrite.[107]

The involvement of cell survival and signaling pathways

Studies using various cellular apoptotic models demonstrated that AChE expression simultaneously accumulated in the nuclei of apoptotic cells.[108] Indeed, there is increasing evidence that AChE might be involved in apoptosis.[109] Transfection with AChE leads to an increase of apoptosis in retinal cells and highly purified AChE has been shown to have toxic effects, both in neuronal- and glial-like cell lines via the apoptotic mechanism.[110] In this context, various AChEIs exhibited neuroprotection properties regulating the expression levels of anti-apoptotic and pro-apoptic genes and proteins.[111,112]

In the extremely neurotoxic model of human neuroblastoma SK-N-SH, ladostigil was recently reported to have a significant neuroprotective activity, including inhibition of caspase-3 activation, induction of Bcl-2, and reduction of Bad and Bax gene and protein expressions[53] (FIG. 2). These neuroprotective properties of ladostigil may be associated with its AChE inhibitory activity. Nonetheless, previous studies demonstrating that rasagiline, as well as its propargyl moiety, promoted neuronal survival mediated by PKC-, MAPK- dependent activation associated with Bcl-2 family members[18] and mitochondrial membrane stabilization,[52,104] and thus might also have a crucial role in the neuroprotective activity of ladostigil. Also, ladostigil induced stimulatory effects on PKC and MAPK cascades[21] (FIG. 2), promoting the phosphorylation of p44 and p42 MAPK, which was abolished by specific inhibitors of MAPK activation.[21] ERK activation in the central nervous system has been implied in mammalian synaptic plasticity and learning.[113] Thus, the effect of ladostigil on ERK activation could make ladostigil a potential valuable drug for the treatment of different types of dementia. It has been demonstrating that ladostigil selectively reverses the behavioral and neurochemical effects induced by prenatal stress,[57] has antagonistic effect on scopolamine-induced impairments in spatial memory,[58] prevents memory deficits of rats induced by intracerebroventricular injection of STZ[59] or administering sodium azide.[114] These findings are allied with the elevation of the neurotrophic factors, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) mRNA expression,[115] and activation of PKC-MAPK signaling pathways by ladostigil,[21] since previous studies demonstrated that neurotrophic factors play critical roles in the development and function of neurons and may serve as regulatory factors for synaptic transmission, learning, and memory.[116,117] Thus, it has been proposed that several neurodegenerative disorders, such as PD, AD, and Huntington's disease are linked to a lack of trophic factor support in those neurons and brain areas associated with these diseases.[116,117] In addition, upon ligand-receptor binding, BDNF and GDNF stimulate intracellular signaling pathways involved in differentiation and survival, including phospholipase C-γ, phosphatidylinositol 3-kinase and MAPK.[116,118,119] Thus, elevation of neurotrophic factors by ladostigil, which may possibly initiate respective cell signaling cascades, might suggest an involvement of neurotrophic factors in the neuroprotective mechanism of action of ladostigil. Furthermore, ladostigil was recently shown to upregulate the brain-specific isoform of the synaptotagmin family, synaptotagmin intravenous in old rat hippocampus.[120] The current hypothesis of synaptotagmin intravenous function states that its upregulation is correlated with a neuroprotective-like activity resulting in neurotransmitter release and thus, it may be suggested that this pathway is associated with the neuroprotective mechanism of action of ladostigil.

NEUROPROTECTIVE MECHANISMS OF ACTION OF M-30

Radical scavenging and iron chelation

The multifunctional, nontoxic, brain permeable iron chelator drug, M-30,[121] was developed for two purposes: 1) it was designed to prevent the ability of iron to induce OS as a consequence of reactive hydroxyl radical generation via its interaction with hydrogen peroxide (Fenton Reaction), and 2) M-30 was designed to inhibit the formation of reactive hydroxyl radical from hydrogen peroxide generated by MAO and potentiate the pharmacological action of accumulated dopamine formed from L-dihydroxyphenylalanine (L-DOPA).

