Targeting Monoamine Oxidases with Multipotent Ligands: An Emerging Strategy in the Search of New Drugs Against Neurodegenerative Diseases

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Abstract: The socioeconomic burden of multi-factorial pathologies, such as neurodegenerative diseases (NDs), is enormous worldwide. Unfortunately, no proven disease-modifying therapy is available yet and in most cases (e.g., Alzheimer’s and Parkinson’s disease) the approved drugs exert only palliative and symptomatic effects. Nowadays, an emerging strategy for the discovery of disease-modifying drugs is based on the multi-target directed ligand (MTDL) design, an innovative shift from the traditional approach one-drug-one-target to the more ambitious one-drug-more-targets goal. Herein, we review the discovery strategy, the mechanism of action and the biochemical evaluation of multipotent ligands exhibiting monoamine oxidase (MAO) inhibition as the core activity with a potential for the treatment of NDs. In particular, MAO inhibitors exhibiting additional acetylcholinesterase (AChE) or nitric oxide synthase (NOS) inhibition, or ion chelation/antioxidant-radical scavenging/anti-inflammatory/A2A receptor antagonist APP processing modulating activities have been thoroughly examined.

Keywords: Multi-target directed ligands, Monoamine oxidase inhibition, Acetylcholinesterase inhibition, Neurodegenerative diseases, Alzheimer’s disease, Parkinson’s disease.

MULTI-TARGET DIRECTED LIGANDS (MTDLs)

Largely spread pathologies such as neurodegenerative diseases (NDs), diabetes, cardiovascular diseases and cancer [1], share a common multi-factorial nature. They display complex and not fully understood onset and progression implying the deregulation of multiple, and often unrelated, cellular physiological events that are caused by genetic, environmental and endogenous pathogenic factors. The complexity of this biochemical landscape suggests that the targeting of a single pathophysiological process among the array of the altered ones may be a challenging goal but it will likely result largely inadequate to prevent, retard, halt or reverse the disease. Over the years, deep efforts have been made in order to discover the key biochemical events triggering these diseases and to identify the most crucial druggable targets. Indeed, many new drugs showing a single-target mechanism have been discovered and are currently used in many multi-factorial pathologies although their therapeutic efficacy is limited to an essentially palliative and/or symptomatic effect. Hence, the discovery of genuine disease-modifying drugs addressing a multi-factorial disease through a single-targeting action can be considered as a limited and generally losing strategy.

The failure of the so-called one-drug-one-target paradigm urged researchers to investigate alternative ways to manage more efficiently diseases triggered by the combination of several cellular aberrations and suggested a new and different approach by addressing simultaneously diverse biological targets that operate independently or cooperatively [2]. This approach might be applied by using a combination of two or more drugs (polypharmacology), each targeting a single biochemical process, or a single molecular entity able to exert beneficial therapeutic effects by modulating different targets.

So far, the polypharmacology approach has represented the most exploited way to tackle multi-factorial diseases and two main strategies have emerged in this field. As a first case, a cocktail of two or more drugs addressing different biochemical targets have been used following appropriate therapeutic protocols. The main problems associated with this approach reside in the low patient compliance and adherence, mostly due to the daily assumption of a high number of drugs often with repeated and varying doses, and in the high chance of drug-drug interactions and multiple competing metabolic transformations. To overcome these drawbacks, a second strategy that combines different drugs with a well defined dose ratio within a unique formulation has been envisaged. Despite both patient compliance and administration regimen may result improved, some disadvantages may arise from undesired drug-drug interactions, extremely high variability of patient responses and an unforeseeable toxicological profile. Within this frame, an emerging strategy is now coming to the attention of the medical and pharmaceutical communities to improve the efficacy of the therapeutic protocol and enhance the drug safety. This alternative strategy is grounded on the assumption that a single, properly designed molecular entity, may be able to interfere with different cellular events and cope with a multi-factorial disease more efficiently [3]. The combination of different biochemical and pharmacological activities in a single molecule (multi-target directed ligand, MTDL) [4] may provide a series of benefits mainly related to unique pharmacokinetic and pharmacodynamic patterns in comparison to drugs cocktails. Indeed, the pharmacokinetic and clinical profiling of a multipotent drug is greatly made easier, being limited to the study of ADMET (adsorption, distribution, metabolism and excretion) and toxicological properties of a single molecular entity. Moreover, the use of a multipotent drug will clearly simplify the administration regimen, improve the patient compliance, and avoid the risk of adverse drug-drug interactions.

Basically, the multiple action of a drug could be unveiled by serendipitous observations and by the repositioning study of old drugs, that is a systematic search of potential additional activities of already-known molecules often revealing an unexpectedly wider pharmacological domain [5]. Next, the discovery of multipotent molecules can be pursued through a knowledge-based or a library screening approach [6]. In the former, the starting point is represented by a bioactive compound already biased towards a specific target of interest. Typically, a series of rational structural modifications are introduced to add further desired activities to the original one. The latter discovery method relies on the sequential screenings of selected molecular libraries on the multiple targets of interest. These libraries are preliminarily screened to shortlist compounds having the most desired/important biological activity. The filtered compounds are then subject to cross-screening towards other possible targets under investigation. Normally, the campaigns of wet screening are anticipated by a cheaper virtual screening performed by using as a query a multi-pharmacophore model integrating the common structural features shared by the pharmacophore models developed for each single target.

When a more traditional, but very rational design strategy is applied, the molecular framework of a multi-target molecule might be built up with three different approaches. One classical way consists in the molecular heterodimerization of two structurally
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Current Medicinal Chemistry, 2011 Vol. 18, No. 30

diverse scaffolds, each responsible for a different biological action. These molecules are structurally joined (conjugated) through an appropriate linker (compound A, Fig. 1), that can be either metabolically stable or cleaved to release the two active moieties (dual pro-drugs).

In a second method, the dual activity might be exhibited in the absence of a linker, by a compound resulting from the “exact fusion” (e.g., through a single bond connection) in a single molecular entity of two molecules with different biological activities (compound B, Fig. 1). A third strategy is based on the preliminary identification of the key structural fragments (pharmacophoric features) responsible for a defined biochemical activity in different molecules. Then these structural elements are combined in a novel unique, appropriately designed, molecular hybrid (compound C, Fig. 1) [7]. The latter is probably the most exploited and feasible design method that can be applied when the two sets of “active” molecular fragments are easily identifiable and the structural similarity between the starting compounds is relatively high.

In most cases, the potential multi-target hit/lead compounds coming out from all of these discovery strategies need further optimization to suitably balance their in vitro and in vivo pharmacological properties.

Monoamine Oxidases (MAOs): A “Druggable” Target in Neurodegenerative Diseases

Monoamine oxidase (MAO, EC 1.4.3.4, amine-oxygen oxidoreductase) is a flavoenzyme localized at the inner side of the outer mitochondrial membrane to which is tightly bound through its C-terminal sequence. This enzyme is ubiquitously distributed in nature, both in human and animal tissues. Its biochemical function resides in the oxidative deamination of xenobiotic [8] and endogenous amines, including monoamine neurotransmitters such as serotonin (5-HT), noradrenaline (NA), and dopamine (DA) [9]. Two distinct mechanisms have been postulated for MAO-A degradation both leading to an iminium intermediate that is further hydrolyzed to the final product, that is an aldehyde. As depicted in Fig. (2), one process is based on a single electron transfer (SET) whereas the other involves a polar nucleophilic attack of the degradation-prone amine to the flavin cofactor (Fl, Fig. 2) [10].

