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Perspective

Multi-target-Directed Ligands To Combat Neurodegenerative Diseases

Andrea Cavalli,* Maria Laura Bolognesi,* Anna Minarini, Michela Rosini, Vincenzo Tumiatti, Maurizio Recanatini, and Carlo Melchiorre*

Department of Pharmaceutical Sciences, Alma Mater Studiorum, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

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1. Introduction

Our understanding of the pathogenesis of diseases has advanced enormously in recent decades. As a consequence, drug discovery has gradually shifted from an entirely human-phenotype-based endeavor to today's reductionist approach centered on single molecular targets. The focus has shifted from the early animal models to isolated proteins via cellular models. This change has led to a decrease in complexity but also to a decrease in relevance to the human condition.¹ In this context, drug research has become (and still is) aimed mainly at the discovery of small molecules able to modulate the biological function of a single protein target thought to be fully responsible for a certain disease. Much effort has been devoted to achieving selectivity for that given target, and indeed, nowadays, many ligands endowed with outstanding *in vitro* selectivity are available. This one-molecule, one-target paradigm has led to the discovery of many successful drugs, and it will probably remain a milestone for years to come. However, it should be noted that a highly selective ligand for a given target does not always result in a clinically efficacious drug. This may be because (a) the ligand does not recognize the target *in vivo*, (b) the ligand does not reach the site of action, or (c) the interaction with the respective target does not have enough impact on the diseased system to restore it effectively. Reasons for the latter might lie in both the multifactorial nature of many diseases and the fact that cells can often find ways to compensate for a protein whose activity is affected by a drug, by taking advantage of the redundancy of the system, *i.e.*, of the existence of parallel pathways.^{2,3} Medicinal chemists are often faced with these

frustrating aspects of drug research. Drawbacks a and b can be addressed through the well-established rational ligand modification approaches. But issue c is more problematic and needs to be carefully discussed. This is one of the aims of the present article.

Drugs hitting a single target may be inadequate for the treatment of diseases like neurodegenerative syndromes, diabetes, cardiovascular diseases, and cancer, which involve multiple pathogenic factors.⁴ Different pharmacological approaches offer possible ways of overcoming the problems that arise from the use of such drugs.⁵ When a single medicine is not sufficient to effectively treat a disease, a multiple-medication therapy (MMT)^a (also referred to as a "cocktail" or "combination of drugs") may be used. Usually, an MMT is composed of two or three different drugs that combine different therapeutic mechanisms. But this approach might be disadvantageous for patients with compliance problems. A second approach might be the use of a multiple-compound medication (MCM) (also referred to as a "single-pill drug combination"), which implies the incorporation of different drugs into the same formulation in order to simplify dosing regimens and improve patient compliance. Finally, a third strategy is now emerging on the basis of the assumption that a single compound may be able to hit multiple targets. Clearly, therapy with a single drug that has multiple biological properties would have inherent advantages over MMT or MCM. It would obviate the challenge of administering multiple single-drug entities, which could have different bioavailability, pharmacokinetics, and metabolism. Indeed, if a single molecular species can show a complex ADMET profile, an MMT/MCM approach might be untenable. Furthermore, in terms of pharmacokinetic and ADMET optimization, the clinical development of a drug able to hit multiple

* To whom correspondence should be addressed. Phone: +39 0512099700. Fax: +39 0512099734. E-mail: for A.C., andrea.cavalli@unibo.it; for M.L.B., marialaura.bolognesi@unibo.it; for C.M., carlo.melchiorre@unibo.it.

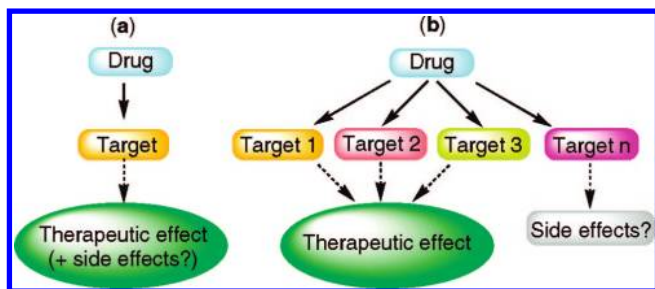


Figure 1. Pathways leading to the discovery of new medications: (a) Target-driven drug discovery approach, that is, the application of the current one-molecule, one-target paradigm. Although this approach has led to many effective drugs able to hit a single target, it is now well-documented that these drugs may represent the exception rather than the rule. (b) MTDLs approach to drug discovery. A drug could recognize (in principle, with comparable affinities) different targets involved in the cascade of pathological events leading to a given disease. Thus, such a medication would be highly effective for treating multifactorial diseases. The design of such a drug may not be easy because it could also bind targets that are not involved with the disease and could be responsible (although not necessarily) for side effects. With MTDLs, the one-medication, one-disease paradigm finds a practical application.

targets should not, in principle, be different from the development of any other single lead molecule. It thus offers a much simpler approach than MMT/MCM. In addition, the risk of possible drug–drug interactions would be avoided and the therapeutic regimen greatly simplified in relation to MMT. All these considerations are of particular relevance, as one of the major contributions to the attrition rate in drug development continues to be the drug candidate's pharmacokinetic profiling.⁶ There is, therefore, a strong indication that the development of compounds able to hit multiple targets might disclose new avenues for the treatment of, for example, major neurodegenerative diseases, for which an effective cure is an urgent need and an unmet goal.

Morphy and Rankovic elegantly discussed this approach in recent articles,^{5,7,8} which was mostly concerned with non-neurodegenerative diseases. They proposed the term “designed multiple ligands” to describe compounds whose multiple biological profile is rationally designed to address a particular disease. Despite our awareness that the use of different terms to illustrate the same concept may obscure developments in the field, we are convinced that the definition “multi-target-directed ligands” (MTDLs) more completely describes those compounds that are effective in treating complex diseases because of their ability to interact with the multiple targets thought to be responsible for the disease pathogenesis (Figure 1).

The excellent perspective by Morphy and Rankovic⁵ covers several aspects of the design strategy leading to MTDLs for different areas such as inflammation, dopaminergic D₂-receptors,

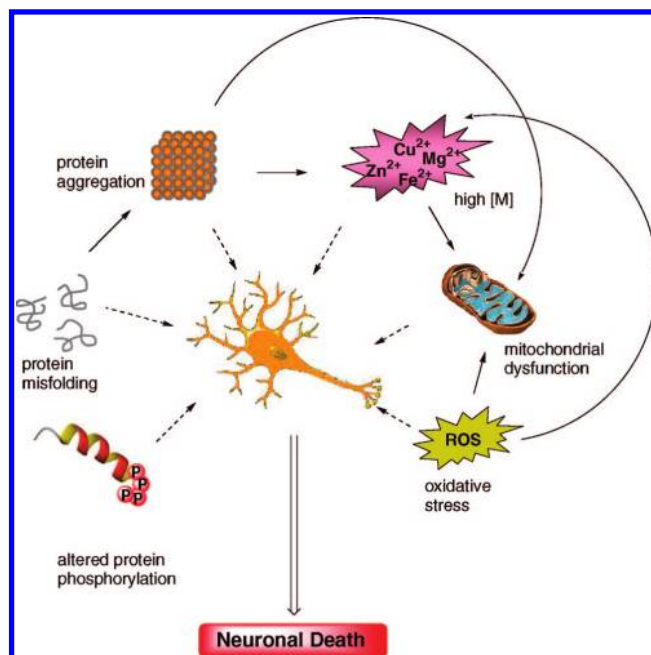


Figure 2. Schematic pathways of the multifactorial events leading to neuronal death. General mechanisms, such as protein misfolding and aggregation, oxidative stress, metal (M) dyshomeostasis, mitochondrial dysfunction, and altered protein phosphorylation, have been identified in several neuronal disorders.

histaminergic H₁-receptors, serotonergic receptors, angiotensin system, peroxisome proliferators activated receptors, kinases, and nitric oxide releasing conjugates. However, there is a paucity of review literature dealing with complex diseases associated with neurodegeneration. The objective of this Perspective is to review those investigations reported in the past decade that illustrate the ability of medicinal chemists to design MTDLs to confront neurodegenerative diseases. We will briefly illustrate the multifactorial nature of these diseases before discussing the possible development of MTDLs in light of the known molecular bases of the main neurodegenerative pathologies.

2. Multifactorial Nature of Neurodegenerative Diseases

Neurodegenerative diseases have long been viewed as among the most enigmatic and problematic issues in biomedicine.⁹ As research on neurodegenerative diseases has moved from descriptive phenomenology to mechanistic analysis, it has become increasingly clear that the major basic processes involved are multifactorial in nature, caused by genetic, environmental, and endogenous factors. The neurodegenerative diseases sharing such multifactorial pathogenic mechanism are, among others, Alzheimer's, Parkinson's, and Huntington's diseases and amyotrophic lateral sclerosis (hereafter referred to as AD, PD, HD, and ALS, respectively). These diseases will be the subject of this article.

Although each disease has its own molecular mechanisms and clinical manifestations, some general pathways might be recognized in different pathogenic cascades. They include protein misfolding and aggregation, oxidative stress and free radical formation, metal dyshomeostasis, mitochondrial dysfunction, and phosphorylation impairment, all occurring concurrently (Figure 2).¹⁰

Protein misfolding followed by self-association and subsequent deposition of the aggregated proteins has been observed in the brain tissues of patients affected by these disorders. Monomeric species initially form small oligomers, which then

^aAbbreviations: A β , amyloid- β ; ACh, acetylcholine; AChE, acetylcholinesterase; AChEI, acetylcholinesterase inhibitor; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; BACE, β -secretase; BBB, blood–brain barrier; BuChE, butyrylcholinesterase; cdk5, cyclin-dependent protein kinase 5; COMT, catechol-O-methyltransferase; CoQ, coenzyme Q10; DA, dopamine; ER, endoplasmic reticulum; GSK-3 β , glycogen synthase kinase-3 β ; HD, Huntington's disease; 5-HT, serotonin; LA, lipoic acid; LBs, Lewy bodies; LPO, lipid peroxidation; MAO, monoamine oxidase; MCM, multiple-compound medication; MMT, multiple-medication therapy; MTDL, multi-target-directed ligand; NFT, neurofibrillary tangle; NQO1, NAD(P)H/quinone oxidoreductase 1; PAS, peripheral anionic site; PD, Parkinson's disease; PHF, paired helical filaments; ROS, reactive oxygen species; SAPK, stress-activated protein kinases; SERT, serotonin transporter; SOD, superoxide dismutase; UPS, ubiquitin-proteasomal system; VDCC, voltage-dependent calcium channels.

nucleate the growth of fibrillar structures in a stepwise fashion.^{11,12} Indeed, soluble proteins of entirely different sequences can fold into stable β -sheet conformations that share conformational epitopes. This finding suggests that assemblies produced by different amyloidogenic proteins might trigger a similar neurotoxic mechanism. Despite presenting widely divergent primary and secondary structures, all proteins, when they form amyloid fibrils, adopt the cross- β -sheet arrangement with the axis of the peptide oriented perpendicularly to the fibril axis.¹³ The final proteinaceous aggregates derived from this pathological event share common structural and histological morphologies. Their biological effects, however, are distinctive and depend on such features as the tissue involved and whether the deposits are intracellular or extracellular.^{14,15} The biophysical behavior of these proteins, leading to their misfolding, aggregation, and deposition, has prompted scientists to group these kinds of neurological disorders under the common name of "conformational diseases".¹⁶ It is worth noting that amyloid oligomers such as amyloid- β (A β) and α -synuclein have been widely reported to permeabilize both cell and mitochondrial membranes.^{17,18} They are therefore probably responsible for calcium dysregulation, membrane depolarization, and impairment of mitochondrial functions, which have been identified as a further common feature of most neurodegenerative disorders (see below).¹⁹

The role of reactive oxygen species (ROS) in many neurodegenerative diseases was deemed to be as important as the role of microorganisms in infectious diseases.²⁰ For years, scientists debated whether oxidative stress was a cause or consequence of the neurodegenerative cascade.²¹ At present, scientists are close to a consensus that imbalance of intracellular oxidation state is an early event of neurodegeneration and is therefore likely to be one of the major factors of neurodegenerative diseases. Neuronal tissue is particularly sensitive to oxidative stress, and imbalance in pro-oxidant vs antioxidant homeostasis in CNS results in the production of several potentially toxic ROS, including both the radical and nonradical species that participate in the initiation and/or propagation of radical chain reactions. In AD, PD, HD, and ALS, oxidative damage is found in every class of biological molecules within neurons, spanning from lipids to DNA and proteins.²² However, the administration of one or a few antioxidants is too simplistic, as demonstrated by the several clinical studies that have shown modest success with antioxidants in the treatment of neurodegeneration.²³

For the above-mentioned neurodegenerative diseases, a direct cause and effect relationship between metal abnormalities and increased oxidative damage has been hypothesized on the basis of various experimental evidence. While transition metals are essential in many biological reactions, alterations in their homeostasis result in increased free radical production, which is catalyzed by iron, copper, or other trace redox active metals.^{11,24} Moreover, all the disease-specific proteins bear metal-binding motifs, and metal ions favor fibril generation and deposition.²⁵ In all cases, metal-mediated oxidative stress is also linked to mitochondrial dysfunction.¹⁹ Notably, abnormal mitochondrial function could follow ROS generation rather than being a cause of oxidative stress in neurons.²⁶ There is substantial evidence of morphological, biochemical, and molecular abnormalities in mitochondria in various tissues affected by AD, PD, HD, and ALS.²⁷ Moreover, an impressive number of disease-specific proteins that interact with mitochondria have recently been disclosed: (i) amyloid precursor protein (APP), A β , presenilin in AD; (ii) α -synuclein, parkin, DJ-1, PINK1, HTRA2 in PD; (iii) superoxide dismutase 1 (SOD1) in ALS;

(iv) huntingtin in HD.¹⁹ Although the chronological hierarchy of events and underlying causes in neurodegenerative diseases with regard to mitochondrial dysfunction and oxidative stress are not yet fully understood, there is overwhelming evidence that either one can lead to the evolution of the other, setting in motion a self-sustaining, amplifying cycle that can ultimately trigger activation of neuronal death processes.²⁶ In addition to mitochondria, the endoplasmic reticulum (ER) also serves as an important apoptotic checkpoint. For instance, it has been shown that, in AD, apoptosis induced by misfolded proteins involves ER impairment.²⁸

As recently disclosed in the specialist literature, a further common mechanism shared by neurodegenerative diseases concerns the alteration of the phosphorylation state of some key proteins involved in the pathogenic cascades. Besides the well-recognized hyperphosphorylated state of τ protein in the neurofibrillary tangles observed in AD brain,²⁹ specific altered patterns of kinase and phosphatase activities are associated with alteration in the phosphorylation state of disease-specific proteins in PD,³⁰ ALS,³¹ and HD.³²

The availability of such extensive molecular evidence raises the issue of cell-type specificity in neuronal disorders. Selective neuron degeneration has been shown to be a fundamental characteristic of each disease. The issue of cell-type specificity is therefore an open question for those studying the pathogenesis and treatment of these illnesses.

However, none of these general mechanisms alone are sufficient to explain the high number of biochemical and pathological abnormalities of neurodegenerative diseases, which involve a multitude of cross-related cellular and biochemical changes that might not be adequately addressed by following the one-molecule, one-target paradigm. In our opinion, there is therefore a growing interest in and urgent need for MTDLs to provide real disease-modifying drug candidates for such neurodegenerative diseases. Since most of these neurodegenerative mechanisms are shared by many neuronal disorders, MTDLs may also potentially be used as medications for more than one illness. Surprisingly, the pharmaceutical industry is not investing heavily in the development of innovative treatments for neurodegenerative diseases, as one would expect on the basis of both growing scientific evidence and patient populations.¹

Polypharmacology is not new. What is new is both the understanding of its relevance in drug discovery and the understanding of the molecular events at its basis.³³

3. Alzheimer's Disease (AD)

AD stands out among the neurodegenerative diseases as the fourth leading cause of death in the Western countries and the most common cause of acquired dementia in the elderly population. Two forms of AD exist: a familial one (multiple family members are affected) and a sporadic one, in which one or a few members of a family develop the disease. In line with an increase in average life expectancy, the number of affected persons is expected to triple by 2050, with immense economic and personal tolls.³⁴ In parallel with this increase, the speed of drug research has accelerated noticeably in recent decades. However, the number of therapeutic options on the market remains severely narrow. The currently registered drugs for AD are not able to alter or prevent disease progression. They are, instead, palliative in alleviating disease symptomatology.³⁵

One-hundred years after the discovery of AD, the scientific consensus is quite firm that although the pathogenesis of AD is not yet fully understood, it is a multifactorial disease caused by genetic, environmental, and endogenous factors, as with the

other neurodegenerative disorders. These factors include excessive protein misfolding and aggregation, often related to the ubiquitin–proteasomal system (UPS), oxidative stress and free radical formation, impaired bioenergetics and mitochondrial abnormalities, and neuroinflammatory processes. These insights, coupled with further ongoing discoveries about AD pathogenesis, have provided the rationale for therapies directly targeting AD molecular causes. New drug candidates with disease-modifying potential are now in the pipeline and have reached testing in clinical trials.