In searching for the superlative neuroprotective agents in a series of multifunctional iron chelators,[71,75] M-30 was found to be a highly potent inhibitor of both MAO-A and MAO-B activities,[73,75] and the most effective inhibitor of lipid peroxidation with higher IC50 values, comparable with that of DFO, which is a potent inhibitor of

lipid peroxidation and strong prototype iron chelator.[75] The ability of chelators to inhibit lipid peroxidation may result from two processes, iron chelation and free radical scavenging. It is well established that strong iron chelators could form inert complexes with iron and interfere with the Fenton reaction leading to a decrease in hydroxyl free radical production, and thus block lipid peroxidation. The novel chelator, M-30, which has been shown to possess high iron-binding capacity,[71,72] may also act by a similar mechanism to inhibit free radical formation. In addition, this chelator may act as radical scavenger to directly block formation of the free radical, as confirmed by the spin trapping of hydroxyl radical by 5.5-dimethyl-I-pyrroline-N-oxide (DMPO) measured in the electron paramagnetic resonance spectra of the resulting DMPO- hydroxyl radical spin adduct. The results showed that the novel chelator can significantly reduce the DMPO- hydroxyl radical signal generated by the photolysis of H_2O_2,[122] suggesting that it works as radical scavenger to directly scavenge the hydroxyl radical, because the photolysis of H_2O_2 generates hydroxyl radical independently from metal ions. The mechanism by which M-30 acts as a radical-scavenging antioxidant is not well understood. Previous studies have shown that some phenolic and polyphenolic compounds, such as vitamin E, catechin gallates, and green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) possess free radical scavenging properties.[123] Their scavenging activities are believed to be due to the presence of the phenolic moiety.[123] Indeed, M-30 contains a phenolic moiety, and thus its mechanism as a radical scavenging antioxidant needs to be further investigated.

APP regulation and Aβ peptide reduction

Iron chelating therapeutic strategy for the treatment of neurodegenerative disorders comprises multifunctional drug candidates designed specifically to act on multiple central nervous system (CNS) targets.[32,124] A recent study[125] has demonstrated that M-30 has a wide range of pharmacological activities, including a significant down-regulation of membrane-associated holo-APP levels in the mouse hippocampus and in SH-SY5Y neuroblastoma cells, presumably by chelating intracellular iron pools. Indeed, M-30 was found to suppress translation of a luciferase reporter mRNA via the APP 5'UTR sequence that includes the APP IRE.[125,126] Furthermore, M-30 markedly reduced the levels of cellular APP and β-C-terminal fragment, and the levels of the amyloidogenic Aβ peptide in the medium of SH-SY5Y cells and Chinese hamster ovary cells stably transfected with the APP "Swedish" mutation (CHO/ΔNL). Levels of the nonamyloidogenic soluble APPα and α-C-terminal fragment in the medium and cell lysate, respectively, were coordinately increased.[125] These results support the implication that M-30 was involved in the regulation of iron-homeostasis-associated protein, such as APP (FIG. 3).

Attenuation of neuronal death

The neurorescue potency of M-30 was identified by the ability of the drug to augment SH-SY5Y neuronal viability, even though it was administered after an extreme apoptotic damage of 3 days of serum deprivation.[125] In this model system, the SH-SY5Y neuroblastoma cells exposed to long-term serum deprivation exhibited a significant increase in apoptotic cells compared with cells grown in full serum. M-30 caused a marked decrease in the level of apoptotic cells and significantly reduced phosphorylated histone H2A.X, a marker of apoptosis and cleaved caspase-3 appearance.[125] Considering the importance of Bcl-2 family proteins in the regulation of the programmed cell death pathway, the authors examined whether M-30 has any effect on the levels of the "BH-3 only" pro-apoptotic protein Bad, which is particularly associated with the response to withdrawal of survival factors.[127] Long-term serum deprivation of SH-SY5Y culture resulted in increased levels of Bad, while M-30 significantly decreased Bad and Bax levels, and conversely increased the levels of Bcl-2.[125] These results indicate that the neurorescue action of M-30 might be associated with a reduction in the levels of the pro-apoptotic proteins and modulation of their neuronal balance (FIG. 3). Consistent with the neurorescue effects of M-30, our previous studies showed the neurorescue/neuroprotective activity of the propargyl moiety and related propargylamines, such as rasagiline and ladostigil, against cell death induced by a variety of insults (e.g., serum withdrawal and the neurotoxins N-morpholinosydnonimine and N-methyl[R-]salsolinol).[6,12,18–23]