Two distinct enzymatic isoforms, namely MAO-A and MAO-B, have been isolated and fully studied. They are characterized by different amino acid sequences, three-dimensional structures [9], organ and tissue distributions [11], sensitivity to inhibitors [12] and substrate specificity. Both enzymes oxidatively deaminate DA, whereas MAO-A preferentially deaminates 5-HT, adrenaline and NA and is selectively inhibited by clorgyline, MAO-B preferentially metabolizes benzylamine and β-phenethylamine (PEA) and is inhibited by selegiline and rasagiline selectively. Isoform ratio and tissue distribution of MAOs may vary according to the species. Thus human and rat liver express almost identical levels of both MAO isoforms. MAO-A predominates in human placenta and intestinal mucosa [13], whereas MAO-B is the prevalent isoenzyme found in platelets [14]. Both isoforms are present in rat and human brain, with proportions depending on the specific neural region [15]. Adrenergic and histaminergic neurons contain prevalently MAO-A and MAO-B isoforms, respectively.
Taking into consideration the isoforms differences, the native role of MAOs, and their consequent implication in the catabolic degradation of many biogenic amine neurotransmitters, these enzymes have been regarded as attractive targets for the treatment of widespread neurological disorders [16]. Several selective MAO-A inhibitors exhibited antidepressant activity (e.g., moclobemide, brofaromine, clorgyline and toloxatone, Chart 1) [17], whereas selective MAO-B inhibitors have been introduced in the therapy of Parkinson’s disease (PD) (e.g., lazabemide, selegiline, and rasagiline, Chart 1). Iproniazid, originally used as an anti-tuberculosis agent, was the earliest unselective MAO inhibitor introduced in the therapy of depressive disorders in the 1950s and later withdrawn because of hepatotoxic side-effects. Other agents acting as MAO inhibitors (MAO-Is) that still exhibited a non-selective inhibition of both isoenzymes (phenelzine, isocarboxazid, and tranylcypromine, Chart 1) have been later introduced. The major drawbacks linked to these antidepressants were represented by severe side-effects such as the hepatotoxicity and the hypertensive crisis triggered by the consumption of tyramine-rich food (e.g., wine, beer and especially cheese and therefore called “cheese-effect”). Since 80% of intestinal MAOs is constituted by the A isoform, the block of this enzyme is responsible for the halted degradation of tyramine and the “cheese effect” can be therefore ascribed to the lack of selectivity of the earliest MAO-Is. As a result, the clinical use of MAO-Is as antidepressants has been progressively reduced because they required severe dietary restrictions and safer drugs such as tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRIs) have been preferred. Currently, MAO-Is constitute the third- or fourth-line treatment of depression [19].

The inhibition of MAOs still represents an interesting field of research thanks to some new emerging and promising therapeutic perspectives. In fact, the ongoing medicinal chemistry research of selective and reversible MAO-A inhibitors (RIMA) with a better therapeutic profile, fewer drug-drug interaction, and lower adverse effects is encouraged by solid evidence enlightening the effectiveness of MAO-Is in the treatment of major depression, treatment-resistant major depressive disorder and bipolar depression [19]. Moreover, MAO-B inhibitors gained also new consensus as safer drugs for the treatment of early-phase PD, providing symptomatic relief of motor deficits and retarding the need of levodopa administration. Interestingly, they exhibited a wide tolerability and may perform a disease-modifying action in the long-term treatment of PD [20].

In addition, a very recent and renewed interest has regarded MAOs as potential targets in other NDs such as Alzheimer’s and Huntington’s disease (AD and HD, respectively), and amyotrophic

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**Chart 1.** Chemical structures of non-selective (NS) and selective MAO-A (A) and MAO-B (B) inhibitors.

**Fig. (2).** Proposed mechanisms of oxidative deamination catalyzed by MAO-A [10].
lateral sclerosis (ALS). Within the complex biochemical portrait compromising neuronal viability in these pathologies, some cellular impairments may be ascribed to the enzymatic activity and peculiar mechanism of action of MAO. The enzymatic action of MAOs sets the reduction of neurotransmitter levels in the synaptic cleft, thus altering the signalling transfer mechanisms. Moreover, as a result of the oxidative deamination of neurotransmitters and xenobiotic amines, hydrogen peroxide (H₂O₂) is produced. H₂O₂ represents a strong oxidant itself and is a precursor of radical reactive oxygen species (ROS), that can promote harmful cellular alterations in pathological conditions. Hydrogen peroxide generated through MAO catalytic cycle can interact with free Fe⁺⁺ cation in the Fenton reaction (Scheme 1), resulting in the production of ROS and neuronal oxidative stress. Therefore, MAO activity is thought to play a key role to the onset of oxidative stress. In physiological conditions, ROS, e.g., superoxide radical anion (-O₂⁻), hydroxyl radical (-OH), and hydrogen peroxide are produced through several processes including mitochondrial respiration, cellular metabolism, and degradation of dietary components. Cellular ROS levels are balanced by the action of an endogenous detoxification system involving glutathione (GSH) and several enzymes, namely glutathione reductase (GRD) and glutathione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD). These redox systems are responsible for the transformation of these reactive species in non-toxic compounds. When the amount of ROS is extensively increased and/or the efficacy of this protective redox system is impaired in many pathological conditions, ROS production becomes potentially harmful. Oxidative radicals may react with proteins and DNA, producing base-pair mutations, deletions and insertions [21], and cause lipid peroxidation, thus affecting cell membrane integrity. Ultimately, these biochemical aberrations induced by ROS lead to cellular damage and death.

The scope of the present review is the collection and discussion of the main results of the research projects focused on the discovery of multipotent ligands showing MAO inhibitory activity as a key biochemical feature. Since MAO inhibition occupies a central role in neurodegeneration, we paid attention to multi-target molecules with a therapeutic potential for AD, PD, HD and ALS. From a literature survey, in contrast with other targets (e.g., AChE) MAO inhibition via multi-target ligands has not extensively attracted the attention of medicinal chemists although convincing experimental evidence demonstrated the pivotal role of these enzymes in NDs. To date, MTDL strategy targeting MAOs among other targets represents an elegant and promising strategy even if it has not led yet to novel clinical therapeutics, with the exception of rasagiline whose multi-targeting profile has been discovered later. Interestingly, some drug candidates emerged from MTDL design showed promising multi-target properties and have been submitted to extensive bio-pharmacological profiling, as described in the present survey.

Rasagiline as a Multipotent MAO Inhibitor

Rasagiline (1) (N-propargyl-l-(-R)-+)-aminoindan) [26], the R-isomer of a molecule originally coded as AGN1135, is a potent, highly selective and irreversible MAO-B inhibitor which has been developed as an anti-Parkinson drug in monotherapy or as an adjuvant drug in levodopa treatment [27]. In comparison to selegiline (2), that is another potent, selective and irreversible “suicide-type” MAO-B inhibitor, the pharmacokinetic profile of rasagiline presents safer features since it is not linked to the in vivo metabolic production of L-amphetamine and L-methamphetamine [28] responsible for some sympathomimetic adverse effects such as increased blood pressure and heart rate [29]. In addition, rasagiline shows a neuroprotective and anti-apoptotic action in PC-12 and neuroblastoma SH-SY5Y cellular lines against different neurotoxins such as SIN-1 (a peroxynitrite donor) [30], MPTP [31], 6-hydroxydopamine (6-OHDA) [32], and L-norepinephrine [33] and protects from neuronal damage caused by several insults in vivo, e.g., global ischemia, anoxia and head injury [34].
the presence of the reactive propargylamino moiety which might interfere with many other cellular processes [36]. It has been demonstrated that rasagiline acts on different key steps of the apoptotic cascade. In fact, it up-regulates the expression of antiapoptotic proteins Bcl-2 and Bcl-xl, belonging to Bcl-2 family, while down-regulates Bad and Bax [37], through the stimulation of PKCα and ε and inhibition of pro-apoptotic PKCδ and γ. Moreover, rasagiline prevents the decline in mitochondrial membrane potential, the activation of caspase 3, and the nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase [38]. The consequence is the block of both the mitochondrial swelling process and the opening of the permeability transition pore, that are two cellular events that initiate the apoptotic cascade. Interestingly, rasagiline and some related propargylamines, promote the non-amyloidogenic processing of amyloid precursor protein (APP) by stimulating the PKC and MAP-kinase mediated cleavage operated by α-secretase [37c, 39]. This proteolytic pathway produces soluble fragments of APP (sAPP) that are unable to initiate the amyloid cascade leading to the neurotoxic fibrillogenesis [40].

Furthermore, ADME studies of rasagiline highlighted the role of its major hepatic metabolite, the 1-(R)-aminoindan, which is still a weak and reversible MAO inhibitor endowed with neuroprotective properties that could contribute to the overall biological effects observed for rasagiline [41].

Taken together, these findings lead to the conclusion that rasagiline could be considered a highly valuable drug against PD not only for a symptomatic dopamine-replacement therapy but also for a promising disease-modifying profile of the severe motor and neurological deficiencies of PD [27a, 42].