3.1. Molecular Causes of AD. Since Alois Alzheimer's seminal report of November 1906,³⁶ pathologists have considered the defining characteristic hallmarks of the disease to be A β deposits in senile plaques and neurofibrillary tangles (NFT), consisting mainly of paired helical filaments of abnormally phosphorylated τ protein. As the disease progresses, neuronal death appears. In particular, cholinergic neurons and synapses of the basal forebrain are selectively lost, accounting for the development of cognitive impairments. These findings constituted the premises for the so-called "cholinergic hypothesis", which proposed cholinergic enhancement as an approach for improving cognitive function in AD.³⁷ This approach has so far produced the majority of drugs approved for treating AD.³⁸

Nowadays, compelling evidence suggests that A β secretion is the triggering event in the pathogenesis of AD and that τ aggregation may be an important secondary event linked to neurodegeneration (Figure 3).³⁹ According to the "amyloid cascade hypothesis", A β would originate from the sequential proteolysis of the APP and it would deposit and aggregate (or aggregate and deposit) in extracellular insoluble plaques. The first cleavage of APP is carried out by β -secretase (also called β -site APP cleaving enzyme or BACE).⁴⁰ The carboxyterminal fragment is then severed by the γ -secretase complex,⁴¹ producing A β . Alternatively, cleavage of the protein by α -secretase allows for the release of a large fragment, α -APPs, which is not amyloidogenic. Central to this hypothesis is the observation that the amount of fibrillogenic A β is increased by the vast majority of mutations causing familial AD and that A β impairs neuronal functions in a variety of experimental models.⁴² Soluble A β is thought to undergo a conformational change to high β -sheet content, which renders it prone to aggregate into soluble oligomers and larger insoluble fibrils in plaques. In this process, the fibrillogenic A β 42 isoform triggers the misfolding of other A β species. Currently, the nature of the neurotoxic A β species is very difficult to define because monomers, soluble oligomers, insoluble oligomers, and insoluble amyloid fibrils are expected to accumulate and exist in dynamic equilibrium in the brain. Initially, only A β deposited in plaques was assumed to be neurotoxic, but more recent findings suggest that soluble oligomers (A β -derived diffusible ligands or ADDLs) might be the central players. Afterward, A β may exert its neurotoxic effects in a variety of ways, including disruption of mitochondrial function via binding of the A β -binding alcohol dehydrogenase protein,⁴³ induction of apoptotic genes through inhibition of Wnt⁴⁴ and insulin signaling,⁴⁵ formation of ion channels,¹⁸ stimulation of the stress-activated protein kinases (SAPK) pathway⁴⁶ or activation of microglia cells leading to the expression of proinflammatory genes, an increase in ROS, and eventual neuronal toxicity and death.⁴⁷ More recently, it has become clear that, in addition to forming extracellular aggregates, A β (or its precursor APP) has complicated intracellular effects involving a variety of subcellular organelles, including mitochondria. Mitochondrial APP has been shown to accumulate in the protein import channels of mitochondria of human AD

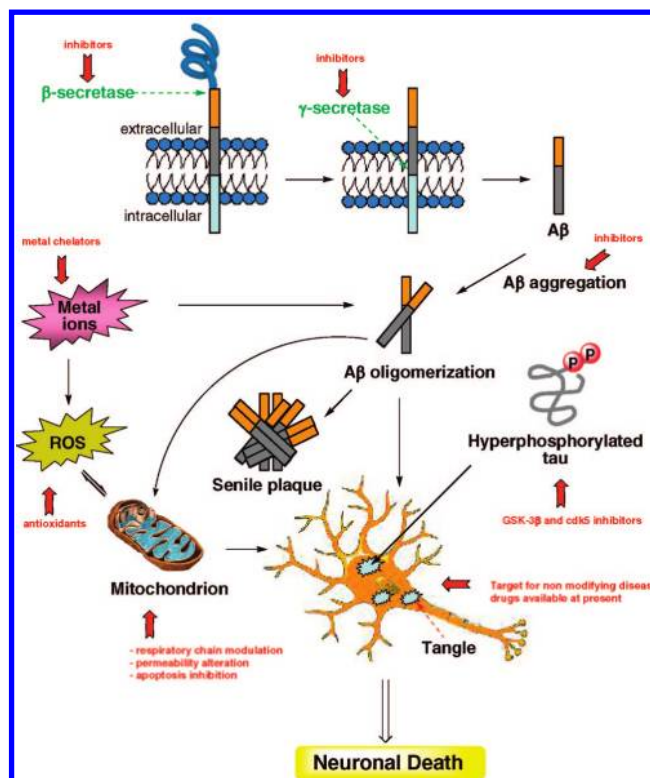


Figure 3. Possible molecular causes of neuronal death and protective mechanisms in AD. The central event in AD pathogenesis is an imbalance between A β production and clearance. The enhanced activity of β - and γ -secretases leads to increased release of amyloidogenic A β 42, which forms oligomers and then extracellular deposits (senile plaques). One way to confront AD pathogenesis may be to combat the oligomerization by means of small molecules. A role for metal ions and ROS in the A β oligomerization has also been advanced. Therefore, metal chelation and antioxidant activity are two general mechanisms to be considered in the search for disease-modifying anti-AD drug candidates. Also, β - and γ -secretase inhibitors may be promising lead compounds because they tackle an early event in AD pathogenesis. Mitochondrial dysfunction plays a fundamental role in the neuronal death associated with AD, as it is likely that intracellular A β could compromise the function of this organelle. τ hyperphosphorylation leading to tangle formation is regarded as a downstream event but could contribute to reinforcing neuronal dysfunction and cognitive impairment.

brains, and this accumulation inhibits the entry of cytochrome *c* oxidase subunits proteins,⁴⁸ with decreased activity of respiratory chain enzymes, increased free radical generation, and impaired reducing capacity. These data provide a potential explanation for the well-established observation that mitochondrial function and energy metabolism are impaired early in AD.⁴⁹

NFTs are composed mainly of paired helical filaments (PHF) that contain an abnormal hyperphosphorylated form of the microtubule-associated protein τ . Phosphorylation within the microtubule binding domain of τ protein results in its reduced ability to stabilize microtubules assembly, leading to the disruption of neuronal transport and eventually to accelerated synaptic loss and cell death. Dephosphorylation of τ protein isolated from NFT restores its ability to bind with neuronal microtubules, indicating that the mechanisms regulating phosphorylation/dephosphorylation kinetics are perturbed in AD. The nature of protein kinases, phosphatases, and τ sites involved in this lesion has recently been elucidated, suggesting that activation of phosphoserine/phosphothreonine protein phosphatase-2A (PP-2A) or inhibition of both glycogen synthase kinase-3 β

(GSK-3 β) and cyclin-dependent protein kinase 5 (cdk5) might be required to inhibit AD neurofibrillary degeneration.⁵⁰

Several other hypotheses have been proposed to explain the pathogenesis of AD, including oxidative stress, metal ion dyshomeostasis, and inflammation.⁵¹ In the context of such a complex disease, it is not trivial to state that these hypotheses are not mutually exclusive. Rather, they complement each other, intersecting at a high level of complexity.

Oxidative damage is present within the brain of AD patients and is observed within every class of biological macromolecules, including nucleic acids, proteins, lipids, and carbohydrates.⁵² Oxidative injury may develop secondary to excessive oxidative stress resulting from A β -induced free radicals, mitochondrial abnormalities, inadequate energy supply, inflammation, or altered antioxidant defenses. Oxidative stress is thought to have a causative role in the pathogenesis of AD.⁵³ Support for this hypothesis has also been provided by the current notion that, while AD is probably associated with multifaceted etiologies and pathogenic phenomena, all these mechanisms seem to share oxidative stress as a unifying factor.

Strictly related to the oxidative damage hypothesis, there is general acceptance that redox active metals can contribute to excess production of damaging ROS through Fenton's chemistry.⁵⁴ Besides creating oxidative stress, copper, together with other metal ions, influences the protein aggregation processes that are critical in most neurodegenerative diseases. For example, APP and A β are able to bind and reduce copper, which forms a high-affinity complex with A β , promoting its aggregation, and A β neurotoxicity depends on catalytically generated H₂O₂ by A β -copper complexes in vitro. Moreover, copper, together with zinc and iron, is accumulated in the amyloid deposits of AD brains, which are partially disassembled by metal chelators.¹¹

Finally, neuroinflammation of CNS cells has been recognized as an invariable feature of all neurodegenerative disorders. In AD, among CNS cells, microglia have received special interest. Microglia are activated by A β to produce cytokines, chemokines, and neurotoxins that are potentially toxic and therefore may contribute to neuronal degeneration. However, recent findings suggest that microglia may play a neuroprotective role in AD. This highlights the potential risk of using the inhibition of monocyte/macrophage recruitment as a therapeutic strategy and argues for caution in the pursuit of this approach.⁵⁵ Despite this, modulation of inflammation is one of the most dynamic areas in the search for new therapeutic targets for AD and related neurodegenerative disorders.⁵⁶

3.2. Current AD Therapies. Of the above-mentioned hypotheses, the cholinergic one is the oldest. It has also had the strongest influence on the development of clinical treatment strategies. In fact, in 1993, it led to the introduction of the acetylcholinesterase inhibitor (AChEI) tacrine (**1**), the first drug to be approved for the treatment of AD, now rarely used because of its hepatotoxicity. Later, three other AChEIs, donepezil (**2**), rivastigmine (**3**), and galantamine (**4**) reached the market, becoming the standard for AD therapy, only later complemented by memantine (**5**), a noncompetitive NMDA antagonist (Figures 4 and 5).

Notwithstanding the diffused clinical practice,⁵⁷ the debate on whether or not AChEIs are effective medications continues. Although beneficial in improving cognitive, behavioral, and functional impairments, they seem unable to address the molecular mechanisms that underlie the pathogenic processes.⁵⁸

Current AD drug development programs focus primarily on agents with anti-amyloid disease-modifying properties. Many different pharmacological approaches to reducing amyloid

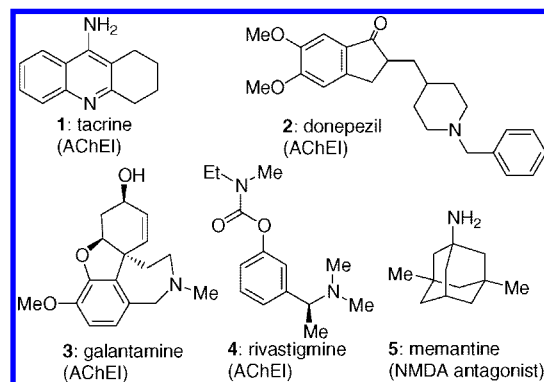


Figure 4. Chemical structures of the five drugs available for AD treatment (mechanisms of action are indicated in parentheses).

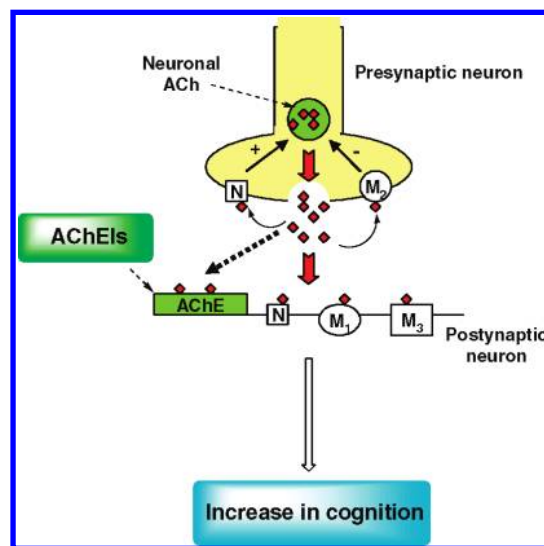


Figure 5. Mechanism of action of ACh at a cholinergic synapse. ACh is released in the synaptic cleft where it activates both postsynaptic and presynaptic cholinergic receptors [nicotinic (N) and muscarinic (M)] leading to an increase of cholinergic transmission, which results in cognition improvement. ACh is removed from the synapse by the action of the enzyme AChE, which is the target of the available AChEIs for AD treatment.

pathology and tauopathy are being studied. Classes of therapeutic modalities currently in the advanced stage of clinical trial testing include forms of immunotherapy, a γ -secretase inhibitor, the selective A β 42-lowering agent R-flurbiprofen, and the antiaggregation agent tramiprosate. Other nontraditional dementia therapies such as the HMG-CoA reductase inhibitors (statins), valproate, and lithium salts are now being evaluated for their clinical benefits as disease-modifying treatments.⁵⁹

In parallel, the recent discovery of the so-called "nonclassical function"⁶⁰ of acetylcholinesterase (AChE) has renewed interest in the search for novel AChEIs, expanding their potential as real disease-modifying agents.^{61–63} In particular, it has been reported that AChE might act as a "pathological chaperone" in inducing A β aggregation through the direct interaction of its peripheral anionic site (PAS) with fibrils of the peptide.⁶⁴ This has led scientists to reconsider this enzyme as a target that mediates two important effects in the neurotoxic cascade, that is, A β fibrils formation and acetylcholine (ACh) breakdown.

3.3. MDTLs for the Treatment of AD. The accumulating insights into AD complexity and the multiple etiologies contributing to the disease allow us to be confident that MMT and/or MCM might result in a more effective treatment strategy.⁶⁵ These medications, which offer the prospect of

additional benefits with respect to single-drug treatments, may represent a way of targeting the multiple pathological processes involved in AD. MMT has already proven successful in the treatment of similarly complex diseases such as cancer, HIV, and hypertension, where it achieves maximum efficacy by attacking several targets simultaneously, exploiting synergy, and minimizing individual toxicity. It is worth noting that the number of patented MCM has overtaken that of single-drug entities for the potential treatment of AD.⁶⁶ In clinics, MMT of memantine (**5**) and an AChEI appears to produce an additional effect resulting in a well-tolerated, effective treatment strategy.⁶⁷

Considering the well-accepted clinical use of MMT as a starting point, the MTDL design strategy might represent a natural evolution, and MTDLs emerge as valuable tools for hitting the multiple targets implicated in AD etiology. Several MTDLs have been developed by academia and industry in recent years. These have been the subject of some interesting review articles.^{68–73}

To obtain novel MTDLs, a design strategy is usually applied in which distinct pharmacophores of different drugs are combined in the same structure to afford hybrid molecules. In principle, each pharmacophore of these new drugs should retain the ability to interact with its specific site(s) on the target and, consequently, to produce specific pharmacological responses that, taken together, should slow or block the neurodegenerative process. One of the most widely adopted approaches in the field has been to modify the molecular structure of an AChEI in order to provide it with additional biological properties useful for treating AD.

Dual Binding Site AChEIs Targeting A β Aggregation. In the MTDL design strategy context, major research efforts have been devoted to the development of the so-called “dual binding site” AChEIs. By simultaneously interacting with AChE catalytic and peripheral sites, these AChEIs might alleviate the cognitive deficit in AD by restoring cholinergic activity. More importantly, they might address the disease mechanisms by reducing A β aggregation. However, the classification of this highly populated class of compounds as MTDLs deserves comment. The fact that these inhibitors are shown to bind to more than one site along the AChE gorge is not a sufficient condition to label them as MTDLs. Rather, the term “multisite inhibitors” seems more appropriate. However, if it can be proven with experiments that their ability to block PAS allows them to retard A β assembly, then they do indeed show concurrent activities deriving from the multiple-binding mode and can therefore be classed as MTDLs.