Induction of neurite outgrowth and regulation of cell cycle

Additional and new aspect of iron chelator compounds in the etiology of AD therapy is related to their ability to abort anomalous cell cycle reactivation in post-mitotic degenerating neurons. Indeed, during the last few years, accumulating evidence for an activated cell cycle in the vulnerable neuronal population in AD has suggested a crucial role for cell cycle abnormalities in AD pathogenesis. Therefore, therapeutic interventions targeted toward ameliorating mitotic changes would be predicted to have a positive impact on AD progression. Previous studies have shown that the reactivation of the cell cycle is an obligatory component of the apoptotic pathway evoked by Aβ peptides.[128,129] Recently, we have found that M-30 significantly reduced the percentage of neurons in the S-phase, while it re-raised the relative cell number in the G_0/G_1 phase and lowered apoptotic levels after exposure to Aβ in primary cultures of rat cortical neurons (unpublished observation). In support, M-30 was shown

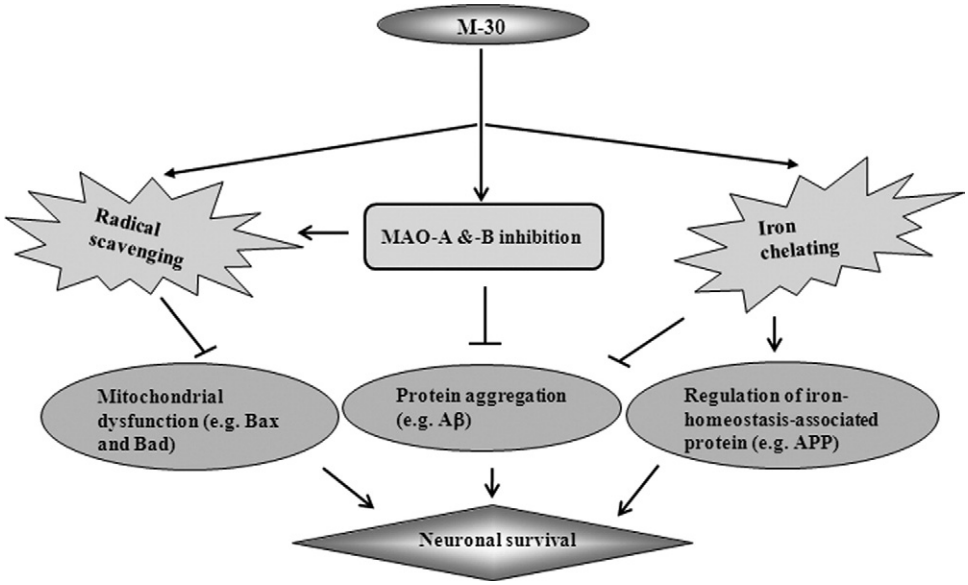

FIG. 3. Schematic illustration of the multifactorial effects involved in the neuroprotective mechanism of action of the iron chelator, M-30. APP = amyloid precursor protein; MAO = monoamine oxidase.

to induce cell cycle arrest, increase the number of PC12 cells in G_0/G_1, decrease the cell number in S-phase, as well as the proportion of cells in the G_2 phase, further indicating that this compound inhibited cell progress beyond the G_0/G_1 phase.[125,126]