Dual MAO-Acetylcholinesterase (AChE) Inhibitors

Alzheimer’s disease (AD) is the most diffuse form of senile dementia and represents the fourth leading cause of death in western countries. More than one century has passed from the discovery of this disease by the German neuropsychiatrist Alois Alzheimer [43] and different studies have been undertaken to shed light into the pathophysiological mechanisms underlying this devastating neurological disorder. Notwithstanding, a complete comprehension of the origin and progression of AD is far to be reached. What is clear is the fact that AD is a complex disease triggered by several cellular impairments regarding, among many others, cholinergic neurons deficit in the basal forebrain ultimately leading to neuronal loss. From a histopathological viewpoint, convincing studies identified some unequivocal hallmarks of AD: i) extracellular fibrillary aggregates of β-amyloid (Aβ) peptide (senile plaques), soluble peptidic fragments mainly located in neocortical regions and derived from sequential β- and γ-secretase proteolytic cleavage of APP; and ii) intracellular neurofibrillary tangles (NFT), paired helical structures mainly constituted of insoluble hyperphosphorylated τ protein, accumulated in the medial temporal lobe [44]. These biochemical hallmarks are associated with an increased oxidative stress, abnormalities in endoplasmic reticulum and deficiencies in the clearance process operated by the ubiquitine-proteasome system (UPS). Both Aβ oligomers and τ self-aggregates are cytotoxic so that oxidative and inflammatory insults seem to be a direct consequence of the misfolding of these proteins in neuronal cells. AD is characterized by the progressive loss of brain capacities including memory loss, learning and language difficulties, and diminished simple problem-solving capabilities mainly controlled by the cholinergic neurotransmission. In basal forebrain cholinergic neurons of late-stage AD patients, it has been documented a dramatically reduced level of neurotrophins, that manage learning, memory and behaviour in physiological conditions [44]. On this ground, the so-called “cholinergic hypothesis” has been proposed [45] which suggested the enhancement of the acetylcholine (ACh) concentration and/or of its action in the CNS, as a viable and winning strategy to solve cognitive and behavioural symptoms. In principle, this goal may be attained throughout the use of AChE-Is [46] or sub-type selective muscarinic agonists [47].

An increased potential efficacy of some AChE-Is stems from the occasional discovery of a non-catalytic action of the enzyme, whose peripheral anionic subsite (PAS) proved to be the structural motif involved in the promotion of the aggregation of Aβ to form the amyloid plaques [48].

Nowadays, inhibition of AChE represents the predominant pharmacological approach that has led to the development of anti-Alzheimer drugs. Three AChE-Is (Chart 2), i.e., donepezil, rivastigmine and galantamine – the hepatotoxic tacrine was withdrawn from the market – are currently used in clinical practice in the moderate form of AD but their therapeutic efficacy is still under debate, since they act symptomatically in relieving the AD-related cognitive decline by restoring adequate levels of ACh without affecting the leading causes of cholinergic neuronal loss [49]. Another drug, memantine, with a different mechanism of action (NMDA antagonism) and a limited efficacy is now available to treat AD in a more advanced stage.

Behind the central role of cholinergic transmission, the complexity of AD advocated many other triggering factors of the neurodegenerative process. One of them is the “amyloid cascade”, whose study has led to a deeper comprehension of the pathology of

![Tacrine](image1.png)

**Tacrine** (Cognex®)

![Rivastigmine](image2.png)

**Rivastigmine** (Exelon®)

![Memantine](image3.png)

**Memantine** (Namenda®)

![Galantamine](image4.png)

**Galantamine** (Reminyl®)

![Donepezil](image5.png)

**Donepezil** (Aricept®)

**Chart 2. Approved drugs for AD.**
AD and has provided insights for the design of new potential therapeutic agents [50]. According to this hypothesis, the increase of Aβ production and the aggregation into low-molecular weight oligomers, pro-tufibils, fibrils and ultimately amyloid plaques, are the leading causes of the onset and progression of AD. The reduction of both Aβ formation (with β- and γ-secretases modulators) [51, 52] and aggregation (with anti-aggregating agents) [53], and the increase of the Aβ clearance (with active and passive immunization [54]) are potential therapeutic strategies for the therapy of AD. Recent studies enlightened also the role of oxidative stress [55], metal ion dyshomestasis, and inflammation in AD pathogenesis. Neuronal death is the result of different insults, comprising bio-macromolecules alteration upon peroxidation reactions. Oxidative damage may arise from MAO-induced free radicals, mitochondrial abnormalities, inflammation, or redox active metals, that can contribute to the excessive production of toxic ROS through Fenton chemistry.

Within this complex scenario, MAO-IIs might play a beneficial neuroprotective action in NDs by reducing oxidative stress conditions [27d, 56]. The following experimental evidences support this research line: i) up to 3-fold increased MAO-B activity in the temporal, parietal and frontal cortex has been observed in patients suffering from AD compared to control healthy patients [57]; ii) the connection between behavioural disorders and the observed modification in other neurotransmitter systems is mediated by biogenic amines (e.g., serotonin, noradrenaline) whose level is influenced by the catabolic action of MAOs.

The high complexity of AD calls for a huge effort to find multiple therapeutic agents. The multi-factorial nature of the disease rules out the use of consolidated mono-therapy and put researchers on the way of multipotent drugs hitting cooperatively different targets underlying the onset and/or progression of the disease. This approach is grounded on the idea that an AChE-I can exhibit a more efficient therapeutic effect in AD when able to express an additional MAO inhibitory activity, limiting the sources of ROS-related neurotoxicity [58].

As already mentioned, one of the most exploited way to obtain a MTDL is to combine in a unique molecular framework different pharmacophoric features responsible for well-defined and different biological actions. Hopefully, the resulting hybrid molecules will retain the biological activities associated to each different pharmacophore and exert beneficial effects from the synergy of these combined mechanisms.

A pioneering work in the field of hybrid multi-target anti-AD drugs was carried out by D. M. Fink and M. G. Palermo [59]. They designed dual MAO-AChE inhibitors by decorating a tricyclic 1,2,3,4-tetrahydrocyclopent[b]indole carbamate scaffold, inspired by the known AChE-I physostigmine, with a propargylamino group, a recurrent pharmacophoric group of different MAO-Is.

The interest for this molecular series might be limited by their irreversible and selective MAO-A inhibition due to the presence of the reactive N-propargylamino group, that is likely associated to adverse side-effects [60]. More interestingly, the synthetic iminic intermediates (3a-3e) were also tested and showed from moderate (3b-3c) to good (3d-3e) dual inhibitory activities. Regarding MAO inhibition, 3b-3e exhibited a reversible mechanism of action and a marked selectivity for MAO-A. Thus structure-affinity and structure-selectivity relationships, SARs and SSRs, respectively, were examined for these derivatives. The introduction of the carbamate moiety induced a weak affinity for AChE while reducing MAO-A inhibition not so drastically (compare 3a and 3b, Fig. 4). A critical influence was exerted by the R 4 substituent at the iminic nitrogen. Increasing the size of R 4 alkyl groups had a slighter influence over AChE than over MAO inhibitory potency (compare 3b and 3c), the ethyl group being the best substituent to maintain a good dual activity as proved by derivative 3c. The R 2 substituent ortho to the carbamate moiety affected both enzymatic activities leading to the very interesting compounds 3d-3e. Unfortunately, a further development of these compounds was halted by the low oral activity and poor brain penetration found during ex vivo studies on brain tissues.

Starting from the observation that ensaculin, a compound bearing a coumarin skeleton, showed an interesting AChE inhibition in vitro (IC₅₀ = 0.36 μM) [61], a set of various 7-substituted coumarin derivatives (Fig. 5), previously synthesized and characterized as potent MAO inhibitors [62], was screened by some of us on AChE. All the tested derivatives inhibited AChE with Kᵵ values in the medium to low micromolar range (3-100 μM) [63]. As for MAO inhibition, they were endowed with micromolar inhibitory activity. The IC₅₀ values for MAO-A are remarkably higher than 1000 μM. The derivatives 3b-3d, which are a dead end in terms of MAO inhibition, exhibited a good dual activity against AChE (IC₅₀ = 0.14, 0.36 μM) and MAO-A (IC₅₀ = 0.92, 3.36 μM), respectively. The introduction of R 4 Me group, a recurrent pharmacophoric group of different MAO-Is, allowed for a more relevant MAO-A inhibition not so drastically (compare 3b and 3c).

The high complexity of AD calls for a huge effort to find molecules able to modulate multiple CNS targets and then to promote a real improvement of AD therapy. The multi-factorial nature of the disease rules out the use of consolidated mono-therapy and put researchers on the way of multipotent drugs hitting cooperatively different targets underlying the onset and/or progression of the disease. This approach is grounded on the idea that an AChE-I can exhibit a more efficient therapeutic effect in AD when able to express an additional MAO inhibitory activity, limiting the sources of ROS-related neurotoxicity [58].