Caproctamine (**6**) represents one of the first examples of a successfully designed AChEI endowed with additional pharmacological effects beneficial in AD. **6** was developed using the universal template approach (Figure 6).⁷⁴ It was argued that ligands having affinity for both AChE catalytic site and PAS and for muscarinic M₂ receptors might be of interest because inhibition of AChE activity would potentiate the remaining cholinergic transmission in affected brain regions while antagonism of muscarinic M₂ autoreceptors would facilitate the release of ACh in the synapse. Furthermore, inhibition of the peripheral binding site would prevent the aggregation of A β induced by AChE. The starting point of this study was the observation that the tetraamine disulfide benextramine (**7**), originally developed as an irreversible α -adrenoreceptor antagonist and then shown to also be a muscarinic M₂ receptor antagonist, turned out to reversibly inhibit AChE activity. Thus, structural manipulations of the tetraamine backbone of **7** led to **6**, which emerged as an effective pharmacological tool in AD because of a well-balanced

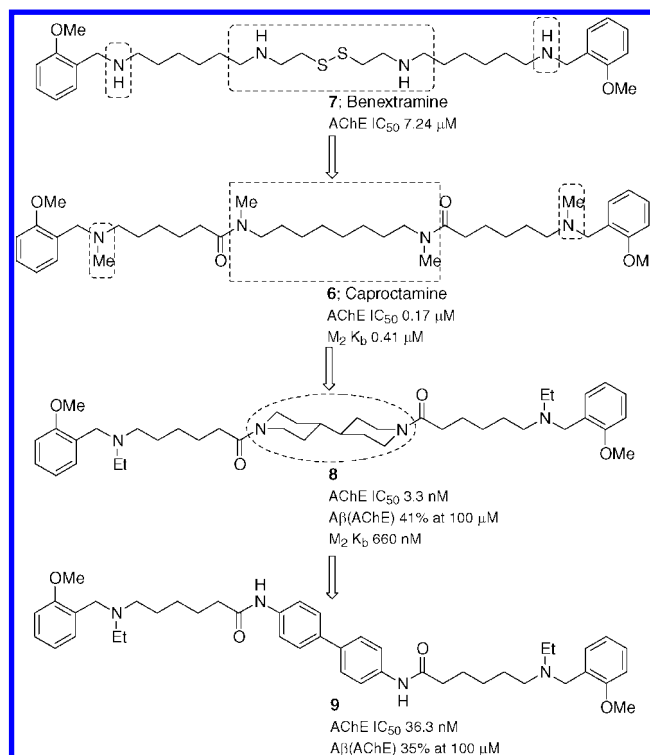


Figure 6. Design strategy leading to the diamine diamide **6** and, subsequently, to its two derivatives **8** and **9** (selected from among many others) through the modification of the cystamine moiety of the tetraamine **7** and then the octamethylene spacer of **6**.

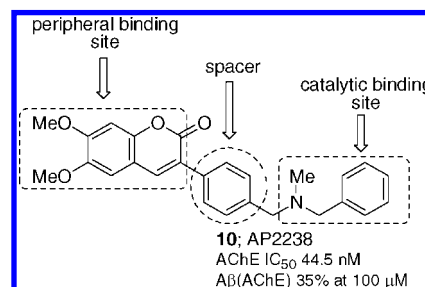


Figure 7. Design strategy leading to **10**, the first AChEI designed to bind both AChE catalytic site and PAS in order to counteract AChE-induced A β aggregation as well as increasing ACh content.

affinity profile as AChEI and competitive muscarinic M₂ receptor antagonist.⁷⁵ Moreover, docking studies aimed at clarifying the binding mode of **6** at the AChE gorge revealed that it may make contact with both AChE sites, even though this behavior was not experimentally verified.

The development of **10** gave direct evidence of the A β antiaggregating action of AChEIs purposely designed to bind at both the catalytic site and PAS of the human AChE. Such a dual inhibitor was designed by combining in the same molecule two moieties optimal for the binding at each enzyme site and linked by an appropriate spacer. The two moieties, a benzylamino group and a coumarin (2*H*-2-chromenone) heterocycle, were selected from previously developed AChEIs (Figure 7). With regard to the spacer, the choice of a phenyl ring was dictated by the fact that it could favorably interact with some of the numerous aromatic residues lining the AChE gorge. Besides having an AChE inhibitory activity comparable to that of **2**, **10** was shown to counteract A β aggregation with a higher potency than other tested AChEIs.⁷⁶

This pioneering work, together with the availability of a detailed experimental protocol for testing the inhibition of AChE-induced

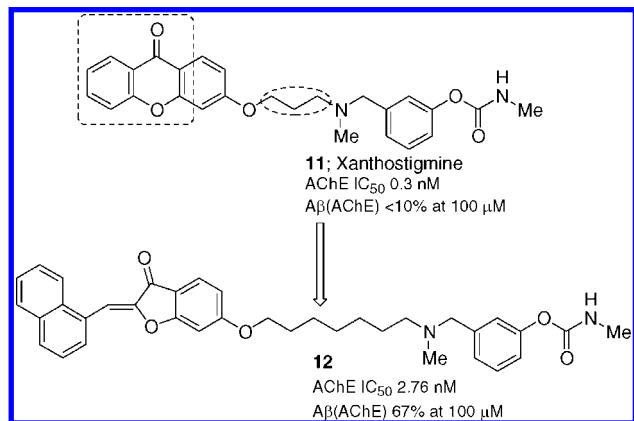


Figure 8. Structural modification of **11** leading to a series of derivatives, of which **12**, unlike the prototype, effectively inhibited AChE-induced Aβ aggregation by interacting with both AChE catalytic site and PAS.

Aβ aggregation,⁷⁷ opened the way for the development of novel AChEIs that emerged as potential new AD therapeutics because of their ability to increase the level of ACh while simultaneously interfering with amyloid processing.^{61–63} In this respect, SAR studies of **6** were expanded by investigating the role of the octamethylene spacer separating its two amide functions. This was achieved with its replacement by more rigid dipiperidine and dianiline moieties (Figure 6). Compound **9** was the most potent AChEI of the series (pIC₅₀ = 8.48 ± 0.02). It also displayed significant muscarinic M₂ receptor antagonism (pK_b = 6.18 ± 0.20). Moreover, the ability of **8** and **9** to inhibit AChE-induced Aβ aggregation was verified in comparison to that of the lead compound **6**. Although all the derivatives caused a mixed type of AChE inhibition (active site and PAS), only **8** and **9**, which bear an inner constrained spacer, were able to inhibit AChE-induced Aβ aggregation to a greater extent than **2**. Clearly, the ability of an AChEI, based on a linear polyamine backbone, to bind both AChE sites may not be a sufficient condition to inhibit AChE-induced Aβ aggregation too.⁷⁸ Notably, the **9** concentration that effectively inhibited AChE-induced Aβ aggregation (35% at 100 μM) was much higher than its IC₅₀ value (36.3 nM) against the AChE catalytic activity. Nevertheless, as previously highlighted,⁷⁷ if “normalized” on the basis of the different amounts of enzyme used in the two experiments, the ratio [inhibitor]/[AChE] is the same in both assays. Consequently, it seems plausible that similar amounts of inhibitors can simultaneously carry out both the anticholinesterase and antiaggregating actions.

Similarly, the structure of the potent AChEI xanthostigmine (**11**), which displays only marginal activity against AChE-induced Aβ aggregation, was modified to obtain derivatives able to simultaneously block both AChE catalytic and proaggregatory activities. The design strategy entailed the replacement of the dibenzopyran-4-one moiety of **11** with groups characterized by greater flexibility and space extension, such as the 2-arylidenebenzofuran-3-one moiety, which provides the ability to bind AChE PAS (Figure 8). Indeed, these structural modifications led to **12**, which was shown to interact with Trp286 of PAS and consequently to effectively prevent AChE-induced Aβ aggregation.⁷⁹

On the basis of the observation that polyamines inhibit AChE activity and that AChE gorge is lined with several aromatic amino acid side chains capable of forming cation–π interactions with basic counterparts, dual binding site AChEIs were designed by linking the molecular features of well-known AChEIs, which

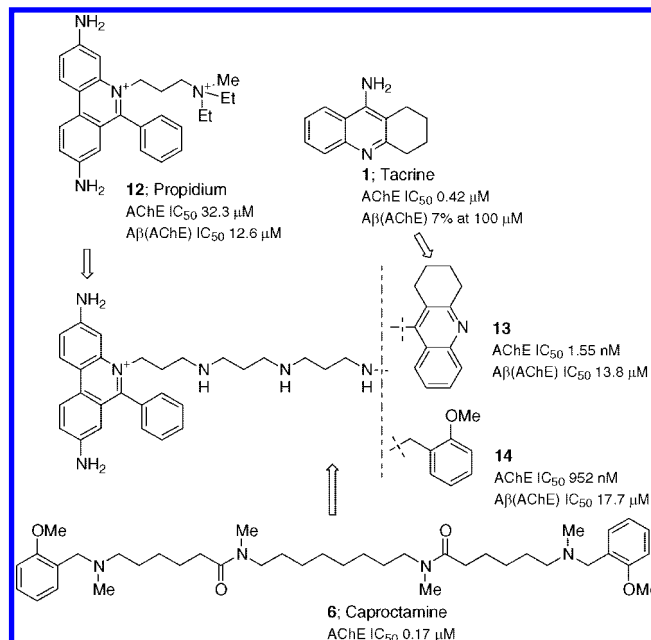


Figure 9. Design strategy leading to **13** and **14** by combining the structural features of **12** with those of **1** or **6**.

inhibit both AChE catalytic site and PAS, through a polyamine chain. In this case, as postulated by the universal template design strategy,⁸⁰ the nature and length of the spacer become critical, since they play an active role in the target recognition process. As proof of principle, the structural motif of propidium (**12**) was linked to either the tetrahydroaminoacridine system of **1** or the methoxybenzylamino group of **6** by way of a triamine backbone, affording novel heterobivalent polyamine ligands (Figure 9). Heterodimerization resulted in a remarkable increase in AChE potency. Compound **13** was nearly 20000-fold more potent than **12** and 300-fold more potent than **1**, consistent with simultaneous binding to both the AChE catalytic site and PAS. In fact, both inhibitors markedly prevented the proaggregating effect of AChE toward Aβ, with IC₅₀ values comparable to those shown by **12**, a specific PAS ligand and the most effective inhibitor available at that time.⁸¹

An improved AChE-induced Aβ aggregation inhibitory profile was shown by a series of heterodimers in which a 1,2,3,4-tetrahydroacridine moiety was linked through a suitable spacer to an indole ring (Figure 10). The indole ring was shown to reach the entrance of the catalytic gorge and interact with the Trp286 by forming a π–π stacking. In particular, compounds **15** and **16** emerged as the most potent AChEIs of the series, displaying IC₅₀ values of 20 and 60 pM, respectively. More importantly, these dual AChEIs inhibit the AChE-induced Aβ aggregation with IC₅₀ values 1 order of magnitude lower than that of **12**. They are thus the most potent derivatives so far reported to inhibit the AChE-mediated Aβ aggregation.⁸²

Recently, a series of pyridinium-bearing AChEIs were evaluated as inhibitors of self-mediated Aβ aggregation. This was done by means of a thioflavin T fluorescence assay to reveal potential additional pharmacological effects that might reinforce their therapeutic application. **17** and its dimeric derivative **18** (Figure 11) inhibited Aβ aggregation by 50% when tested at an equimolar concentration with Aβ40. Although these compounds were not investigated to verify their ability to inhibit AChE-induced Aβ aggregation too, the ability to block both AChE- and self-mediated Aβ aggregation makes **17** and **18** valuable tools for developing drugs targeting Aβ pathogenesis in AD.⁸³ However, concern arises about the ability of these

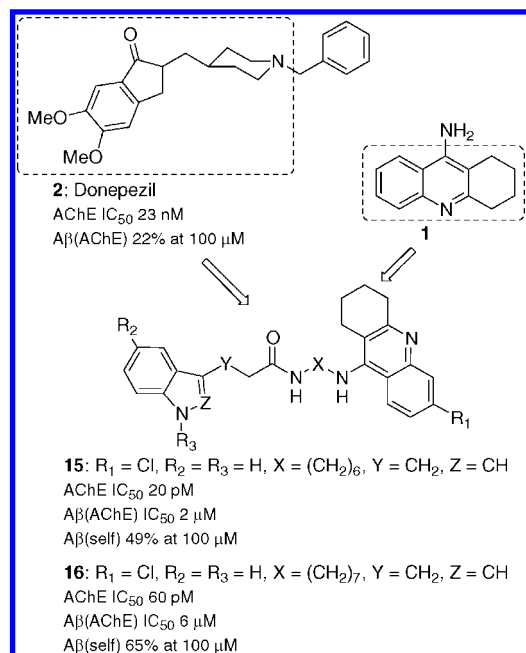


Figure 10. Design strategy leading to dual binding site AChEIs by combining an isosteric group of the indanone moiety of **2** with the 1,2,3,4-tetrahydroacridine system of **1** through a spacer of variable chain length.

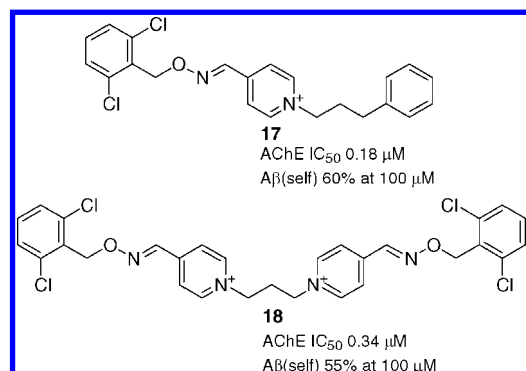


Figure 11. Structures of the AChEIs **17** and **18** with the additional property of inhibiting self-mediated Aβ aggregation.

quaternary ammonium derivatives to cross the blood–brain barrier (BBB).

AChEIs Targeting Other Neurotransmitter Systems. There is a well-documented link between neurotransmitter changes occurring in the brain of AD patients and clinically observed symptoms, such as cognitive decline and neuropsychiatric abnormalities. It is widely acknowledged that cognitive decline relates most closely to loss of cholinergic and glutamatergic neurons. However, behavioral change relates not only to the severity of cholinergic loss but also to alterations in the serotonergic and noradrenergic systems. For example, the noradrenergic deficits of AD stem from locus ceruleus atrophy and are linked to depression.⁸⁴ Serotonergic deficits, in contrast, have their source in raphe atrophy and are linked to depression and psychosis.⁸⁵

Opportunities therefore arise for therapeutic intervention with drugs that increase the activity of biogenic amines, either alone or as MMT/MCM with compounds acting on the cholinergic system. Recent clinical trials have combined the monoamine oxidase (MAO) inhibitor selegiline (**19**) with either **1** or physostigmine (**20**). Early data suggest possible synergistic effects^{86,87} and point to MAO inhibition as an interesting

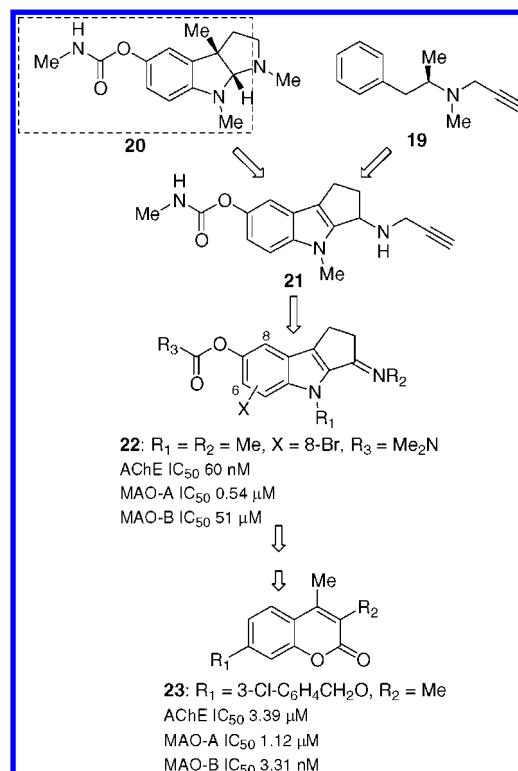


Figure 12. Design strategy leading to selected dual inhibitors of AChE and MAO.

property to be taken into account when designing MTDLs against AD. In fact, MAO, during its catalytic activity of deamination of neurotransmitters [noradrenalin, dopamine (DA), and serotonin (5-HT)], produces H₂O₂, which is a possible source of oxidative stress for vulnerable neurons affected by AD.⁸⁸