We recently presented a novel neuroprotective target for iron chelators regarding the aberrant cell cycle reentry of postmitotic neurons in AD. Accordingly, similar to cancer drug therapy, a newly therapeutic strategy for neurodegenerative diseases is currently directed at interfering with mitogenic signaling and cell cycle progression to ameliorate cell death. Because iron chelators have been shown to affect critical regulatory molecules involved in cell cycle arrest and proliferation,[130] a therapeutic intervention with M-30 is assumed to have a profound impact on neuronal preservation and AD progression. Indeed, our studies[125,126] revealed that M-30 has a profound impact on neuronal differentiation features in neuroblastoma SH-SY5Y and PC12 cells, including cell body elongation, stimulation of neurite outgrowth, and upregulation of the growth associated protein-43 (GAP-43). Taken together, the data suggest that iron chelators may be considered potential therapeutic agents in AD, targeting early cell cycle anomalies and re-establishing the synaptic connection loss in the injured neuronal cells.

CONCLUSIONS AND PERSPECTIVES

The challenge of designing polypharmacological drugs has to link multiple *in vitro* activities to *in vivo* models and clinical settings.[131,132] Drug combinations, mixing target-acting compounds, provide a practical way to design specific polypharmacology. Nonetheless, developing a combination of therapy strategies raises complexity of drug-drug interaction, dosage ranging, and metabolic shunt effects more than those that are derived from a single multifunctional drug.[131,132] A growing number of compounds have been specifically designed by conjugating two or more distinct pharmacophores, exhibiting safe dosage use and interaction with multiple molecular pathways targets, which are different from the unconjugated pharmacophore.[32,124] The multifunctional "dirty" drug, ladostigil, was designed to possess the neuroprotective activity established for rasagiline, and address the therapeutic requirements needed to delay the progression of neurodegenerative diseases (PD with dementia, Lewy Body disease, and dementia Lewy Body disease with extrapyramidal symptoms) with features of dementia, behavioral abnormalities, depression, and extrapyramidal symptoms. Thal and co-workers[133] introduced the notion that these alterations are restricted to specific regions of the brain and may be associated with several phases of premorbidity (or preclinical debut stages) of neurodegenerative diseases. The unique feature of ladostigil and M-30 is to produce brain-selective inhibition of both MAO-A and MAO-B and increase brain levels of dopamine, serotonin, and noradrenaline. Thus, the design of ladostigil and M-30 was to address multiple CNS etiology in various dementias, which can have beneficial effects, compared with their parental drugs or to a combination therapy. For example, rivastigmine is neither an MAO-A nor an MAO-B inhibitor, whereas ladostigil is an inhibitor of both MAO-A and MAO-B, leading to an increase in brain dopamine and functional activities.[134,135]

In summary, the several targets and diverse pharmacological properties of ladostigil and M-30 make these

compounds potentially valuable for clinical therapy for AD to delay further neurodegeneration. Ladostigil possesses a neuroprotective activity and regulatory effects on holo-APP and sAPPα levels, hence reducing the possibility of generating the amyloidogenic pathway, its stimulatory effects on PKC and MAPK cascades (FIG. 2).[21,53,136] In addition, the observation that ladostigil induces both BDNF and GDNF expression[115] may suggest a linkage between neurotrophic factors and the neuroprotective mechanism of action of ladostigil, as described previously for rasagilne and its propargyl moiety (FIG. 2).[18,20] These positive outcomes of our studies led to the design of the multimodal drug, M-30. Indeed, M-30 was shown to possess a wide range of neuroprotective activities, including pro-survival/neurorescue effects, induction of neuronal differentiation, and regulation of APP and Aβ levels (FIG. 3).[125,126] It is apparent that this compound targets a number of pharmacological sites involved in neurodegeneration processes and thus might serve as a potential neuroprotective/neurorescue drug for the treatment of various neurodegenerative diseases, including AD. Future preclinical experiments in the animal model of AD will improve our understanding of the multimodal molecular mechanisms of ladostigil and M-30 and will be value for our knowledge of these drugs potential in future clinical setting.

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