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to low nanomolar affinities and a marked selectivity for the B isoenzyme. The benzylxylo group linked to C7 of coumarin was the main molecular determinant of the high MAO-B affinity and its absence reduced, albeit less drastically, the AChE inhibition (see compound 4a in Table 1). To ascertain the mechanism of AChE inhibition by compounds 4, a kinetic analysis of selected 7-benzylxylo coumarins was undertaken. Lineweaver-Burk and Hanes plots highlighted the non-competitive character of the AChE inhibition, that could be of great interest in the context of Alzheimer’s disease as this kinetic mechanism may be ascribed to the interaction of the inhibitor at the PAS. This kinetic behaviour was later confirmed on a series of potent and selective heterodimeric AChE-Is containing the coumarin ring [64]. The coumarin nucleus resulted optimal for the dual AChE-MAO affinity and selectivity over MAO-A. Compound 4g would deserve further optimization and investigation as a potential anti-Alzheimer agent.

Youdim and coll. studied a very large series of N-propargylaminoidans and N-propargyl-phenethylamines (Fig. 6) as dual MAO-AChE inhibitors [65]. The molecular design was based on the combination in the same molecule of a propargylaminic fragment, characterising well known MAO-Is such as rasagiline and selegiline, with a carbamatic moiety, which could add the AChE inhibitory property. In addition to AChE and MAO-B inhibition these molecules might display a potential antidepressant action, through MAO-A inhibition, and induce a neuroprotective effect unrelated to MAO inhibition, due to the presence of the propargylamino group. MAO-A inhibition showed by derivatives 5-6 constitutes only an apparent drawback linked to possible tyramine-potentiation because a stronger and more specific inhibition of central with respect to the peripheral (liver and intestine) MAO-A was claimed [66]. In this research project, two hit compounds belonging to the indan family (5a-b) have been identified along with an additional candidate bearing a phenethylamine system (6). Ladostigil (5b, TV3326) has comparable equine butyrylcholinesterase (BChE) and human AChE

![Fig. (5). Ensaculin and coumarin derivatives 4 with dual MAO-AChE inhibitory activity.](image)

**Table 1. MAO and AChE Inhibition Data of Coumarin Derivatives 4a-g [62]**

<table>
<thead>
<tr>
<th>Compd</th>
<th>R₁</th>
<th>R₂</th>
<th>AChE* (Kᵢ)</th>
<th>MAO-A* (IC₅₀)</th>
<th>MAO-B* (IC₅₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>Me</td>
<td>H</td>
<td>100 μM</td>
<td>35%</td>
<td>18%</td>
</tr>
<tr>
<td>4b</td>
<td>Me</td>
<td>Bn</td>
<td>4.07 μM</td>
<td>0.69 μM</td>
<td>4.37 nM</td>
</tr>
<tr>
<td>4c</td>
<td>H</td>
<td>Bn</td>
<td>30.9 μM</td>
<td>1.95 μM</td>
<td>18.2 nM</td>
</tr>
<tr>
<td>4d</td>
<td>Me</td>
<td>(3'-Me)Bn</td>
<td>9.33 μM</td>
<td>3.31 μM</td>
<td>4.37 nM</td>
</tr>
<tr>
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<td>(3'-OMe)Bn</td>
<td>17.8 μM</td>
<td>1.51 μM</td>
<td>3.63 nM</td>
</tr>
<tr>
<td>4f</td>
<td>Me</td>
<td>(3'-F)Bn</td>
<td>7.76 μM</td>
<td>0.58 μM</td>
<td>2.82 nM</td>
</tr>
<tr>
<td>4g</td>
<td>Me</td>
<td>(3’-Cl)Bn</td>
<td>3.39 μM</td>
<td>1.12 μM</td>
<td>3.31 nM</td>
</tr>
</tbody>
</table>

* Electric eel AChE (EC 3.1.1.7) was used. † Rat brain mitochondrial suspensions were used. ‡ Percentage of inhibition at [inhibitor] = 30 μM.

![Fig. (6). Carbamate derivatives 5-6 with dual MAO-AChE inhibition activity [65].](image)
inhibitory activity, with a greater potency against the former and a duration of action longer than rivastigmine. At a preliminary stage, the introduction of the carbamate moiety resulted in the loss of MAO inhibitory activity, as assessed by in vitro assays on rat brain homogenates. Notwithstanding, ladostigil, submitted to in vivo studies showed a dose-dependent and not selective MAO-A/B inhibition in rats, mice and monkeys after chronic oral administration (25–150 mmol/kg dose once daily, for 14 days) [67]. Fortunately, MAO-A inhibition activity was markedly less pronounced in liver and small intestine than in CNS and this result was attributed to the action of local active metabolites in the CNS. The 6-hydroxy derivative, produced by the metabolic degradation of the carbamic group, has weak in vitro activities (IC50 against MAO-A and B of 0.46 mM and 0.35 mM, respectively). Thanks to these central effects, ladostigil is able to elevate dopamine, noradrenaline and serotonin levels and these synergic actions result in a promising antidepressant profile. Moreover, by lacking peripheral activity on both MAO isoforms, this molecule is devoid of the cardiovascular side-effects associated with tyramine-induced “cheese-effect”. Ladostigil prevents MPTP-induced Parkinsonism in mice model [68], as a typical MAO-B inhibitor, and shows neuroprotective functions similar to those of rasagiline in several in vivo [69] and in vitro models [70], likely due to the presence of the propargylaminic moiety. As demonstrated for the well-known propargylaminic MAO-Is selegiline and rasagiline, additional neuroprotective mechanisms might be exerted through the block of cellular death signalling process and the production of different neurotrophic factors, namely neurotrophins and ligands of glial cell line-derived neurotrophic factor [71]. Further investigations for these novel classes of multipotent molecules are warranted to meet the following goals: (a) closer AChE and MAO-B affinities, (b) good to moderate AChE inhibition and (c) improved MAO-B affinity and MAO-B over MAO-A selectivity.

![Thiocarbamoyl-3-phenyl-5-thienyl-2-pyrazoline derivatives](image)

Fig. (7). Thiocarbamoyl-3-phenyl-5-thienyl-2-pyrazoline derivatives with dual MAO-AChE inhibitory activity [73].

Aiming at the discovery of new drugs with multiple actions able to perform a disease-modifying effect in NDs, Gökhan and co-workers evaluated a series of racemic 1-N-substituted-thiocarbamoyl-3-phenyl-5-thienyl-2-pyrazoline derivatives (Fig. 7), previously investigated as MAO-Is [72], for their ability to block ChEs activity. Their research project was based on the assumption that AChE inhibitory properties might be conferred to these molecules by the thiocarbamoyl moiety, supposedly acting as an isosteric substitution of a carbamic functionality [73]. The analysis of SARs for this small series of congeners indicated that the electron-releasing p-methoxy group in the phenyl ring plays a crucial role in enhancing both affinity and selectivity towards AChE and MAO-B compared to BChE and MAO-A, respectively. Some of these inhibitors were reported as moderate and selective MAO-BIs with an IC50 ranging from 22 to 92 μM on bovine liver homogenates assays but they displayed an irreversible-type mechanism of action. Interestingly, these N-substituted pyrazolines were also active against human erythrocyte and plasma AChE and exhibited a selective, reversible and non-competitive inhibition profile. The comparison of compounds 7a and 7b shows that AChE affinity was notably decreased by the increasing size of the R1 substituent of the thiocarbamoyl exocyclic nitrogen. The optimal substitution of the phenyl ring and the thioureidic moiety, represented by a p-methoxy and a methyl group, respectively, furnished a promising derivative (7a) exhibiting a strong AChE affinity with an IC50 in the nanomolar range and a moderate MAO-B inhibitory potency and selectivity.

Despite the outstanding innovations in synthetic organic chemistry that could have expedited the preparation of new bioactive compounds, nature still remains a great source of structurally diverse and promising molecules for the drug discovery process. To perform a medium/high-throughput screening of naturally-derived products, an efficient microtitre plate method based on Ellman’s assay was developed and validated for the study of both electric eel AChE inhibition and kinetic behaviour [74]. When this microplate technique was applied to the biological screening of a library of 45 non-alkaloidal natural compounds in the search of novel AChE-Is, several xanthones were identified as good to moderate inhibitors. Four of them (8a-c and 9, Fig. 8) are worth noting because previous studies had highlighted their promising MAO affinity [74b], thus representing dual AChE-MAO ligands. Electrosorption spectroscopy revealed that the mechanism of these reversible MAO-Is is likely mediated by the formation of charge-transfer complexes with FAD cofactor. Actually, these xanthones showed interesting inhibitory activities towards AChE and MAO, albeit no pronounced MAO isoform selectivity was observed with the exception of 8a and 8b that were considerably more active towards the A isoform. In the kinetic analysis, xanthones 8a-c behave as mixed-type inhibitors of AChE and compound 9 shows a non-competitive profile. These data suggested a partial binding at PAS, and a possible consequent inhibition of β-amyloid formation induced by AChE.