The initial design strategy for combined MAO/AChE agents resulted in a series of inhibitors (e.g., **21**) that, because of the incorporation of the propargylamine pharmacophore of **19** in **20** (Figure 12), were still irreversible MAO inhibitors.⁸⁹ Biological testing of imine intermediates of target compounds showed some weak combined activities. More importantly, the compounds appeared to be MAO reversible inhibitors, potentially devoid of the undesirable side effect associated with the old irreversible inhibitors. Therefore, the imino 1,2,3,4-tetrahydrocyclopent[*b*]indole carbamate scaffold was selected for further SAR studies. Introduction of a bromine atom in a position ortho to the carbamate function afforded **22**, which showed a dramatic improvement in affinity for both AChE and MAO-A. Unfortunately, following oral administration in animal model, **22** showed low activity due to poor brain penetration and poor bioavailability and was considered unsuitable for further development.⁸⁹ However, this work provided researchers with the structural starting point for the design and synthesis of several dual AChE/MAO inhibitors. Thus, some coumarinic derivatives, already characterized as MAO-A and MAO-B inhibitors, were investigated as potential AChEIs.⁹⁰ All compounds inhibited AChE with IC₅₀ values in the micromolar range (3–100 μM). In a kinetic study, most of them acted as noncompetitive AChEIs.⁹¹ This finding was of particular interest for AD because AChE inhibition, as already pointed out, might also decrease Aβ deposition. Indeed, this program led to the MTDL **23**, which made it possible to propose an experimental and virtual screening pathway to generate MTDLs as hits for the treatment of AD and other aging-related neurodegenerative

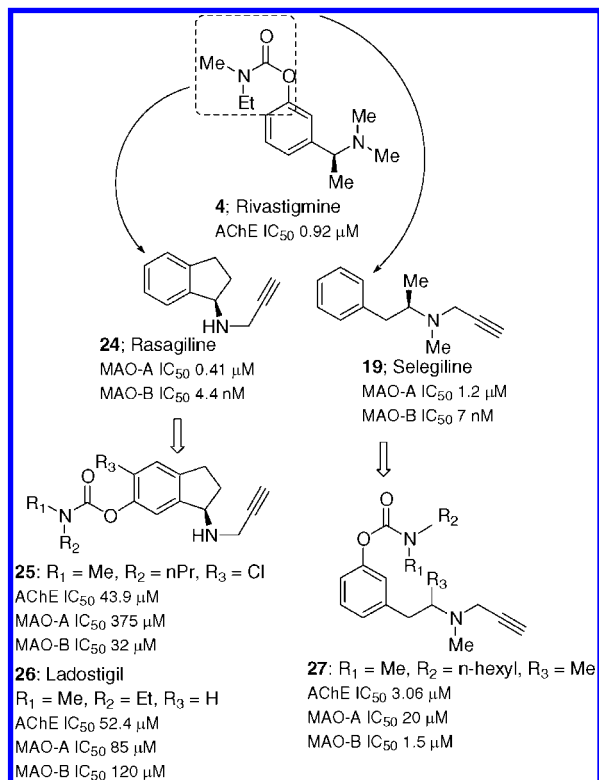


Figure 13. Design strategy leading to AChE and MAO-B inhibitors by combining the structural features of the MAO-B inhibitors **24** and **19** with a carbamate moiety of AChEIs, for example, **4**.

disorders.⁹² The application of this screening pathway to a series of non-alkaloid natural compounds led to the identification of four xanthone derivatives as interesting dual AChE/MAO inhibitors.⁹³

A large series of MAO/AChE inhibitors were rationally designed by assuming that the ability to inhibit AChE activity (Figure 13) might be conferred by the introduction of a carbamate moiety in the structure of either rasagiline (**24**) or **19**, both of which are MAO-B inhibitors with neuroprotective activity in vitro and in vivo (see below). These new ligands would hold promise as effective MTDLs because, in addition to reducing oxidative stress, the inhibition of the MAO-A activity could also have a direct effect on cognition and could induce an antidepressant action. Several compounds based on the indane scaffold (**25**, **26**) and one phenethylamine (**27**) were identified as possible leads for further development based on a well-balanced profile of AChE and MAO-B inhibitory activities.⁹⁴ Ladostigil (**26**) is now finishing phase II clinical studies for the treatment of dementia with PD-like symptoms and depression.⁹⁵ One major feature of the action mechanism of **26** is its neuroprotective activity in neuronal cell cultures. In vivo, its molecular basis was clarified by employing an apoptotic model of neuroblastoma cells. In this assay, **26** significantly decreased apoptosis via inhibition of the cleavage and prevention of caspase-3 activation through a mechanism related to regulation of the Bcl-2 family proteins. This resulted in reduced levels of Bad and Bax and increased levels of Bcl-2. Regarding the regulation of APP processing, **26** markedly decreased apoptotic-induced levels of APP. It also stimulated the release of the nonamyloidogenic soluble APP via an established protein kinase C-MAP kinase dependent pathway. Thus, **26** may reasonably be expected to contribute positively to the cognitive benefits of AD patients. The clinical development of such an MTDL, hitting several targets and endowed with diverse pharmacological

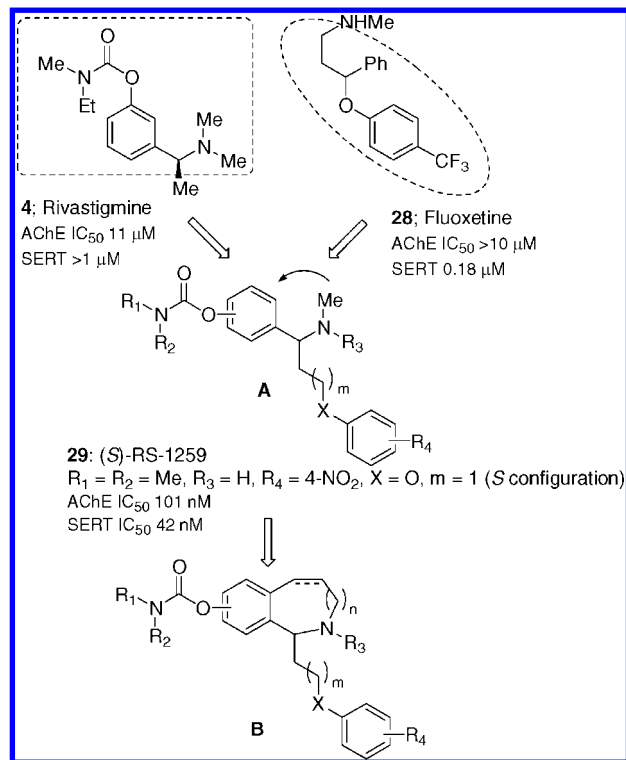


Figure 14. Design strategy leading to dual inhibitors of AChE and SERT by combining the structural features of **4** and **28**.

properties, may conclusively demonstrate the validity of the MTDL approach as a most promising prospective therapy against neurodegeneration.

In addition to MAO inhibitors, depression in AD patients has been successfully treated with inhibitors of serotonin transporter (SERT), antidepressants that lack anticholinergic action. Thus, it was reasoned that combining SERT and AChE inhibitory activities could offer greater therapeutic benefits in AD because the antidepressant effect of SERT inhibitors might reduce the disorder's related symptoms, such as irritability, anxiety, and depression. Previous efforts had failed to provide any compound able to demonstrate simultaneous activation of both cholinergic and serotonergic nervous systems in vivo probably because of the inappropriate combination of activities.^{96,97} A successful design strategy was based on a model, built by means of crystallographic and molecular modeling studies, using **4** (AChEI) and fluoxetine (**28**, SERT inhibitor), which were chosen as lead compounds to design AChE/SERT inhibitors (Figure 14).⁹⁸ Compounds of the A series were designed by linking the methyleneoxyphenyl moiety of **28** to the ethylamine function of **4**,⁹⁹ whereas ring-closed compounds of the B series were designed to explore the effect of the conformational restriction.¹⁰⁰ Of the obtained compounds, (S)-RS-1259 (**29**) exhibited potent inhibitory activities against AChE and SERT in vitro (IC₅₀ values of 101 and 42 nM, respectively) and, following oral administration in mice, in the brain as well. Actual simultaneous elevation of extracellular levels of 5-HT and ACh in vivo was confirmed by different rodent models.¹⁰¹

Another series of compounds, simultaneously targeting cholinergic and serotonergic pathways for a synergistic effect against AD, stemmed from the observation that 5-HT₃ receptors mediate the tonic inhibitory control of ACh release in the cortical tissue and do not seem to be significantly impaired in AD.¹⁰² In this approach, an optimized 5-HT₃ ligand was used as an anchor for receptor recognition and conjugated (by means of a spacer) to **1** in order to obtain a 5-HT₃ receptor–ligand (**30**)

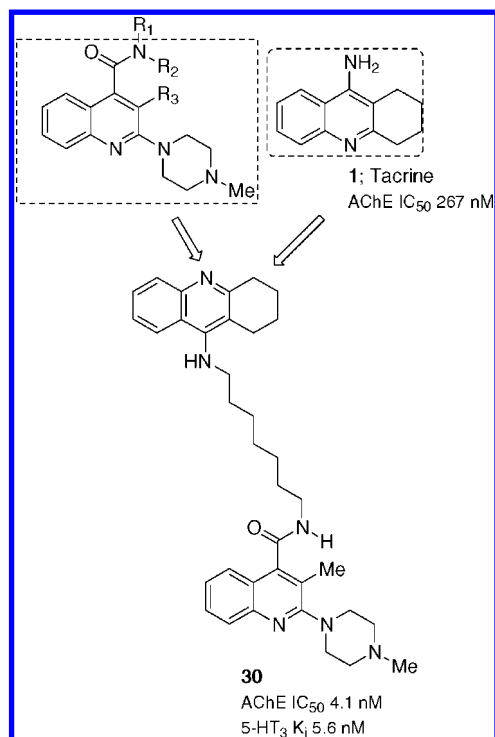


Figure 15. Design strategy leading to compounds endowed with activity toward 5-HT receptors and AChE.

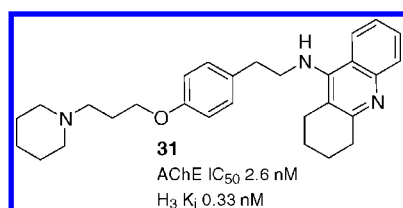


Figure 16. Structure of **31**, which was designed to combine, in the same molecule, the pharmacophoric moieties to interact with both histaminergic and cholinergic systems.

endowed with AChE inhibitory properties (Figure 15). Very interestingly, **30** displayed a nanomolar affinity ($K_i = 5.6 \pm 0.02$ nM) for the 5-HT₃ receptor. It also displayed a nanomolar potency in inhibiting the human AChE ($IC_{50} = 4.1 \pm 0.6$ nM) and a lower potency in inhibiting butyrylcholinesterase (BuChE) ($IC_{50} = 40 \pm 5.0$ nM). Molecular modeling studies showed that **30** may interact with 5-HT₃, AChE, and BuChE in a comparable fashion, since the two heteroaromatic moieties are accommodated by the three target proteins into suitable pockets located at approximately the same distance (14–17 Å).¹⁰³

As mentioned above, it seems that several neurotransmitter systems, which probably subserve the various components of memory and cognitive ability, are affected to varying degrees in AD. Blockade of histaminergic H₃ heteroreceptors with selective antagonists can increase the release of the neurotransmitters (such as ACh) that are involved in cognitive processes.¹⁰⁴ Thus, the possibility that the combined blockade of histaminergic H₃ receptors and AChE might produce added therapeutic benefit in the symptomatic treatment of AD led to the development of a new class of **1**-based compounds combining histaminergic H₃ receptor antagonism and AChE inhibitory potency. This is exemplified by **31** (Figure 16).¹⁰⁵

AChEIs with Antioxidant Properties. As already highlighted, oxidative stress is recognized as a central feature of AD pathogenesis. Treatments that specifically target sources of

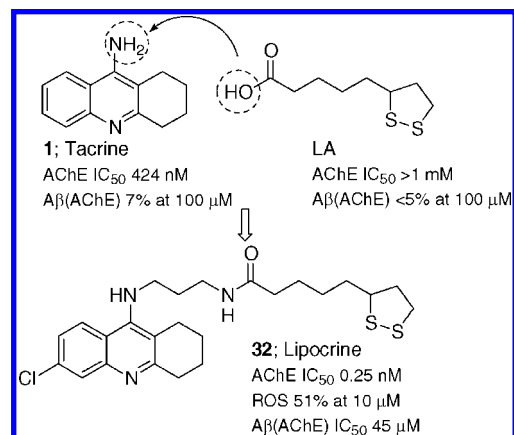


Figure 17. Design strategy leading to **32** by combining the structural features of **1** and LA.

ROS have therefore attracted particular attention. In the search for MTDLs able to act as far upstream as possible in the neurodegenerative cascade, the structure of lipoic acid (LA), an antioxidant exerting different protective effects in neurodegeneration underlying AD,¹⁰⁶ was combined with a pharmacophore endowed with well-established biological properties, namely, the ability to inhibit AChE activity.¹⁰⁷ It was also argued that the cyclic moiety of LA could interact with AChE PAS, which is associated with Aβ aggregation. Thus, this strategy allowed the antioxidant properties of LA to be combined into the same molecule with the AChEI **1** to improve cholinergic transmission, inhibit Aβ aggregation, and control oxidative damage.¹⁰⁸ This led to a new class of compounds (Figure 17) whose prototype is lipocrine (**32**). Although all the synthesized analogues showed nanomolar affinities against AChE, **32** was selected for further development because of its superior AChE inhibition profile. Once kinetic studies verified that **32** also interacts with the AChE PAS, its ability to inhibit AChE-induced Aβ aggregation was tested. It emerged as significantly more potent than all the other AChEIs investigated at the time. Moreover, cellular assays showed that LA and **32** did not affect the neuronal viability while **32** was able to protect neuronal cells against ROS formation evoked by oxidative stress to a higher extent than the parent compound LA.

The same strategy was followed to obtain MTDLs in which the structure of **1** was linked through a spacer of appropriate length to another high-quality antioxidant moiety, such as melatonin (**33**) (Figure 18). Furthermore, recent studies have shown that **33**, an indoleamine secreted by the pineal gland, may play an important preventative role in aging and AD as antioxidant and neuroprotector. The recently documented impact of **33** on τ and Aβ pathology further enhances its potential in the prevention or treatment of AD.¹⁰⁹ The most potent inhibitor of the series (**34**) exhibited an outstanding potency ($IC_{50} = 0.008$ nM) and selectivity (1000-fold) for AChE inhibition relative to BuChE. It also showed a potency 2.5-fold higher than trolox, a vitamin E analogue universally used as a standard for the assessment of antioxidant properties. In addition, this MTDL was also predicted to cross the BBB without difficulty.¹¹⁰

AChEIs and Calcium Channel Blockers. The pivotal role of altered calcium homeostasis in the pathogenesis of AD and other aging-related dementias is well-supported.¹¹¹ Therefore, identification of new compounds that combine both a moderate calcium channel blockade effect and AChE inhibition has been advanced to be useful in the treatment of AD.¹¹² **1** was shown to inhibit voltage-dependent calcium channels (VDCC) in dorsal root ganglionic cells.¹¹³ A series of **1** derivatives were therefore

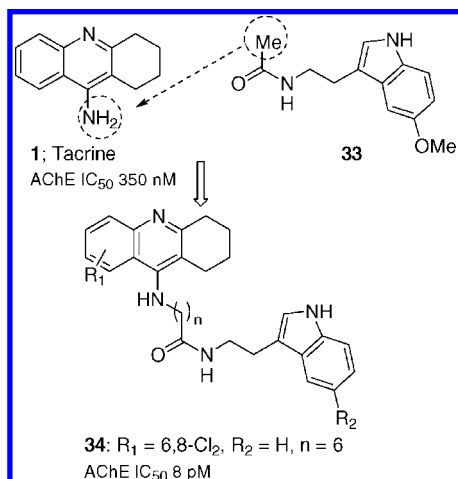


Figure 18. Design strategy leading to AChEIs with antioxidant properties by combining the structural features of **1** and **33**.