**MAO-Is with Additional Ion-Chelating and/or Antioxidant Activities**

The combination of different biochemical actions in a single molecular entity sounds as a profitable approach to combat another widespread ND with a multi-factorial nature, that is Parkinson’s disease. As for AD, no therapy is currently available to stop or reverse the neurodegenerative decline observed in PD patients. Patients suffering PD manifest typical motor symptoms, including postural instability, tremor, bradykinesia and rigidity [75]. These clinical manifestations are the consequence of the CNS depletion of dopamine, the neurotransmitter modulating the motor circuits in the basal ganglia. Therefore, the mainstay of the current PD therapy is based on the dopamine-replenishment strategy chiefly accomplished with levodopa and/or dopamine agonists, and with MAO-BIs as adjuvants [76]. This therapy can be seen as a palliative symptomatic treatment of motor deficits associated with PD as it lacks the potential to alter the neurotoxic cascade. Beside the loss of dopaminergic neurons mainly located in the substantia nigra pars compacta, an additional histological hallmark in PD is the accumulation of misfolded α-synuclein into intraneuronal fibrillar aggregates, known as Lewy bodies (LBs) [77].

The prevention of α-synuclein-mediated neuronal toxicity has been proposed as an alternative and valuable disease-modifying approach in PD. Several biochemical events have been supposed to promote α-synuclein fibrillogenesis, such as post-translational modifications [78], oxidative stress [79], and the dyshomeostasis of bioactive metal ions such as zinc, copper [80] and iron [81]. In addition, the free iron ion seems to be one of the metals promoting dopaminergic nigral neurodegeneration leading to PD [82]. On the
basis of such observations, iron chelation has the potential to prevent ROS formation through Fenton reaction [83], the consequent oxidative stress and the aggregation of α-synuclein [84]. Therefore metal chelation might represent a profitable approach to efficiently target PD. Moreover, antioxidant compounds that are able to reduce the rate of formation of toxic ROS might play a role in PD.

Over the years the pivotal role of MAO-Is in PD therapy has gained a growing consensus, since these drugs are able to limit dopamine depletion. More recently, a strict connection of increased MAO activity with high iron levels and oxidative stress conditions has been postulated. Whether these mechanisms are really causative or are manifestations of an ongoing neurotoxic cascade is still under debate.

The multifaceted pathogenesis of PD prompted researchers to develop therapeutic agents able to interfere simultaneously with different targets in order to ameliorate the unsatisfactory current pharmacological treatments. Indeed, a multipotent molecule with a potential in PD can be obtained by the combination of appropriate biological activities including an iron-chelating activity and a ROS level reduction by radical scavenging mechanisms or throughout MAO inhibition.

A multi-target ligand design approach has been applied by Youdim and co-workers to obtain MAO-Is endowed with additional iron-chelating and/or antioxidant properties (Fig. 9) by combining the iron-chelating and antioxidant 8-hydroxyquinoline scaffold of VK-28 (10) with a propargylamino group, which, along with MAO inhibition, could offer an additional neuroprotective effect [85]. VK-28 has a potency for iron chelation equivalent to that of the prototype iron chelator, desferal (Table 2) [85]. In contrast to desferal, this iron-chelator was able to protect in vivo neurons from toxicity deriving from 6-OHDA and MPTP neurotoxins in rats, showing a favourable blood-brain barrier crossing capability [86]. Among these novel hybrid derivatives, two compounds, namely HLA20 (11) and M30 (12) stand out for their highly relevant biological activities [87]. In addition to their chelating power very close to that of desferal, as measured in Fe²⁺-induced calcein fluorescence quenching assays [85], both compounds inhibited lipids peroxidation with IC₅₀s in the low micromolar range (likely through iron complexation) and showed higher MAO inhibitory potency and brain permeability than VK28. The relative cell permeability of 11 and 12 was measured through a chelator-induced calcein fluorescence enhancement within the iron-quenched calcein assay [85]. These multipotent ligands had a good permeability in K562 cells that could be correlated to their favourable lipophilicity as suggested by experimental logD determination. Further analyses are needed to clarify their antioxidant profile that could be ascribed to two possible mechanisms, that is the interference with Fenton reaction and the radical scavenging properties.

Fig. (9). Natural non-alkaloidal dual MAO-AChE inhibitors 8-9 [74].

<table>
<thead>
<tr>
<th></th>
<th>AChE Kᵢ (µM)</th>
<th>MAO-A IC₅₀ (µM)</th>
<th>MAO-B IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a</td>
<td>53.0</td>
<td>0.040</td>
<td>33.0</td>
</tr>
<tr>
<td>8b</td>
<td>15.4</td>
<td>29.0</td>
<td>28% at 30 µM</td>
</tr>
<tr>
<td>8c</td>
<td>28.7</td>
<td>19.0</td>
<td>14.7</td>
</tr>
<tr>
<td>9</td>
<td>26.8</td>
<td>37.4</td>
<td>65.5</td>
</tr>
</tbody>
</table>

Fig. (9). 8-Hydroxyquinolines 10-13 with MAO inhibitory, metal-chelating and/or antioxidant activities.
complexing property was more selective for iron than copper as detected by spectrophotometric methods [85]. To investigate the radical-trapping capability of 11, electron paramagnetic resonance (EPR) spectra were registered with different concentrations of scavenger in the presence of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap [87]. The concentration-dependent reduction of the DMPO-OH spin adduct signal in a H2O2 photolysis system clearly indicated the ability of 11 to trap directly hydroxyl radicals.

Compounds M30 (12) and M30A (13) showed radical-scavenging properties (data not shown) similar to 11 [87]. HLA20 (11) was a moderate MAO-I, less active but more MAO-B selective than M30. On the other hand, both M30 and its demethylated derivative M30A showed moderate selectivity for MAO-A in vitro and in vivo. Their inhibition was time-dependent and of a suicide type. Within this 8-hydroxyquinoline series, M30 is worth noting. It proved to be a very potent albeit not selective MAO inhibitor with potencies in the nanomolar range. Interestingly, M30 showed an in vivo MAO inhibition profile similar to ladostigil (5b). M30 acts as a brain permeable MAO-A and -B inhibitor without significant effects on MAO activity in the liver and small intestine [88]. As a consequence, M30 is able to enhance 5-HT and adrenaline neuronal transmission, adding an antidepressant effect to its pharmacological profile. The prominent, but still unclarified, central and unselective MAO inhibition of M30 explains the lack of cardiovascular adverse effects linked to tyramine potentiation.

Both M30 and M30A are metabolically transformed into active propargylaminic metabolites that block the MAO catalytic activity upon binding irreversibly and covalently to the prosthetic flavin cofactor, FAD. The well-documented neuroprotective activity associated to a propargylamino group, and unrelated to MAO inhibition [89], claimed for a deeper exploitation of the potential use of these multifunctional compounds to prevent neuronal cell death on different in vitro and in vivo models. Concerning the neuroprotective activity of HLA20, cell death induced by 6-OHDA under oxidative stress conditions was studied in vitro using a PC12 cells model [87]. The neuroprotective power of HLA20 might be ascribed to the combination of its multiple and different biochemical properties, namely iron-chelation, MAO-B inhibition and antioxidant activity. As for HLA20, a similar neuroprotective ability against 6-OHDA toxic insults was observed for M30 [87]. In a neurorescure trial performed on SH-SY5Y neuroblastoma cell lines under serum deprivation conditions, M30 decreased apoptosis likely through a reduced iron-promoted ROS production. In addition, M30 is able to induce neuronal differentiation, blocking cell cycle in G0/G1 phase [90]. Furthermore, in SH-SY5Y cells M30 proved to reduce APP expression and shift APP processing preferentially towards the non-amyloidogenic pathways [90]. Taken together, these interesting features suggested M30 as a very promising molecule for the treatment of NDs, such as PD and AD, characterized by oxidative stress and iron dysregulation. In addition to the neuroprotective and neurorescue effects mainly triggered by the presence of the propargylamino moiety, the combination of MAO-inhibitory activity with the iron chelating and antioxidant properties by limiting two leading causes of ROS-related oxidative stress might result in clinically relevant beneficial activities even superior to rasagiline.

A further development of these 8-hydroxyquinoline-containing metal chelators was focused on the improvement of their pharmacokinetic properties, such as CNS permeability and on the amelioration of their toxicity profile. Moreover a higher metal selectivity was pursued to avoid an indiscriminate interference with different metal ions pools, limiting their several important physiological functions.

### Table 2. Biological Data of 8-Hydroxyquinoline Derivatives 10-13 [85, 87]

<table>
<thead>
<tr>
<th>Compd</th>
<th>Enzymatic inhibitory activity</th>
<th>Antioxidant activity</th>
<th>Chelating activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAO-A (IC50)</td>
<td>MAO-B (IC50)</td>
<td>LPO (Fe3+) (IC50)</td>
</tr>
<tr>
<td>10</td>
<td>&gt; 200 μM</td>
<td>&gt; 200 μM</td>
<td>13.0 μM</td>
</tr>
<tr>
<td>11</td>
<td>300 μM</td>
<td>64.2 μM</td>
<td>12.0 μM</td>
</tr>
<tr>
<td>12</td>
<td>0.037 μM</td>
<td>0.057 μM</td>
<td>9.22 μM</td>
</tr>
<tr>
<td>13</td>
<td>2.39 μM</td>
<td>4.24 μM</td>
<td>n.d.</td>
</tr>
<tr>
<td>desferal</td>
<td>&gt; 1000 μM</td>
<td>&gt; 1000 μM</td>
<td>8.0 μM</td>
</tr>
</tbody>
</table>

*Rat brain homogenates were used. Lipid peroxidation (LPO) was induced by [ascorbic acid] = 50 μM and [FeSO4] = 1.5 μM. [M]<sup>1/2</sup> is the half maximal dequenching concentration of chelators in Fe2+-quenched calcine fluorescence test. Fe2+ binding affinity is inversely proportional to [M]<sup>1/2</sup> value. n.d. = not detected.

**Fig. (10).** Multifunctional pro-chelator and MAO-AChE inhibitor 14 [91].

A rational strategy to improve the drug-like properties of metal-chelators is based on a prodrug or on a neutral amino acid conjugation approach. To reduce citotoxicity and enhance lipophilicity, a site activated pro-chelator of M30 was designed by masking the 8-OH group of the quinoline moiety with a carbamate group (Fig. 10) [91]. In addition, this structural modification holds a potentially increased AChE targeting. The resulting compound 14 was evaluated to determine its chelating properties and its ability to inhibit both MAO and AChE in rat brain homogenates. As expected, compound 14 per se was not able to chelate biometals but was a potent and selective inhibitor of AChE (versus BChE) and MAO-A (versus MAO-B). The central action of AChE, that is located in different brain areas, allows to unmask the hydroxyl group of 14 upon the carbamylation of the catalytic serine thus releasing M30 and restoring the metal-chelating ability of the molecule. UV-visible spectral bands shifts of 14 were observed upon AChE-activation of the pro-chelator in the presence of metal salts, thus proving its ability to chelate Fe, Cu and Zn ions. The pronounced selectivity of this pro-chelator towards AChE may be indicative of a safer profile, since it could reduce the toxicity associated to the administration of non-selective AChE inhibitors as tarecine, whose hepatotoxicity is attributed to some extent also to its
low selectivity. In addition, the increased lipophilic character allows the compound to cross the blood-brain barrier massively thus obtaining a high CNS targeting while the low affinity for BChE minimizes the peripheral cleavage and the unwanted interference with metal homeostasis in peripheral tissues.

Compounds with Dual MAO Inhibitory-Antioxidant (Radical Scavenging) Activity

As already discussed, one of the main factors triggering or sustaining NDs may be the formation of free toxic radicals that contributes to neuronal impairments by promoting abnormal oxidized species in different biomacromolecules (e.g., lipids, proteins and nucleic acids). The oxidative stress cascade can be broken upstream, preventing the formation of hydroxyl radicals, or, as an alternative, by sequestering intermediate radicals with appropriate radical scavengers. Since a runaway free radical production begins working in brain tissues suffering from neurotoxicity and basal MAO catalytic activity contributes to ROS production, the simultaneous inhibition of MAOs and the free radical-scavenging activity through a single molecular entity could potentially represent synergic activities that might result useful in the treatment or prevention of NDs.

Flavonoids represent a class of compounds widely diffuse in nature that are able to mediate different pharmacological actions, often through antioxidant mechanisms. Four flavonoids, quercitrin (15a), isoquercitrin (15b), rutin (15c) and quercetin (15d; Fig. 11), have been isolated from the leaves of Melastoma candidum D. Don and investigated for their ability to inhibit MAOs and to scavenge free radicals [92]. The kinetic analysis of MAO-B inhibition indicated a mixed-type mechanism, with Ki values in the low micromolar range. Flavonoids 15 were endowed with hydroxyl radical scavenging activity as assessed by an EPR spin trapping technique. More recent results show that quercetin (15d) is a potent and selective MAO-A inhibitor with an IC50 = 0.010 μM [93]. In contrast with previous findings, it has been recently reported that 15d weakly inhibits MPTP bioactivation (33% at 10 μM concentration) and this suggests also a limited inhibition of the MAO-B activity [94].

Eugenol (16)

MAO-A \( K_i = 26.0 \mu M \)
MAO-B \( K_i = 211 \mu M \)

Fig. (12). Eugenol (16), a MAO inhibitor with antioxidant activity [96].

Eugenol (4-allyl-2-methoxyphenol, 16) is the major component of clove, cinnamon, basil, and nutmeg oils and the major active principle of Rhizoma acori graminei (RAG), a botanical remedy described in Chinese pharmacopoeias and used as a palliative treatment of AD cognitive dysfunctions. This compound holds an antioxidant activity that could be ascribed to the phenolic structure. It has been reported that 16 inhibits Fe2+-ascorbic acid induced lipid peroxidation in a dose dependent manner, producing a 76% reduction at a concentration of 250 μM [95]. To clarify the mechanisms driving the antioxidant properties of 16, EPR spin trapping experiments were performed. Eugenol antioxidant activity is linked to its ability of trapping directly some ROS, such as superoxide and hydroxyl radicals, rather than by acting as a free radical scavenger. In addition, eugenol is linked to its ability of trapping directly some ROS, such as superoxide and hydroxyl radicals, rather than by acting as a free radical scavenger. In addition, eugenol can reduce ROS production and relieve oxidative stress conditions through another mechanism, that is a moderate and competitive inhibition of MAO catalytic activity. In fact, 16 inhibits preferentially human recombinant MAO-A (Ki = 26.0 μM) over MAO-B (Ki = 211 μM) [96]. This dual activity of eugenol may represent a valuable feature in the treatment of NDs.

Compounds with Dual MAO-NOS Inhibitory Activity

Different studies highlighted the effect of oxidative stress in promoting mitochondrial dysfunction, inflammation processes and nitric oxide (NO) toxicity in PD. Nitric oxide is a cellular signalling mediator involved in several physiological and pathological conditions [97]. Among others, NO regulates smooth muscle relaxation, platelet aggregation, penile erection, macrophages activities, brain functionalities (e.g., learning and memory). Notwithstanding, NO is a free radical species in nature and its overproduction may lead to harmful oxidative damages. NO can act either as a weak oxidant or as reductive species and can exist in three redox forms, namely nitric oxide (NO), nitrosonium (NO+) and nitrosoy anion (NO−) [98]. It has been hypothesized that NO toxicity is exerted through the production of reactive nitrogen species (RNS; e.g., ·NO2, ·N2O3, HNO2) and by the reaction with superoxide to form peroxynitrite anion (ONOO−), that is a strong oxidizing agent involved in protein oxidation under physiological conditions (Scheme 2) [99].