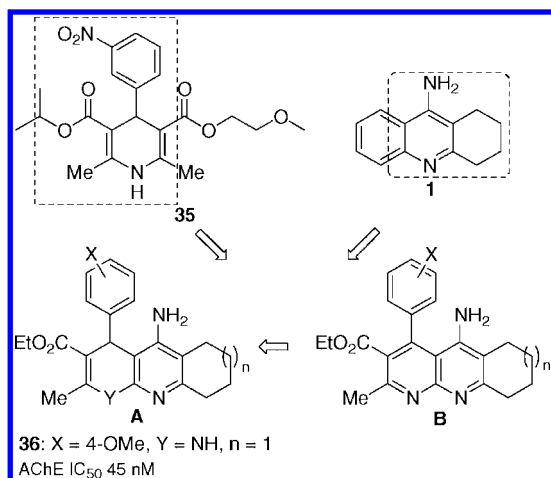


Figure 19. Design strategy leading to AChEIs with neuroprotective activity, exemplified by tacripyrine (**36**).

investigated as AChE/BuChE inhibitors and VDCC modulators as potential neuroprotectants in AD.^{114–119} The structure of **1** was modified by replacing the benzene ring with different substituted heterocyclic systems and by altering the size of the cyclohexane ring to afford two series of compounds (A and B, Figure 19). The compounds of series A are 1-dihydropyridine hybrids. Termed “tacripyrines”, they are obtained by combining the tetrahydroaminoquinoline system of **1** with a 4-substituted dihydropyridine moiety as in nimodipine (**35**), a well-known calcium channel blocker. Tacripyrine **36** was the most potent AChEI (IC₅₀ = 45 nM), being 4-fold more potent than **1** and displaying high selectivity toward BuChE.¹¹⁹ In addition, it gave a significant Ca²⁺ blockade and afforded neuroprotection in a cellular model of calcium overload. Furthermore, in neuroblastoma cells exposed to H₂O₂, the protection afforded by **36** was 1.5-fold higher than that of the prototype **35**.

Metal Chelators with Additional Properties. Dyshomeostasis of cerebral metals is another clear-cut factor contributing to the neuropathology of AD. Recent reports have described the development of potential therapeutic agents based on the modulation of metal bioavailability. The metal chelator clioquinol (5-chloro-6-iodo-8-hydroxyquinoline) has been successfully used in vitro, as well as in animal models and small clinical trials, and several metal chelation based therapeutics are currently under development.¹²⁰

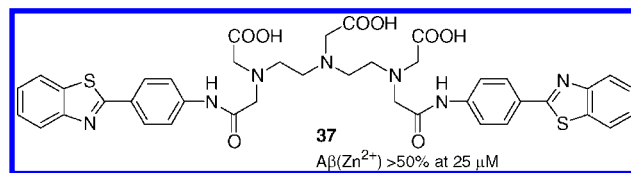


Figure 20. Structure of the metal chelator **37**, which also targets Aβ.

However, the poor target specificity and the concurrent safety problems of presently available metal-complexing agents have limited their widespread clinical use. The long-term use of such agents is likely to perturb the homeostasis of many metals as well as the normal physiological functions of essential metal-requiring biomolecules. Within this context, however, the MTDL approach has revealed itself to be particularly successful for developing a new generation of metal-complexing agents.

On the basis of a novel “pharmacophore conjugation” concept, the bifunctional molecule XH1 (**37**) (Figure 20) has been reported as an innovative metal-complexing agent that specifically targets amyloid. **37** contains in its structure one metal-chelating and two amyloid-binding moieties. These can be envisaged as the pharmacophores of thioflavin T and EDTA-like compounds, respectively, linked by amide bonds. A putative binding geometry between **37** and Aβ peptide was described by a computational ligand/receptor docking procedure. This showed that the hydrophilic metal-chelating moiety of **37** may interact preferentially with one of the α-helices in Aβ, while its lipophilic amyloid-binding moieties stretch toward the hydrophobic C-terminal of Aβ. It transpired that **37** reduced Zn^{II}-induced Aβ precipitation and APP expression in vitro and attenuated cerebral Aβ amyloid pathology in PS1/APP transgenic mouse model in a 4-week treatment period. Furthermore, from pilot studies, it appeared that **37** did not have significant cellular and animal toxicity at the tested doses. These preliminary findings strongly support the potential of **37** as a candidate metal chelator targeting AD amyloidogenesis.¹²¹

A molecule with both a radical scavenger and iron-chelating properties might protect living tissue from oxidative stress to a greater extent than a compound with a single property.¹²² Therefore, the approach of combining metals’ protein-attenuating ability with radical scavenging potential may have relevance for the treatment of neurodegeneration. First, such an MTDL could have the potential to behave synergistically. Second, the antioxidant component (typically lipophilic in nature) of such a molecule would confer more lipophilicity to the iron-chelating component (typically hydrophilic in nature). This would give the resulting compound a greater potential to penetrate and sequester iron from areas susceptible to oxidative damage. This reasoning led to the development of **38**, in which the structural units of the iron chelator deferiprone (**39**) and the antioxidant food additive BHT (**40**) were linked via a simple alkyl chain (Figure 21). This pioneering MTDL proved its neuroprotective effect via inhibition of oxidative stress in pharmacological assays. In two models of chemical-induced cell toxicity, it displayed a neuroprotective action superior to that obtained with the separate administration of the two compounds.¹²²

The preliminary successes of both radical scavengers and metal chelators in clinical therapy of AD supported the development of novel MTDLs with this combined profile. Starting from some SOD mimics, which are metal chelators, it was argued that an SOD-mimic ligand with the metal-binding ability would afford the expected MTDL. On the basis of quantum chemically calculated frontier orbital energies, two

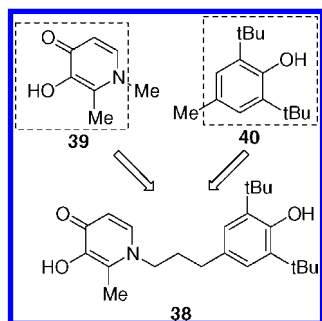


Figure 21. Design strategy leading to **38** by combining the structural features of the lipophilic antioxidant **40** with those of the iron chelator **39**.

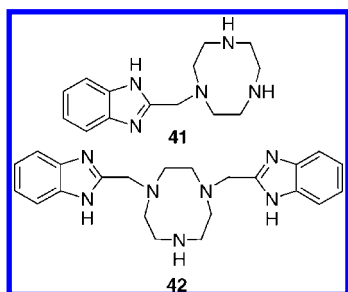


Figure 22. Structures of the metal chelators targeting Aβ **41** and **42**.

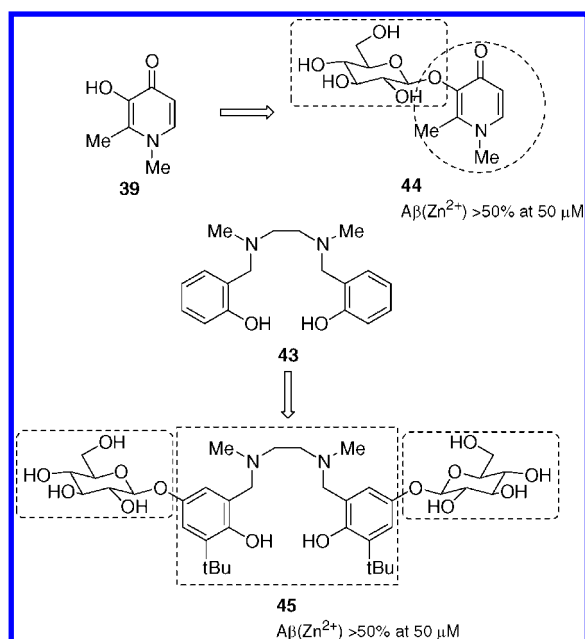


Figure 23. Glucopyranosyl conjugates of the metal chelators **39** and tetrahydrosalicylic acid.

metal chelators, 1-BYT (**41**) and 1,4-BYT (**42**), were considered candidates to fulfill this strategy (Figure 22).^{71,123}

Linking a carbohydrate moiety to drug molecules to form new derivatives and/or prodrugs offers the potential for transporter-facilitated drug delivery for increased brain access of drug molecules. In the case of metal chelators, this strategy appears particularly promising. By use of carbohydrates as both masking and directing substituents, this strategy might solve the potential problem of premature and indiscriminate metal binding. In this respect, a series of **39** and tetrahydrosalicylic acid (**43**) glucosylates (for example, **44** and **45**) have been reported (Figure 23).^{124,125} Once enzymatically deprotected, they can act as selective, tissue-

dependent metal binders and ROS scavengers. This is because the hydroxypyridinone group is a well-known metal binder moiety and the free OH, after deprotection, can efficiently trap radicals. It was claimed that a trifunctional approach to AD therapy had been pursued for the first time. The synthesized prodrugs were demonstrated to cross the BBB using an in situ rat brain perfusion technique to lose the pendent carbohydrate by enzymatic cleavage and to passivate excess metal ions in the brain. Moreover, they showed antioxidant properties similar to those of vitamin E and **40**. Metal-mediated aggregation of Aβ, which occurred following addition of Zn²⁺ or Cu²⁺, was significantly attenuated by the subsequent ligand addition. In summary, the experimental results strongly supported the designed mechanism of action and demonstrated the potential of these multifunctional agents in combating AD.¹²⁴

Development of Memoquin (46). Most of the previous examples provide a convincing argument for shifting the quest for new and disease-modifying agents for AD from single-target-directed ligands to MTDLs. With this in mind, a new drug candidate (**46**)¹²⁶ has recently been described. **46** was derived from an academic program^{75,76,81,107,108} aimed at exploring the possibility of creating multifunctional molecules that possessed some of the above-mentioned activities relevant to AD, such as the ability to inhibit AChE, Aβ processing and aggregation, and the ability to counteract oxidative stress. **46** was rationally designed by incorporating a radical scavenger function into the polyamine skeleton of the previously reported series of cholinergic derivatives (see Figure 6).⁷⁵ Among the possible carriers of radical scavenger activity, attention was focused on the benzoquinone fragment of coenzyme Q10 (CoQ), as this natural antioxidant had offered promise against AD both in vitro¹²⁷ and in vivo.¹²⁸ Moreover, CoQ and different benzoquinone derivatives had been shown to modulate AD molecular targets, directly inhibiting Aβ aggregation.^{129,130} Previous SAR and docking studies revealed that in AChE the biophoric space around the octamethylene spacer that separates the two amide functions of **6** is quite "tolerant" and can accommodate a variety of cyclic moieties.⁷⁸ We therefore decided to introduce the benzoquinone nucleus to replace the inner polymethylene chain. In the resulting prototypic structure 2,5-bis-diamino-1,4-benzoquinone, the two nitrogen atoms in positions 2 and 5 of the benzoquinone moiety are amide-like in character, precisely mimicking the amide bonds of **6**. Moreover, because of the resonance effect, a hydrophobic and planar π system is generated, which is able in principle to bind Aβ and to perturb protein-protein interactions in the fibrillogenesis process.¹³¹

The biological profile of **46** was then widely explored by means of both in vitro and in vivo assays to assess its therapeutic potential as an MTDL for combating AD.

First, the antioxidant properties of **46** were verified by testing both its ability to neutralize free radicals¹³² and to act as a substrate of the enzyme NAD(P)H/quinone oxidoreductase 1 (NQO1). The results, shown in Figure 24, indicate that **46** is a fairly good antioxidant per se and, more interestingly, that **46** might be transformed by NQO1 from the quinone to the hydroquinone form. The latter has been shown to be the actual antioxidant species of other 1,4-benzoquinone derivatives, such as CoQ and idebenone.¹³³ This molecular mechanism was also confirmed in cellular assays, using SH-SY5Y cells. It should be noted that NQO1 activity is increased in hippocampal pyramidal neurons of AD patients and colocalizes closely with AD pathology. This supports its role as an antioxidant system up-regulated in response to the oxidative stress of the AD process.^{134,135} Second, **46** maintained a nanomolar inhibitory

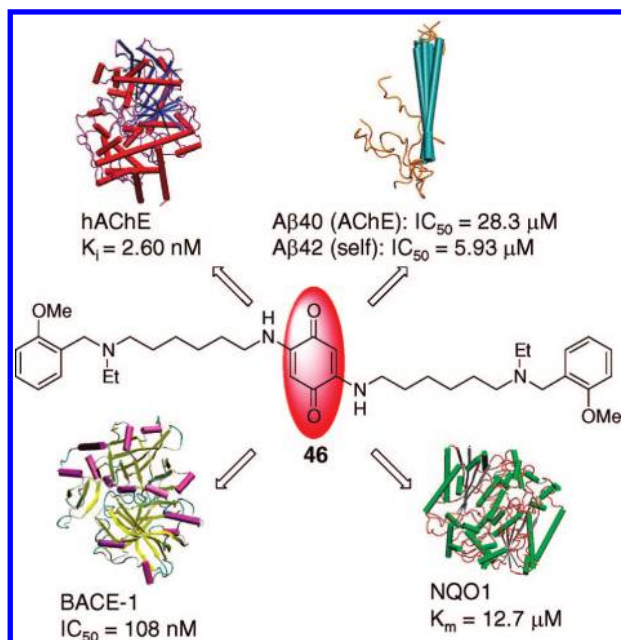


Figure 24. Memoquin (**46**) is able to modulate several mechanisms relevant to AD: the formation of ROS, the aggregation of A β peptides, and AChE and BACE-1 activities. It is also a good substrate of the NQO1 enzyme, which in vivo converts it into the corresponding hydroquinone form.

potency against human AChE. It was also able to inhibit the AChE-induced A β aggregation (Figure 24). Third, **46** inhibited self-assembly of A β 42, which is the most amyloidogenic A β fragment found in AD plaques. Finally, the anti-amyloidogenic profile of **46** was also investigated by testing its ability to act as an inhibitor of BACE-1. The compound was found to have an IC_{50} value of 108 ± 23 nM. Comment is required on the different concentrations at which **46** displays its activities. The relevance of these results have to be referred to in vitro systems, where, for instance, the AChE-induced A β aggregation is tested. The high inhibitor concentration used in this experiment is derived from the need to use a high concentration of AChE to induce the A β aggregation in an experimentally suitable time. Only proof of concept by means of in vivo experiments will shed light on this matter.

46 was then tested in vivo by employing the anti-NGF transgenic mouse (AD11 mouse), which has been shown to be a comprehensive animal model for AD.^{136,137} **46** prevented the AD-like neurodegeneration at three stages (at 2, 6, and 15 months of age). At all ages, **46** rescued the cholinergic deficit in the mice basal forebrain. Interestingly, it was also able to prevent or rescue τ hyperphosphorylation in the cortex. Finally, it showed itself capable of rescuing the behavioral deficits linked to attention and memory in different animal models. Remarkably, besides its in vivo effectiveness, **46** showed other promising properties, such as good oral bioavailability, efficacy in crossing the BBB, and a favorable safety profile, being well-tolerated after prolonged administration. In conclusion, in a scenario where currently available drugs appear to be palliative rather than curative, **46** emerged as a new chemical entity able to confront AD neurodegeneration at different levels. It can therefore be considered a breakthrough MTDL capable of addressing the biological complexity of AD.

4. Parkinson's disease (PD)

In Western countries, PD is the second most common neurodegenerative disorder. Like AD, it is currently an incurable

disease. The available pharmacological therapies are unable to arrest or reverse the neurodegeneration associated with PD. The need for a cure for this debilitating condition is therefore urgent. PD is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta and other subcortical nuclei and by the presence of intraneuronal aggregates known as Lewy bodies (LBs), which are enriched in filamentous α -synuclein and other proteins that are often ubiquitinated. Depletion of DA causes dysregulation of the motor circuits that project throughout the basal ganglia, resulting in the clinical manifestations of PD, which include tremor, bradykinesia, rigidity, and postural instability. However, additional neurotransmitter systems are also involved in PD and, consequently, nonadrenergic, serotonergic, and cholinergic neurons are also lost. This loss is responsible for nonmotor symptoms such as cognitive decline, sleep abnormalities, and depression. These progressive symptoms dominate the later stages of PD.

4.1. Molecular Causes of PD. Until recent advances in the identification of some of the genes that underlie rare familial forms of PD, little was known about its molecular pathogenesis.¹³⁸ Although there are differences between some of the clinical and pathological features observed in familial and nonfamilial forms of PD, there are sufficient similarities that could be exploited to characterize some molecular determinants of PD. This could help in understanding the pathogenetic mechanism of a disease that, although not completely clarified yet, implicates mitochondrial dysfunction, protein misfolding, protein phosphorylation, oxidative stress, and impairment of UPS.

The crucial molecular step of PD pathogenesis is the formation of LBs whose key constituent is represented by α -synuclein, a 140-amino-acid-long neuronal protein. α -synuclein is a cytoplasmic soluble protein that contains a highly amyloidogenic domain within its mid-region.¹³⁹ In pathogenic conditions, α -synuclein misfolds and is converted into pathological oligomers and higher-ordered aggregates, which fibrillize and deposit into LBs as β -pleated sheets. Mutations in the α -synuclein gene have been identified in sporadic familial forms of PD, which are responsible for fibrils formation. However, α -synuclein fibrillization could also be enhanced by post-translational modifications, such as C-terminal truncation and phosphorylation, oxidative and nitrative stress, and interaction with metals, such as aluminum, copper, and iron.¹³⁹ As with AD, some studies propose that small protofibrillar oligomers of α -synuclein, rather than the larger fibrils of LBs, are the toxic species that lead to neuron dysfunction and degeneration by forming structures with porelike morphologies that can disrupt organelle membranes and alter, among others, mitochondrial, ER, and Golgi network functions.¹³⁹ This molecular mechanism is reminiscent of that of bacterial pore-forming toxins, which can generate vesicles on the membrane.¹⁴⁰

Therefore, maintaining α -synuclein in the native and soluble random coil conformation, and consequently preventing α -synuclein-mediated neuron toxicity, emerges as a clear goal of innovative disease-modifying PD therapies. This could be achieved by developing molecules acting at different levels in the cascade of α -synuclein fibril formation (Figure 25). First, prevention of protein misfolding might be obtained by enhancing the activity of chaperones, such as DJ-1. In this respect, it has been shown that in α -synuclein-expressing flies model, treatment with geldanamycin might offer a new way to protect neurons against α -synuclein toxicity by augmenting chaperone activity.¹⁴¹ Second, it is possible to counteract the accumulation of

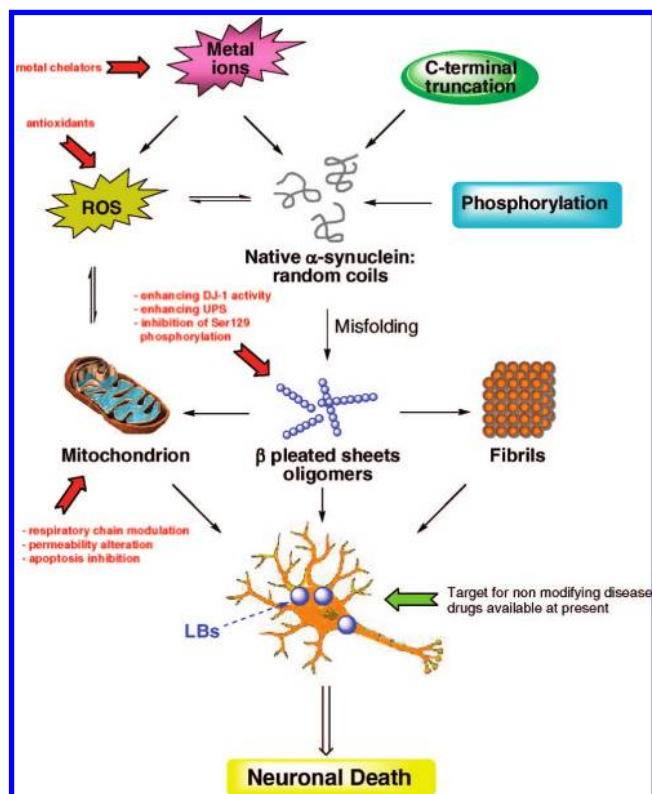


Figure 25. Possible molecular causes of neuronal death and protective mechanisms in PD. Native α -synuclein is a highly soluble protein adopting mainly random coil conformation. Its misfolding leads to oligomers that fibrillize and deposit into LBs. Innovative approaches to the discovery of disease-modifying anti-PD drug candidates might be (i) to prevent α -synuclein misfolding by means of chaperones such as DJ-1, (ii) to enhance UPS activity and in turn to eliminate misfolded proteins, and (iii) to inhibit Ser129 phosphorylation. Oxidative stress and metal-ion-enhanced misfolding are further general mechanisms on which to focus to discover more efficacious anti-PD drug candidates that provide antioxidant and metal-chelating activities. Mitochondrial dysfunction has often been associated with neuronal apoptosis. A straightforward approach to the discovery of small molecules able to recover mitochondrial activity, in terms of (i) respiratory chain modulation, (ii) permeability alteration, and (iii) apoptosis inhibition, has not yet been outlined. Remarkably, mitochondrial dysfunction may be a common pathway in most neurodegenerative diseases by which neuronal death is eventually achieved.

intracellular misfolded α -synuclein through proteolytic pathways, such as UPS and autophagic systems.¹⁴² Finally, although the role of phosphorylation in fibril formation has yet to be fully clarified, abnormal protein phosphorylation might represent a possible innovative pathway to be addressed for the discovery of innovative anti-PD drug candidates. Indeed, it has been shown that in LBs α -synuclein is extensively phosphorylated at Ser129. As a matter of fact, it has been shown that casein kinase 2 phosphorylates α -synuclein on Ser129 in vitro, but it remains unclear which enzyme phosphorylates α -synuclein in vivo.¹⁴³ In addition, phosphorylation plays a role in the process of NFT formation because phosphorylation of τ inhibits binding to microtubules and increases the levels of soluble τ in the cytoplasm. Soluble τ is the species that fibrillizes to form NFTs, which might also be implicated in neuronal death in PD.¹⁴⁴

Besides the above-reported strategies, based on the α -synuclein fibril formation pathway, treatment with antioxidants might theoretically be of benefit in preventing neurodegeneration. However, as discussed, whether oxidative stress is causative or symptomatic of neuronal death is still an open question.

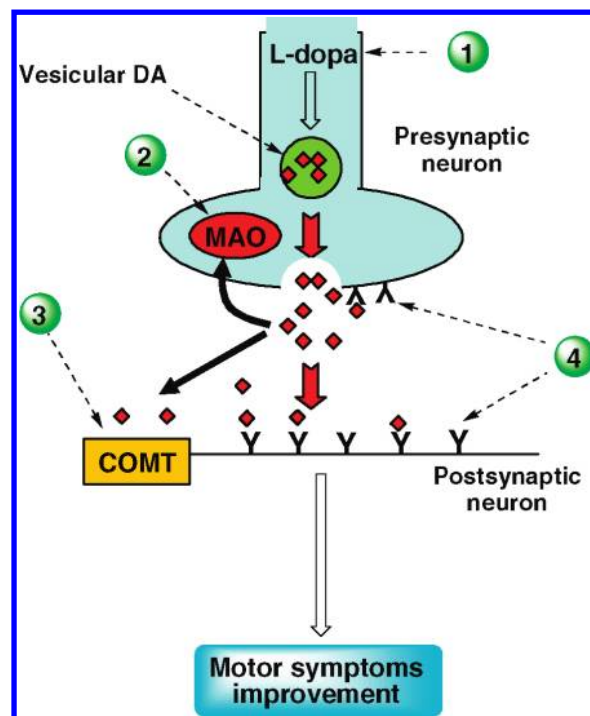

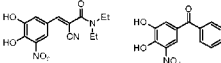
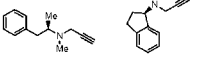
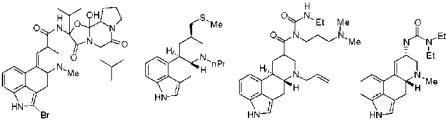
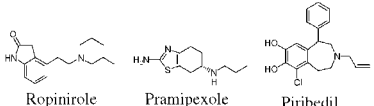
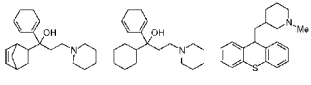

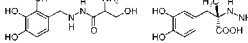


Figure 26. Mechanism of action of DA at a dopaminergic synapse. DA is released in the synaptic cleft where it can interact with dopaminergic receptors at both postsynaptic and presynaptic levels, leading to an improvement in motor symptoms associated with PD. Currently available anti-PD drugs act at points 1–4 as indicated in the picture. L-Dopa (1), the endogenous precursor of DA, is presently the main drug used for the treatment of PD. The others, MAO (2) and COMT (3) inhibitors as well as dopaminergic receptor agonists (4), are often used either in MMT or in MCM along with L-dopa (see also Table 1).

Systematic treatment of rats with rotenone, a pesticide known to inhibit mitochondrial functions, has been shown to cause selective nigrostriatal dopaminergic degeneration with associated inclusion of α -synuclein fibrils.¹⁴⁵ This sheds light on a possible link between mitochondrial dysfunction, oxidative stress, and α -synuclein fibril formation. Therefore, another possible innovative approach would be to target mitochondrial dysfunction. This could be achieved by modulating the respiratory chain, altering mitochondrial permeability, and/or inhibiting mitochondrial-induced apoptosis. The last point should be carefully addressed, since targeting nonspecific cell-death pathways might cause cancerogenesis. Concerning other possible approaches to PD therapy, the evidence that increased levels of iron in the PD brain are responsible for oxidative stress by enhancing the formation of hydroxyl free radicals¹⁴⁶ has prompted scientists to investigate metal chelators as possible drug candidates for the treatment of PD.¹⁴⁷

4.2. Current PD Therapies. Despite an increased understanding of the molecular causes of PD, current PD therapies are based mainly on exogenous replacement of DA within the striatum. This improves the symptoms but without halting the progression of the neurodegenerative process or reversing the neuronal degeneration. Furthermore, although PD also involves degeneration of nondopaminergic neurons, the treatment of the resulting predominantly nonmotor features remains a challenge. Depletion of striatal DA from the loss of nigral projections is the main target for the currently available drugs (Figure 26). The classes of compound that still hold a prominent position in current anti-PD drug discovery are L-dopa and dopaminergic receptor agonists (both used alone or as MMT)

Table 1. Targets for Classic PD Drug Therapies

Mechanism of action	Drugs
DA precursor (prodrug)	 L-dopa
COMT inhibition	 Entacapone Tolcapone
MAO-B inhibition	 Selegiline (19) Rasagiline (24)
Dopaminergic receptor agonism (ergot derivative)	 Bromocriptine Pergolide Cabergoline Lisuride
Dopaminergic receptor agonism (non-ergot derivative)	 Ropinirole Pramipexole Piribedil
Muscarinic receptor antagonism	 Biperiden Trixyphenidyl Metixene
Ion channel blockade	 Amantadine
Peripheral dopa decarboxylase inhibition	 Benserazide Carbidopa

and MMT/MCM of L-dopa with DA modifying drugs, such as (i) peripheral dopa decarboxylase inhibitors, (ii) catechol-*O*-methyltransferase (COMT) inhibitors, and (iii) selective monoamine oxidase type B (MAO-B) inhibitors.¹⁴⁸ The chemical structures and mechanisms of action of the currently available anti-PD drugs are reported in Table 1. L-Dopa is the key compound in the treatment of PD, acting as a precursor of DA. However, besides offering only symptomatic relief for patients, this drug shows substantial side effects at the high doses required for therapeutic action.¹⁴⁹ Certain other available drugs, like MAO-B (**19** and **24**)¹⁴⁸ and COMT [entacapone and tolcapone (Table 1)]¹⁵⁰ inhibitors, are used mainly as MMT with L-dopa, since they alter the *in vivo* metabolism of DA by increasing its plasma half-life. Amantadine (an antiviral agent that also bears actions as an ion channels blocker) is believed to release brain DA from nerve endings, making it more available for activating dopaminergic receptors. Anticholinergic compounds (such as biperiden, trixyphenidyl) were among the first drugs used for PD therapy. They were intended to correct the imbalance between DA and ACh brain levels.¹⁵¹ Dopaminergic receptor agonists may be used either alone to delay the need for L-dopa or as MMT with L-dopa to increase its effectiveness. More recent therapeutic approaches to PD are represented by nicotine, anti-inflammatory agents, melatonin, selenium, iron chelators, and vitamins A, C, and E.¹⁴⁹

None of the currently available pharmacological therapies are able to arrest or reverse the progression of PD. This is probably because these compounds treat only the symptoms of the disease rather than tackling the actual molecular PD causes. Moreover,

the currently available drugs have been shown to interact with a single molecular target, instead of confronting the multifactorial nature of PD neurodegeneration. Comment is required, however, for the MAO-B inhibitor **24**, which, *in vitro* and *in vivo*, can protect neurons from a variety of neurotoxic assaults. Unlike **19**, **24** is not derived from amphetamine. As with other general neuroprotective mechanisms, **24** specifically activates enzymes playing a key role in cellular events including mitochondria viability, modulation of apoptotic processes, and neuronal plasticity. In AD (see above), it induces the release of nonamyloidogenic α -secretase-derived soluble amyloid.¹⁵² This pharmacological action is also associated with (i) the prevention of both the neurotoxin-induced fall in mitochondrial membrane potential and opening of mitochondria permeability transition pore, (ii) activation of the proteasome-ubiquitin complex, (iii) inhibition of cytochrome *c* release, and (iv) prevention of caspase 3 activation.¹⁵³ The molecular mechanism of neuroprotective antiapoptotic activity of **24** has been attributed to its ability to modulate Bcl-2 protein family, up-regulating the antiapoptotic Bcl-2 and Bcl-xL while down-regulating Bad and Bax.^{154–156} SAR studies on **24** have demonstrated that its neuroprotective activity is directly dependent on the propargyl moiety, since the enantiomer of **24** is a very poor MAO inhibitor, but bears similar neuroprotective activity both *in vitro* and *in vivo*.^{157–159}

4.3. MDTLs for the Treatment of PD. To overcome the major limitations of a single-medication therapy, MMT and MCM are currently adopted for the treatment of PD.¹⁶⁰ In particular, the use of L-dopa plus either an MAO-B or a COMT

inhibitor allows patients to decrease the administration of L-dopa. For instance, an MCM has recently been developed by combining, in a single tablet, L-dopa, the dopa-decarboxylase inhibitor carbidopa, and the COMT inhibitor entacapone.¹⁶¹ A similar result can also be achieved by coadministering a dopaminergic receptor agonist and L-dopa. The latter MMT has also been shown to be effective in improving bradykinesia, rigor, and tremor and in ameliorating the abilities of daily living and depressive mood. This has been demonstrated in particular for ropinirole, a modern, nonergot dopaminergic receptor agonist (Table 1).¹⁶²

Clearly, in PD too, MTDL design and discovery emerge as a possible alternative strategy to MMT. Such an approach has recently been applied by some research groups to the discovery of new drug candidates for the treatment of PD.^{69,70,72,73} A common approach has been the combination, in a single molecule, of the pharmacophoric features responsible for modulating the biological activity of a validated molecular target with the chemical functions able to confer metal-chelating and/or antioxidant properties. In particular, in the first series of derivatives, iron-chelating and antioxidant features were combined with MAO-B inhibitory properties.⁷⁰ As previously mentioned, the rationale for this design strategy was related to the observation that iron concentrations are significantly elevated at the neurodegenerative neuronal site¹⁶³ and iron chelators result in a neuroprotective action in PD animal models.^{147,164} The role of MAO enzymes in deaminating (and thus deactivating) DA has been previously mentioned, and MAO-B selective inhibitors are currently in clinics for the treatment of PD. Moreover, a link between high iron concentration and MAO activity, and their involvement in oxidative neuronal stress and ROS production, has also been advanced.¹⁶⁵ MAO is considered one of the major enzymes that generate H₂O₂ as a consequence of its ability to oxidatively deaminate monoamines, especially DA in the substantia nigra pars compacta and striatum. According to Fenton's reaction, either Fe²⁺ or Fe³⁺ free cations can interact with H₂O₂ to generate hydroxyl radicals. Clearly, an increase in free iron concentration and MAO activity results in an increase in radical production with consequent cell-membrane lipid peroxidation, DNA damage, and neuronal death.⁷⁰ Free iron may also be responsible for α -synuclein aggregation, which in turn contributes to the neurodegenerative process. Therefore, chelating iron and decreasing H₂O₂ production by means of a MAO inhibitor dramatically reduce Fenton's reaction and hydroxyl radical formation.