RNS and NO represent potentially mutagenic species able to react with nucleic acids bases through nitration, nitrosation and deamination reactions [100]. Thus the block of the production of nitric oxide in appropriate brain areas might be an important goal in the search of new agents able to halt or reverse neurodegenerative cascades. To this end, one feasible approach has been envisaged to discover molecules with RNS scavenging property. In addition, attention has been focused also on the design of molecules able to inhibit the enzyme responsible for NO endogenous production, that is the nitric oxide synthase (NOS) [101]. This enzyme catalyzes the endogenous NADPH-dependent transformation of L-arginine to citrulline and exists in three different isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and cytokine-inducible NOS (iNOS). Thus, the reduction of the harmful NO-related biochemical
effects along the MAO-B inhibition might represent an original way to slow down the rate of neurodegeneration.

\[
\begin{align*}
&PF9601N \quad (17) \\
&\text{R}_1 = \text{R}_2 = \text{H} \\
&\text{R}_3 = \text{CH}_2\text{C}=\text{CH} \\
&\text{eNOS IC}_{50} = 187 \text{ M} \\
&\text{iNOS IC}_{50} = > 1000 \text{ M} \\
&\text{LA oxidation IC}_{50} = 82.8 \text{ M} \\
&(\text{rat liver}) \quad \text{MAO-A IC}_{50} = 1250 \text{ nM} \\
&(\text{rat liver}) \quad \text{MAO-B IC}_{50} = 22.0 \text{ nM}
\end{align*}
\]

\[
\begin{align*}
&FA72 \quad (18) \\
&\text{R}_1 = \text{R}_2 = \text{H} \\
&\text{R}_3 = \text{CH}_2\text{C}=\text{CH} \\
&\text{eNOS IC}_{50} = 192 \text{ M} \\
&\text{iNOS IC}_{50} = > 1000 \text{ M} \\
&\text{LA oxidation IC}_{50} = 60.2 \text{ M} \\
&(\text{rat liver}) \quad \text{MAO-A IC}_{50} = \text{n.d.} \\
&(\text{rat liver}) \quad \text{MAO-B IC}_{50} = \text{n.d.}
\end{align*}
\]

Fig. (13). Indolyl dual MAO-I and NOS-I PF9601N (17) and its metabolite FA72 (18) [103, 106].

Indole has been shown as a relevant scaffold in the inhibition of MAOs [102]. SARs of a series of substituted indol-alkylamines [103, 106], indicated that the introduction of a benzyl group in position 5 of the indole ring remarkably shifted the selectivity towards the B isomor. The suicide-type inhibition mechanism of compounds of general structure described in Fig. (13) is due to the presence of the propargylamino group. Among these indole derivatives PF9601N (17) demonstrated a neuroprotective effect in different models of PD [104]. Its ability to prevent the induction of mitochondrial permeability transition pore seems to be independent from MAO inhibition [105]. Therefore, to gain a deeper comprehension of the neuroprotective activity, a study of the antioxidant features and of the effect on NO-system of PF9601N showed enzymatic and antioxidant activities comparable to its parent compound, suggested PF9601N as a promising neuroprotective agent for the prevention and/or cure of PD.

The discovery of a dual-targeting drug able to inhibit both MAO-B and NOS was pursued also by Malan and co-workers [107]. They took inspiration from the MAO-B inhibitory potency observed in many xanthine derivatives, above all (E)-8-(3-chlorostyryl)-caffeine (CSC, Fig. 17). For the design of dual MAO-NOS inhibitors (Fig. 14), they followed a ring enlargement approach of the bicyclic xanthine ring of caffeine to a pteridine-2,4-dione ring that, while maintaining the structural features for the molecular recognition of the MAO active site, might have promoted NOS affinity because it structurally resembles the essential tetrahydrobipterin (BH4) cofactor. A potent and reversible MAO-B inhibitor was identified (19) bearing a 6-tert-butyridine-2,4-dione structural framework, that was endowed with a nanomolar potency towards MAO-B as proved by in vitro assays using baboon liver mitochondrial fractions and measuring spectrophotometrically the oxidation rate of MMTP to MMTP. Unfortunately, 19 showed a quite low NOS affinity as assessed in rat brain homogenates containing several enzymatic isoforms. The authors ascribed the failure of their design to the unforeseen steric hindrance of the steryl group that would have hampered the targetting at the BH4 binding site of NOS. The iminic synthetic precursor (20) of the target pteridine-2,4-dione 19 appeared less active towards MAO-B but proved more effective in the inhibition of NOS.

The potent non-aminocacidic neuronal NOS inhibitor (nNOS-I), 7-nitroindazole (21, 7-NI, Fig. 15) [108], showed neuroprotective properties against MPTP-induced parkinsonism in animal models [109].
7-Nitroindazole (21)

(human) MAO-B IC\textsubscript{50} > 50 \mu M
(rat) nNOS IC\textsubscript{50} = 0.71 \mu M

Fig. (15). 7-Nitroindazole (21, 7-NI): dual MAO-NOS inhibitor \[108, 109\].

To gain deeper insights into the biochemical mechanisms underlying this effect, 7-NI was evaluated for its ability to interfere with different steps of the biochemical cascade leading to MPTP neurotoxicity. The neuroprotective action of 21 might be due to some extent also to the reduced bioactivation of MPTP to MPP\textsuperscript{+} as a result of a weak and competitive MAO-B inhibition \[110\]. However, the main contribution of 7-NI to neuroprotection can arise from its antioxidant activity exerted through a powerful hydroxyl radical scavenging property besides NOS inhibition \[111\]. Similarly, regioisomeric 5- and 6-nitroindazole can be considered promising neuroprotective agents since they showed greater human MAO-B inhibitory potencies (IC\textsubscript{50} = 2.5 \mu M and 6.8 \mu M, respectively) \[109\] along with a similar -OH radical trapping ability and a weaker \textit{in vitro} inhibition of rat cerebellar NOS compared to 7-NI (IC\textsubscript{50} = 47.3 \mu M and 31.6 \mu M for 5- and 6-nitroindazole, respectively) \[112\]. Both these regioisomers were able to inhibit reversibly and competitively MAO-B mediated production of MPP\textsuperscript{+} with higher potencies than 7-NI (IC\textsubscript{50} values equal to 0.99 \mu M, 2.52 \mu M, and 27.8 \mu M for 5-, 6-, and 7-nitroindazole, respectively) \[109\].

**Compounds with Dual MAO Inhibitory and Anti-Inflammatory Activity**

In view of the growing number of patients affected by AD, nowadays many novel molecular weapons are under investigation for a neuroprotective or neurorestorative application, e.g., antioxidants, anti-amyloid agents, vitamins, estrogens, antagonists of APO E4, growth factors, selective phosphodiesterase (PDE4) inhibitors, and agents targeting neurotransmitters or neuropeptides alterations \[113, 114\]. One of the most recently explored research line involves the use of non-steroidal anti-inflammatory drugs (NSAIDs), that in murine models of AD showed an interesting activity \[115\]. Though their usefulness in a long-term administration regimen against AD is still a matter of debate because of possible side-effects \[116\], a recent multi-target approach to obtain novel anti-AD agents is focused on the synthesis and biological evaluation of compounds with a dual MAO inhibitory and anti-inflammatory activity.

Since pyrazole is the structural core of different NSAIDs, e.g. celecoxib (a COX-2 inhibitor), and the same heterocyclic scaffold was present in several classes of MAO-I/s, a series of 1-N-substituted thio carbamoyl-3-phenyl-5-(pyrrol-2-yl)-4,5-dihydro-(1\textit{H})-pyrazole derivatives were properly designed and evaluated, as racemic mixtures, for their ability to inhibit MAO in rat liver homogenates and exert an anti-inflammatory action (Fig. 16) \[117\]. The analysis of SARs highlighted the key role of the substituent in the \textit{para} position of the phenyl ring. The compounds bearing a p-chloro substituent were selective MAO-AIs with K\textsubscript{S} in the range 13–61 \mu M. By replacing the chlorine atom in \textit{para} position with an electron-releasing group such as methoxy, a series of derivatives that selectively inhibit MAO-B isofrom with K\textsubscript{S} in the range 13–37 \mu M were obtained. The inhibition profile of all these compounds indicated a non-selective, irreversible and time-dependent mechanism of inhibition. The presence of ethyl or allyl groups on the thio carbamoyl moiety strongly increased both analgesic and anti-inflammatory activity. Within this series, compound 22 is worth noting because it combines moderate MAO-B potency (K\textsubscript{S} = 33.7 \mu M) with promising analgesic and anti-inflammatory activities comparable to those of indomethacin. Its analgesic and anti-inflammatory profile was assessed in the p-benzoquinone-induced writhing in mice and in carrageenan-induced hind paw oedema model. As for the former, the analgesic activity was experimentally proved by a 60\% reduction in the number of writhings in mice. MAO-I 22 reduced markedly hind paw oedema promoted by carrageenan administration. As a difference with known NSAIDs that show ulcerogenesis as a major side-effect, this derivative displayed a promising low ulcerogenic liability.