In light of this, the first series of bifunctional derivatives¹⁶⁶ (Figure 27) was purposely designed to combine, in a single chemical entity, the iron-chelating properties of VK-28 (**47**)¹⁴⁷ and the propargylamine group of a MAO-B inhibitor, **24**.^{158,167} As previously discussed, the propargyl moiety of **24** is responsible not only for its MAO-B inhibitory profile but also for a wide neuroprotective profile unrelated to MAO inhibition. These observations highlighted the need to test the new bifunctional derivatives using whole cell assays.¹⁶⁸ In PC12 cells, some of these derivatives [(HLA20 (**48**)) and M30 (**49**)] were also found to be potent lipid peroxidation inhibitors. This is possibly as a consequence of two different mechanisms: (i) strong iron-chelating compounds interfere with Fenton's reaction, thus decreasing hydroxyl free radical production; (ii) metal chelators can also directly act as radical scavengers, blocking the formation of free radical species.¹⁶⁸ In cell assays, **48** and **49** possessed a neuroprotective activity comparable to that of **24**. It also prevented 6-hydroxydopamine- and iron-induced cell death. Finally, since **49** and its N-desmethylated derivative

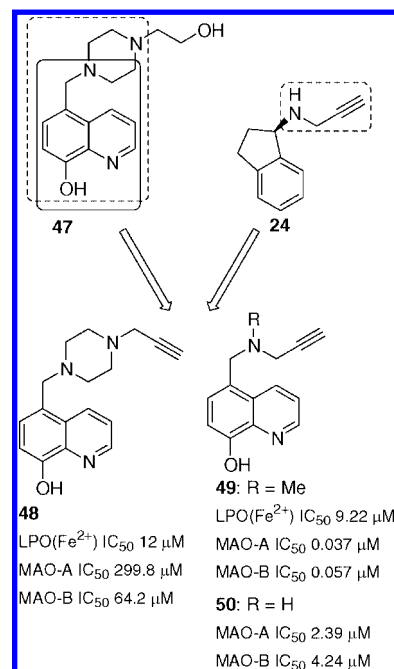


Figure 27. Design strategy leading to the bifunctional derivatives **48–50** by inserting into the same molecule the pharmacophoric groups of **47**, an iron chelator, and **24**, a MAO-B inhibitor.

M30A (**50**) were good MAO inhibitors, they were investigated in vivo.¹⁶⁹ It turned out that **49** preferentially inhibits MAO-A and MAO-B in the CNS while it is a weak inhibitor in the liver and small intestine.¹⁶⁹ It thus prevents potentiation of tyramine-induced cardiovascular activity, which is a major side effect of other nonselective MAO inhibitors. The exact mechanism underlying the preference of **49** for CNS MAO enzymes has not yet been definitively clarified. Conversely, in vivo, **50** inhibited MAO enzymes at concentrations that were 2–3 orders of magnitude higher than those of **49**. This is in line with the profile of N-desmethylated derivatives of other MAO-B inhibitors such as **19** and **24**.¹⁷⁰ Because of its nonselective MAO-inhibiting profile, **49** was also able to increase 5-HT and adrenaline in the CNS, in addition to DA, providing a probable adjunct profile as an antidepressant. This nonselective profile of **49** toward MAO enzymes adds value to the compound. In this respect, it should be mentioned that as early as 1983, it was demonstrated that DA is equally well metabolized by both MAO isoforms^{169,171} and that when one enzyme form is inhibited, the other can continue to metabolize DA.¹⁷² Therefore, a molecule that is able to simultaneously inhibit both MAO-A and MAO-B, without potentiating the tyramine-mediated cardiovascular activity, holds a promising profile for the treatment of PD.

A second class of potential anti-PD MTDLs was developed by combining MAO inhibition and adenosine A_{2A} receptor antagonism. The rationale for the design strategy was based on the observation that caffeine (**51**) consumption is associated with a reduced risk of developing PD^{173,174} and that **51** can reduce dopaminergic neuron toxicity in a PD mouse model.^{175,176} Furthermore, it was observed that such neuroprotective effect could be mimicked by specific adenosine A_{2A} receptor antagonists.^{175–177} These antagonists are currently being investigated as possible therapeutic agents for the symptomatic treatment of motor deficit in PD.¹⁷⁸ One compound is currently undergoing clinical trials for this purpose.¹⁷⁹ In addition, it has recently been shown that adenosine A_{2A} receptor antagonists can also protect against neuronal degenerative processes.^{176,177}

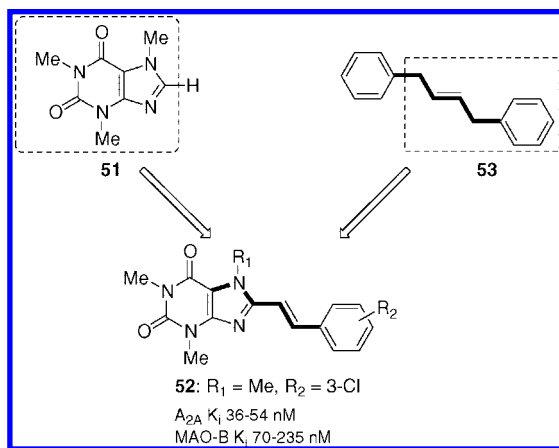


Figure 28. Design strategy leading to **52** by combining, into the same structure, caffeine (**51**), an adenosine A_{2A} receptor antagonist, and 1,4-diphenyl-2-butene (**53**), a reversible MAO-B inhibitor. **52** is clearly recognizable as a combination of the structural features of both **51** and **53**. The caffeine structure is fully incorporated, whereas in the structure of **53**, one phenyl ring is replaced by the heterocyclic ring of **51**. The two terminal rings of **52** are at a comparable distance to that found in **53**, as shown by molecular modeling calculations (unpublished results).

A selective and potent adenosine A_{2A} receptor antagonist, 8-(3-chlorostyryl)caffeine (**52**)¹⁸⁰ (Figure 28), was tested in vitro against MAO-B mitochondrial activity to assess a potential bifunctional profile, showing a K_i of about 100 nM.¹⁸¹ It was then characterized in vivo using the MPTP (1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine) animal model of PD.¹⁸² To cause neurotoxicity, MPTP requires its oxidation to 1-methyl-4-phenylpyridinium (MPP^+) by MAO-B. The effects of **52** on the MPTP metabolism were therefore also investigated in vivo. The inhibition of MPTP metabolism by **52** suggests that adenosine A_{2A} receptor does not regulate MAO-B activity. It also suggests that the two biological profiles, MAO-B inhibition and adenosine A_{2A} receptor antagonism, are indeed unrelated, acting on two parallel biochemical pathways.¹⁸¹ Conversely, the metal-chelating MAO-inhibitor MTDLs discussed above reduced the hydroxyl radical formation synergistically by modulating steps within the same linear biochemical pathway. In the design of MTDLs for complex multifactorial diseases, it is still unclear whether it is more beneficial for an MTDL to act at different points within the same biochemical pathway or to modulate targets belonging to parallel pathways.

Following the observation that a bifunctional adenosine A_{2A} receptor antagonist and MAO-B inhibitor might bear enhanced therapeutic potential for the treatment of PD,¹⁸¹ two series of **52** derivatives (Figure 28) were synthesized and biologically evaluated against both molecular targets.^{183,184} The main aim was to define the structural requirements of an adenosine A_{2A} receptor antagonist to act as inhibitor of the MAO-B enzyme. An initial study showed that structural features relevant to the MAO-B inhibiting profile are the trans configuration of the styryl moiety and the 1,3,7-trimethyl substituents on the xanthinyl ring system of **52**.¹⁸³ A second SAR investigation, carried out on the lead compound **52**, showed that the C-3 and C-4 substitution of the phenyl ring has a considerable effect on the MAO-B inhibiting profile.¹⁸⁴ A possible explanation is that substituents at these positions can interact with the side chains of hydrophobic residues located at the enzyme entrance cavity. SAR and molecular modeling studies indicated that the bulkier is the substituent, the higher is the inhibiting potency against MAO-B enzyme.¹⁸⁴ Moreover, electron-withdrawing groups at the C-3

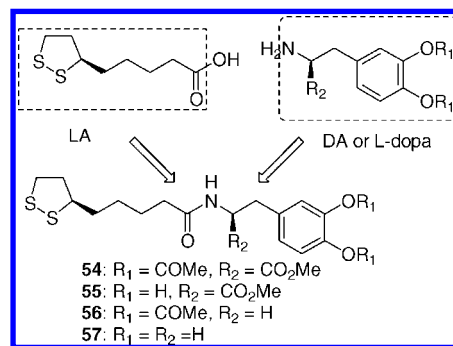


Figure 29. Design strategy leading to compounds **54–57** by inserting into the same molecule the structural features of DA or L-dopa and LA.

position could cause charge localization on the phenyl ring with possible further interactions with entrance residues. In summary, the two studies explored in depth the SAR of adenosine A_{2A} receptor antagonists toward a second validated target (MAO-B) for the treatment of PD.

As discussed, current MTDLs for PD have been based on MAO inhibition combined with a second activity, such as iron chelation and antioxidation and adenosine A_{2A} receptor antagonism. In addition to these compounds, small molecules derived by conjugating L-dopa and DA with LA have recently been reported.¹⁸⁵ The rationale for the development of these molecules was derived from the well-recognized observation that ROS play a role in the progressive and selective loss of the nigrostriatal dopaminergic neurons that occur in PD. However, it is noted that low molecular weight free radical scavengers, such as glutathione, vitamin E, carnosine, and ascorbic acid, have limited antioxidant properties because of their marginal efficiency in crossing BBB and/or affecting iron accumulation. Conversely, LA readily crosses BBB, accumulates in neuronal cell types,¹⁸⁶ and is reduced by mitochondrial dehydrogenases to dihydrolipoic acid (DHLA), which lowers the redox activities of free iron cations.¹⁸⁷ Furthermore, LA per se is also a good metal chelator and can therefore contribute to the tackling of the adverse effects of iron accumulation in aging brains. In light of these observations, new derivatives were synthesized combining DA, L-dopa, and LA (Figure 29). The requirements for gastrointestinal absorption were evaluated, showing that all the derivatives could efficiently overcome the gastrointestinal barrier. Further experiments have also shown that compounds **54–57** pass unhydrolyzed through the stomach and can be absorbed still intact from the intestine. DA, L-dopa, and LA are then released in human plasma after enzymatic hydrolysis. Compounds **54–57** were observed to display antioxidant activities when compared to L-dopa, highlighting their potential in brain diseases where an involvement of DA concentration and free radical damage has been observed. This was demonstrated by comparing the total antioxidant status, the activity of SOD, and glutathione peroxidase in the plasma of rat treated with L-dopa and with **54** or **55**.¹⁸⁵ Earlier, it was shown that SOD and glutathione peroxidase play an important role in the protective mechanisms against oxidative stress. Their activities are therefore very important for protecting against oxidative stress in PD.^{188,189} Overall, **54** and **55** seem to partially protect against oxidative stress derived from auto-oxidation and MAO-mediated metabolism of DA. This finding, together with the observation that in human plasma they release the parent drugs, suggests that these codrugs are better candidates than L-dopa alone in the treatment of PD.

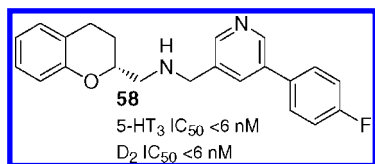


Figure 30. Chemical structure of **58**, displaying both serotonergic 5-HT_{1A} receptor agonism and dopaminergic D₂ receptor antagonism/partial agonism.

The concept of a codrug is somewhat different from the original meaning of MTDLs. An MTDL is a single chemical entity able to simultaneously modulate different molecular targets responsible for a multifactorial disease. A codrug, however, is essentially a prodrug made by two parent compounds linked together by a chemical bond. It has to be stable at the gastrointestinal level, but then it has to be hydrolyzed to provide two (or more) different drugs. The final biological effects of an MTDL and a codrug are essentially the same. Conceptually, however, they represent different approaches to the discovery of multifunctional compounds.

The last MTDL discovered for the treatment of PD is represented by sarizotan (**58**), a chromane derivative that exhibits dual serotonergic 5-HT_{1A} receptor agonism and dopaminergic receptor antagonism/partial agonism (Figure 30).¹⁹⁰ The rationale for its discovery is related to the fact that serotonergic 5-HT_{1A} receptor agonists can reduce some relevant side effects of L-dopa therapy such as wearing-off and L-dopa-induced dyskinesia.¹⁹¹ **58** showed high binding affinity for human dopaminergic D₂, D₃, D₄ and serotonergic 5-HT_{1A} receptors, with IC₅₀ values less than 6 nM. For the latter activity, in vivo characterization demonstrated that **58** can modulate both pre-synaptic and postsynaptic 5-HT_{1A} receptors. Finally, the new drug candidate was introduced in clinical trials and its promising clinical profile showed that **58** could improve DA-induced motor complications by reducing striatal serotonergic nerve impulse activity without altering L-dopa efficacy. **58** therefore emerges as an innovative bifunctional drug for the treatment of dyskinesias associated with L-dopa treatment in PD.¹⁹⁰

Despite enormous efforts at academic and industrial levels to discover new anti-PD drug candidates, with some MTDLs designed purposely to combat this neurodegenerative disease, a real disease-modifying small molecule able to confront the multifactorial nature of PD has not yet been discovered.

5. Other Neurodegenerative Diseases

5.1. Huntington's Disease (HD). HD is a neurodegenerative disorder involving progressive cognitive, psychiatric, and motor symptoms. Cognitive impairment is manifested by difficulties in concentration, decline in language skills with disorganized speech, and perceptive decline. Psychiatric symptoms that usually precede the motor symptoms include depression, anxiety, and sleep disorders. Motor symptoms are characterized by chorea and "dancelike" movements.¹⁹² The brain regions affected in HD are mainly the cerebral cortex and striatum.¹⁹³

The molecular causes of HD were elucidated with the discovery that an expansion of a trinucleotide (CAG) was responsible for encoding an abnormally long polyglutamine tract in the huntingtin (htt) protein, which in turn induces dysfunction at molecular and cellular levels. Since CAG codes for glutamine, expanded CAG results in htt protein containing an expanded polyglutamine tract (more than 40 CAG, while the normal number of CAG repeats is less than 36). This induces molecular processes of protein misfolding and protein aggregation. The

mechanisms by which the mutated htt induces a cascade of molecular and cellular events leading to cell dysfunction and death have not yet been fully elucidated.¹⁹⁴ The caspase cleavage of mutant htt, generating toxic polyglutamine-containing N-terminal fragments of the protein, appears to play a role in the pathogenic mechanism.¹⁹⁵ The truncated htt can interact with other intracellular proteins to form intraneuronal aggregates that the proteosomal machinery is unable to degrade. This is because mutant htt is more resistant to proteolysis than normal htt.¹⁹⁴ As with AD and PD, it has been suggested that in HD too the small aggregates of mutated htt might be responsible for toxic effects, prior to the formation of larger aggregates.¹⁹⁶ Furthermore, oxidative stress and mitochondrial dysfunction again play a role in the pathogenesis of the disease.^{196,197} It has been advanced that oxidative stress might be proportional in intensity to the number of CAG repeats in the mutated htt polypeptide.¹⁹⁸ In addition, experimental observations have shown that mutant htt is physically present on neuronal mitochondrial membranes.¹⁹⁷ This probably leads to mitochondrial depolarization, calcium-induced permabilization, and cytochrome *c* release. Moreover, it has recently been suggested that htt aggregates can be centers of iron-dependent oxidative events, pointing to the metals' possible role in the neuronal death associated with HD.¹⁹⁹

In addition to these general mechanisms, studies with transgenic mice have also disclosed that many changes in neurotransmitter receptors occur in HD. These include alterations in metabotropic glutamate (AMPA, kainate, mGluR₁, and mGluR_{2/3}), NMDA, GABA_A, adenosine A_{2A}, dopaminergic (D₁ and D₂), and cannabinoid CB₁ receptors.^{192,194} In addition, significant reductions in the binding of muscarinic receptors in both the striatum and the cortex have also been observed. These changes frequently precede the onset of disease symptoms in transgenic mouse models, supporting a causative role for selective receptor dysregulation in HD pathogenesis.

Despite an increased understanding of the molecular causes of HD and a plethora of possible molecular targets for potentially efficacious and innovative therapies, there is no effective treatment for HD at present. Additionally, the search for MTDLs is almost absent from the anti-HD drug discovery field.

5.2. Amyotrophic Lateral Sclerosis (ALS). ALS, also known as Lou Gehrig's disease, is a progressive and lethal neurodegenerative disease characterized by a loss of motor neurons in the brain and spinal cord, leading to progressive muscle loss and paralysis.²⁰⁰ In the U.S., there are approximately 25 000 patients with ALS. It is therefore considered an orphan disease (a condition that affects less than 200 000 people nationwide). The pathological hallmark of ALS is the atrophy of dying motor neurons. Swelling of perikarya and proximal axons is also observed, as is the accumulation of phosphorylated neurofilaments, Bunina bodies (i.e., proteinaceous inclusions in motor neurons), LBs like inclusions, and strands of ubiquitinated material in these axons. The activation and proliferation of astrocytes and microglia, as well as fragmentation of Golgi apparatus in motoneurons, are also observed.²⁰¹

Although the molecular causes of ALS are not yet fully disclosed, multiple perturbations of cellular functions have been detected. Protein misfolding and aggregation, alteration of cellular oxidative states, and mitochondrial dysfunction, molecular mechanisms shared by AD, PD, and HD, have been observed in ALS too. Studies with transgenic mouse models have also identified the possible molecular targets involved in ALS pathogenesis.²⁰² These studies point to SOD1 as one protein involved in familial ALS. SOD1 is a 153-amino-acid

cytosolic protein that functions as a homodimer. Each monomer binds one zinc and one copper cation. Through dismutation of copper, SOD1 converts the superoxide anion, which is a byproduct of oxidative phosphorylation in the mitochondrion, to H_2O_2 .²⁰³ More than 125 SOD1 mutations have been identified in ALS patients, but it is not yet clear how diverse mutants gain a common toxic property relevant to the disease pathogenesis.²⁰⁴ Transgenic mice overexpressing human SOD1-G93A mutant are the most widely used model for therapy development. Two hypotheses related to SOD1 malfunctioning have been advanced: oxidative damage and protein misfolding.^{203,205} The oxidative damage hypothesis focuses on the impaired SOD1 catalytic activity, which leads to the formation of toxic species such as peroxynitrite, superoxide, and decomposition products of H_2O_2 . This unbalances the neuron redox state with consequent damage to vital cellular function. The protein misfolding hypothesis states that mutants of SOD1 are prone to misfolding and forming harmful aggregates that may be toxic. Aggregates in ALS transgenic mice are evident at the time of disease onset.^{206,207} With disease progression, they increase in abundance.^{206–208} However, the role of such aggregates in the neurodegenerative cascade is still controversial. As with other neuronal disorders, the larger SOD1 aggregates can be protective, while conversion of soluble SOD1 into insoluble aggregates in the mitochondria might be the actual molecular mechanism of SOD1 toxic effects.²⁰⁴ In this respect, *in vitro* studies have also shown that mutants of SOD1 form small porelike structures that are similar to some forms of amyloid proteins.^{209,210} Interestingly, in addition to other molecular mechanisms, neuronal apoptosis in ALS seems to be related to both an impairment of the association of cytochrome *c* with the inner membrane of mitochondrion and a gradual reduction of the intramitochondrial cytochrome *c* concentration that correlates with disease progression.²¹¹ It is not yet clear whether SOD1 participates in increasing mitochondrial permeability to cytochrome *c* release. It has been suggested that SOD1 accumulates and aggregates in the outer mitochondrial membrane and hinders the protein importation machinery, eventually leading to mitochondrial dysfunction.²¹² The observation that both wild-type and mutated SOD1 bind the antiapoptotic Bcl2, trapping it into a detergent resistant aggregate, highlights a second possible toxic effect of SOD1 toward the mitochondrial functions, which probably leads to a mitochondrion-mediated neuronal apoptosis.²¹³

Another component of ALS neuronal degeneration is related to excessive glutamate-induced stimulation of postsynaptic glutamate receptors (mainly, AMPA receptors). The consequent massive calcium influx is potentially harmful because of its ability to activate processes such as proteases, nucleases, and lipases activities. In ALS, a 3-fold increase in CNS glutamate levels has been observed.^{214–216} Glutamate-induced excitotoxicity provides a possible explanation for the selective vulnerability of motor neurons in ALS. Relative to other types of neurons, motor neurons have a diminished capacity to buffer calcium.²¹⁷

Despite increased knowledge about the molecular mechanisms that underlie ALS, the only drug approved for ALS, riluzole (**59**), a glutamate receptor antagonist (Figure 31), is not a disease-modifying drug. Indeed, it is able to only slightly prolong patient survival.²¹⁸ In this respect, it was reported in 2001 that around 50 different medications had been tested for the treatment of ALS since 1941. Only one, **59**, had any effect on patient survival.²¹⁹ As with other neurodegenerative diseases, there is a growing consensus that new avenues for promising

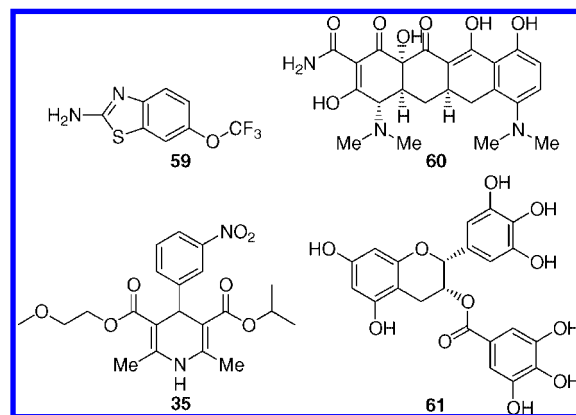


Figure 31. Chemical structures of compounds **35** and **58–61** used or currently under investigation for the treatment of ALS.

therapeutic approaches to ALS can be derived from MMT treatments.²²⁰ In this respect, a three-drug MMT was recently proposed to delay the onset of the disease, slowing the loss of muscle strength and increasing the average longevity of SOD1-G73R transgenic mice.²²¹ This MMT is composed of minocycline (**60**), an antimicrobial agent that inhibits microglial activation, **59**, and nimodipine (**35**), a voltage-gated calcium channel blocker (Figure 31). The rationale for this MMT is justified by the molecular (and/or cellular) causes of ALS as described above. However, it is not yet clear whether this MMT is able to definitively confront the molecular causes and progression of ALS.

Concerning MTDLs for the treatment of ALS, a recent study reports on the neuroprotective effects of (–)-epigallocatechin-3-gallate (**61**) (Figure 31) in SOD1-G93A transgenic mice.²²² **61** is the major polyphenolic constituent of green tea. It has been found to possess great chemopreventive potential due to its ability to interact with an array of molecular targets.²²³ In particular, **61** delayed onset of ALS and prolonged survival of SOD1-G93A mice. This is probably a consequence of its preservation of motor neurons in the anterior horns and reduction of microglial activation. At a molecular level, a reduction in both activation NF- κ B and caspase-3 activity was observed. While in ALS NF- κ B is strongly activated in astrocytes of the spinal cord (probably as a consequence of oxidative stress), the caspase-3 enzyme mediates neuronal apoptosis. The link between microglial and NF- κ B activation is probably the inflammation process, which plays an important role in the pathogenesis of ALS. It has been shown that neuroinflammation occurs before there is a clear evidence of motoneurons loss. It has also been suggested that it can act as a defense in the early stages of the disease, whereas in later stages it can become a crucial propagator of damage.^{224,225} The results obtained with **61** suggest that it is a potent inhibitor of neuroinflammatory processes associated with ALS, acting at multiple steps of the neuroinflammatory cascade. It therefore emerges as a potentially effective drug candidate for the treatment (and even prevention) of ALS.²²²

6. Perspectives: Designed MTDLs and Systems Biology

In light of recent achievements in the fields of AD, PD, HD, and ALS, neurodegenerative diseases appear to share several common multifactorial degenerative processes that could contribute to neuronal death, leading to functional impairments.¹⁹² From the above evidence, it appears that a bioactive compound, whose efficacy is based on interference with a single target, should no longer be considered a good drug candidate for

combating such complex diseases. This is because, as a result of cellular redundancy in many biological pathways, the desired therapeutic effect may not be produced by modulating a single-protein activity.⁷ Indeed, a better candidate might be a molecule able to hit several targets at once (an MTDL). To strengthen this concept, it is worth noting that some of the most interesting compounds under investigation for the treatment of neurodegenerative diseases are those endowed with multifunctional mechanisms. For instance, curcumin and other polyphenols would appear to be useful in AD not only because of their dual function as anti-inflammatory and antioxidant agents but also because they can structurally interfere with A β aggregation and metal dyshomeostasis.^{226,227} Furthermore, estradiol, besides its classic function as a sexual hormone, has recently been linked to neurodegenerative diseases including AD and PD, where it would exert neuroprotective effects through enhancement of neurotrophin signaling and synaptic activities and protection of neurons against oxidative injuries and A β toxicity.²²⁸ A further example is represented by statins, which might be exploited against a variety of CNS illnesses because of their multiple beneficial neuroprotective effects. These effects are linked not only to cholesterol lowering but also to several other mechanisms such as heat-shock proteins and PI-3K/Akt pathways.²²⁹

However, such multifunctional profiles have been discovered serendipitously (mainly because of the wide industrial availability of HTS techniques). A systematic strategy to design and optimize MTDLs does not yet constitute an established approach in traditional medicinal chemistry research.²³⁰ The rational design of compounds that simultaneously modulate different protein targets, either positively or negatively, at a comparable concentration for each target remains a challenging task. As shown by the reported examples, the most frequently adopted MTDLs design strategy has been based mainly on linking two properly selected pharmacophores through a spacer. During the subsequent optimization phase, analogues are made to appropriately balance the desired activities for the two targets. Indeed, very similar affinity values should be obtained for an MTDL toward two or more targets. While this might be reasonable for a compound hitting two targets, the tuning of different activities at comparable concentrations might not be so straightforward in the case of a triple ligand. Moreover, designing a triple MTDL by conjugating three distinct pharmacophores might also be disadvantageous from a pharmacokinetic point of view, since it might lead to high molecular weight compounds with an intrinsic lower probability of optimal druglikeness.^{33,231} Obviously, the classic meaning of SAR should be revisited in the field of MTDLs. For instance, ameliorating the biological profile of a molecule toward a first target could decrease its activity toward a second one. In this respect, multivariate statistical analyses could be of great usefulness for defining quantitative SARs and for driving the design of new MTDLs.

In our opinion, gaining a superior understanding of the cellular mechanisms at which the drug candidate is aimed might represent a step toward rationally designing a balanced MTDL.²³² To tackle this, researchers are turning to the emerging field of systems or network biology. In contrast to the reductionist paradigm, systems biology considers the description of the system, that is, the complex interactions between the molecular constituents of a living cell.²³³ A union between systems biology and medicinal chemistry would certainly help overcome the limits of the one-molecule, one-target approach. In target-centric drug discovery strategies, potential leads are identified and optimized for activity against specific molecular targets. In

contrast, approaches based on systems biology are aimed at the generation of leads that potentially impact interconnected pathways. In view of such goals, human cell models of diseases can be exploited.²³⁴ Because they allow us to directly measure disease-relevant cellular responses, these models represent a practical means of carrying out drug discovery based on systems biology. In these models, disease complexity is engineered into cell systems by activating multiple pathways simultaneously to elicit network regulation, thus casting a much wider net than target-based approaches.²³⁵ In so doing, one can try to identify those biological functions and processes that are altered in the disease, highlighting the actual molecular effectors of such a malfunctioning. This can help to create new hypotheses about the pathogenesis of complex diseases. It can help to identify new putative drug targets by investigating how these proteins are interlinked to each other and how they are relevant to the disease etiology. Finally, it can help to establish whether it is better to design leads able to modulate multiple points within a linear biochemical pathway or within parallel and independent pathways.² These innovative strategies represent a departure from current target-centric activities. They offer the promise of opening up an entirely new era in medicinal chemistry, providing compounds able to modulate systems activity rather than a single target. Notably, a systems biology-based approach has already been proposed to facilitate our understanding of the mechanism of action of drug combinations.^{236,237}

From the observations above, it is clear that, analogous to drug combination, MTDLs call for a strategy to properly address their discovery. It must be emphasized, however, that rationally designed MTDLs are conceptually very different from unselective drugs of the past or from the so-called "dirty drugs", which are found serendipitously and which often exhibit off-target activities irrelevant to the target disease but giving rise to undesired side effects. Conversely, rationally designed MTDLs should only affect their targets partially. This corresponds well with the low-affinity interaction that these compounds usually show against the different targets they modulate. The low affinity of MTDLs may not be a disadvantage. Instead, their weak interactions stabilize the systems, buffering the changes after system perturbation, thus lowering the side effects.^{3,238}

The use of MTDLs in neurodegeneration has been addressed in this review article, justifying their development on the basis of the multifactorial nature of this group of diseases. However, we mention, in conclusion, that a similar scenario is also present in other fields of CNS disorders such as depression²³⁹ and schizophrenia.²⁴⁰

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Biographies

Andrea Cavalli received his degree in Medicinal Chemistry in 1996 and his Ph.D. in Pharmaceutical Sciences in 1999 from the University of Bologna (Italy) under the supervision of Maurizio Recanatini. He was then a Postdoctoral Fellow in Biophysics at the International School for Advanced Studies (SISSA/ISAS) of Trieste (Italy), where he worked with Vincent Torre and Paolo Carloni. In 2001–2002, he was Visiting Scientist at the Swiss Federal Institute of Technology (ETH) of Zürich (Switzerland), working with Gerd Folkers and Leonardo Scapozza. At present, he is Researcher at the Department of Pharmaceutical Sciences at the University of Bologna. His research interests are focused on the development and application of computational methods in drug design. In 2003, he was awarded the Farindustria Prize for Pharmaceutical Research.

Maria Laura Bolognesi received her degree in Medicinal Chemistry (with Highest Honors) in 1990 from the University of Bologna. In 1994, she received her Ph.D. in Pharmaceutical Sciences from the same university under the supervision of Prof. Carlo Melchiorre, working on the design, synthesis, and biological evaluation of selective muscarinic antagonists. After enjoying a postdoctoral year in Prof. Philip S. Portoghesi's research laboratory at the Department of Medicinal Chemistry in Minneapolis, she returned to the University of Bologna where she is now Associate Professor of Medicinal Chemistry. Her current scientific interest lies on the design and synthesis of new chemical entities against neurodegenerative diseases.

Anna Minarini graduated with a degree in Medicinal Chemistry from the University of Bologna in 1987 and received her Ph.D. in Pharmaceutical Sciences in 1992 from the same university. In 1990–1991, she was a Visiting Scientist at the University of Buffalo, New York. In 1998, she was appointed Associate Professor of Medicinal Chemistry at the Department of Pharmaceutical Sciences of the University of Bologna. Her current research interests are devoted to the design and synthesis of new ligands against neurodegenerative diseases.

Michela Rosini obtained her degree in Medicinal Chemistry in 1997, followed by a Ph.D. in Pharmaceutical Sciences in 2001 from the University of Bologna. In 1998 and 2000, she spent some months at The Royal Danish School of Pharmacy of Copenhagen. At present, she is an Assistant Professor in the Department of Pharmaceutical Sciences of the University of Bologna and is focusing her research on the design and synthesis of multitarget-directed ligands for the treatment of Alzheimer's disease.

Vincenzo Tumiatti obtained his degree in Medicinal Chemistry in 1986. In 1991–1992, he was a Visiting Scientist at the University of Frankfurt/M. In 2005, he was appointed Full Professor of Medicinal Chemistry in the Department of Pharmaceutical Sciences of the University of Bologna. His current scientific activity is focused on the design and synthesis of new chemical entities against neurodegenerative diseases and cancer.

Maurizio Recanatini received his degree in Chemistry from the University of Bologna in 1978. He worked as Postdoctoral Fellow until 1983, when he joined the Faculty of Pharmacy of the same university. In 1984–1985, he was a Visiting Scientist at the Department of Chemistry of the Pomona College, Claremont, CA, in the laboratory of Corwin Hansch. Since 2000, he has been Full Professor of Medicinal Chemistry at the University of Bologna, carrying out his research at the Department of Pharmaceutical Sciences. His research interests are focused on the application of computational methods to drug design and discovery.

Carlo Melchiorre received his degree in Chemistry from Camerino University (1966) where he was appointed Full Professor (1980). In 1988, he joined the Faculty of Pharmacy at the University of Bologna where he is presently Professor of Medicinal Chemistry. He has long-standing interests in neurotransmitter receptors. From 1975 to 1979, he joined Professor Bernard Belleau's research group at McGill University (Montreal, Canada) where he developed a series of irreversible α -adrenoceptor antagonists, whose prototype, benextramine, has become a valuable tool in α -adrenergic pharmacology. In 1987, his group designed one of the first selective muscarinic M_2 receptor antagonists, methoctramine, which has been widely used in the characterization of muscarinic receptor subtypes. He is currently focusing his research efforts on the identification of MTDLs for the treatment of neurodegenerative disorders.

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