**Compounds with Dual MAO-I and Adenosine A\textsubscript{2A} Receptor Antagonist Activity**

Currently, the main therapeutic strategy to cope with neuronal dopamine deficit in patients suffering PD consists of a dopamine-replacement approach administering the direct biochemical precursor of the neurotransmitter, namely levodopa (Chart 3), or using receptor agonists that mimic the action of dopamine (e.g., pramipexole and ropinirole, Chart 3) \[76\].

**Chart 3.** Dopamine bio-precursor and agonists used in PD.

Such a therapeutic approach offers a symptomatic relief of the characteristic motor deficits of PD but it is not able to target the mechanisms underlying the dopaminergic dysfunction. Moreover, as the disease advances, progressive neuronal loss is observed and these drugs become ineffective. The inadequacy of dopamine-replacement therapies to perform a disease modifying action and the side-effects of long-term use (e.g. choreiform, dystonic and dyskinetic motor fluctuations) \[118\] prompted researchers to look for alternative strategies to find new, and druggable, biological...
targets that could be modulated in order to prevent, retard or halt the progression of the neurodegenerative cascade leading to PD.

Antagonists of adenosine receptors that are selective for the A2A subtype displayed a neuroprotective action [119] and were able to prevent the onset of dyskinesia associated with long-term levodopa treatment [120]. Thereby, their effects are additive to those of dopamine-replacement therapy and may allow a reduction of levodopa dosage regimen thus limiting the occurrence of adverse effects. For these reasons, A2A antagonists emerged as a new class of compounds with a potential value for neuroprotection and symptomatic relief of motor deficits associated with PD [121]. As previously mentioned, since MAO-B is the prevalent isoform located in human basal ganglia and evidence of its, age-related, increase has been reported by many authors [122], also MAO-B inhibitors were considered a therapeutic opportunity and indeed are used as adjuvants of levodopa in the replenishment of dopamine levels.

On this basis, research efforts have been directed to the discovery of novel molecular entities with a dual action, that is MAO-B inhibition and A2A receptor antagonism, that holds a discovery of novel molecular entities with a dual action, that is used as adjutants of levodopa in the replenishment of dopamine replacement therapy and may allow a reduction of levodopa dosage associated with PD [121]. As previously mentioned, since MAO-B is the prevalent isoform located in human basal ganglia and evidence of its, age-related, increase has been reported by many authors [122], also MAO-B inhibitors were considered a therapeutic opportunity and indeed are used as adjuvants of levodopa in the replenishment of dopamine levels.

On this basis, research efforts have been directed to the discovery of novel molecular entities with a dual action, that is MAO-B inhibition and A2A receptor antagonism, that holds a potential in the treatment of PD. Since experimental evidence showed that caffeine, a non-selective A1/A2A receptor antagonist, is able to protect from MPTP-induced neurotoxicity in mouse model of PD [111], closely related heterocyclic derivatives were evaluated too. Among them, (E)-8-(3-chlorostyryl)-caffeine (23, CSC, Fig. 17) [123] emerged as a potent and selective A2A antagonist endowed with neuroprotective properties, that could be related to its high affinity for MAO-B. Interestingly, CSC effect on the inhibition of MAO-A was not significant [124]. Thus, different compounds bearing a (E)-8-styrylbutane scaffold were synthesized and evaluated for a dual MAO-B/A2A targeting activity [125]. From the analysis of SARs, it emerged that both biological actions were favourably influenced by the introduction of a methyl group on the xanthine N-7. The presence of an electron-donating group on the styryl moiety strongly enhances MAO-B affinity, while reducing A2A receptor antagonism not so dramatically. Since these novel compounds proved to be from moderate to good MAO-B inhibitors and A2A receptor antagonists, they deserved further investigations to gain deeper insights into the molecular determinants of their dual-targeting activity. With this purpose, the molecular framework of the lead compound (CSC) was properly modified paying particular attention to the effects of the styryl group and its substitution pattern [126]. Within this new series of compounds, the phenyl and benzyl congeners resulted less active towards MAO-B. The application of a vinlogous approach led to the (E,E)-8-(4-phenylbutadien-1-yl)caffeine derivatives, among which 3'-halosubstituted compounds showed outstanding MAO-B affinity and potent A2A receptor antagonism, being both Ks in the nanomolar range. The introduction of bromine in 3'-position led to the most active dual-targeting derivative (24), that showed an impressive high potency at both targets (Ks = 21.9 and 59.1 nM at MAO-B and A2A, respectively). Further developments of these compounds may be hampered by their likely low configurational stability and high photosensitivity.

**MAO-Is with APP Processing Modulatory Activity**

The proteolytic cleavage of APP can be modulated at different levels by intracellular second messengers. In this context, a pivotal modulating role seems to be played by MAPK through the activation of MEK/ERK signalling pathways [127]. It has been reported that rasagiline (1) and ladostigil (5b) can stimulate the release of the neuroprotective soluble form of amyloid precursor protein α (sAPPα) by influencing the rate of PKC- and MEK-related processing [128].

On the other hand, the reduction of toxic Aβ1-42 production can be achieved in a straightforward manner, through β- and γ-secretase inhibitors, or indirectly, through the activation of PKC- and MEK-dependent cleavage. The combination in one compound of some of these biological activities, along with MAO-related ROS reduction, may synergistically favour the APP non-amyloidogenic proteolysis, or block the amyloidogenic one, thus deserving great attention for the prevention and treatment of AD.

To achieve this goal, two series of 7-carbamoyl-1,2,3,4-tetrahydroisoquinoline derivatives (25) were designed and synthesized as cyclic congeners of ladostigil (Fig. 18) [129]. These sets of derivatives differ for the substituent at basic nitrogen, where the propargyl group was replaced by a more sterically hindered and lipophilic cyclopropylmethyl group. All the synthesized derivatives were evaluated as MAO inhibitors showing moderate IC_{50} values ranging from 11.6 to 21.5 μM by using rat brain homogenates (Table 3). Since cyclopropyl derivatives (25b-e) were the most active...
compounds for the inhibition of γ-secretase enzymatic activity, some of these compounds were tested in γ-cells to detect their influence on ERK signalling pathways. Interestingly, the most potent MAO-B inhibitor (31b) was also able to inhibit γ-secretase and activate ERK pathways more strongly than rasagiline, showing promising potential against AD [129].

**Repositioning of Known Drugs as Multitarget Ligands with MAO Inhibitory Activity**

A recent research strategy in drug discovery, named “drug repositioning” or “drug repurposing” and focused on the discovery of new drugs for the therapy of neglected or orphan diseases, consists in the analysis of approved drugs in the search of novel and “hidden” pharmacological activities [4b]. Following this approach, two known drugs have been proved to act as multi-target ligands with interesting MAO inhibitory activities and a potential in NDs: the antiepileptic drug zonisamide and the antidiabetic agent pioglitazone (Fig. 19).

Zonisamide (26) is an FDA-approved drug for the treatment of epilepsy that is also being used as an adjuvant in the therapy of PD [130]. Its antiepileptic action is exerted through the blockade of voltage-dependent Na⁺ channels and T-type Ca²⁺ channels [131]. It has been reported that zonisamide is able to protect nigrostriatal neurons from neurotoxic 6-OHDA insults [132], likely through a radical-scavenging mechanism [133]. Its ability to reduce oxidative stress prompted researchers to examine the effects of zonisamide in animal models of PD and to define its mechanisms of action [134]. Zonisamide showed neuroprotection against MPTP toxicity in male Swiss Webster mice and moderately inhibited mouse brain homogenates MAO-B activity in vitro with an IC₅₀ = 25 μM and a reversible mechanism of action as assessed by ex vivo studies in mice [134]. No inhibition of MAO-A activity was observed. More recently, zonisamide complex with human MAO-B has been solved, thus allowing a deeper comprehension of the binding interactions at the active site of the enzyme [135]. Taken together these findings underline the multi-target activity of zonisamide and its potential use in PD, for a symptomatic improvement of motor deficits and a neuroprotective action.

The peroxisome proliferator-activated receptor-γ (PPARγ) agonist pioglitazone (27) is a brain-permeable antiobinetic drug belonging to the thiazolidinedione family. It has been demonstrated that it performs a neuroprotective effect in animal models of AD showing an anti-inflammatory action [136]. In addition, pretreatment of mice with pioglitazone reduced MPTP toxicity [137] and dopaminergic neuronal loss [138]. The protective action against MPTP toxicity is mediated by the inhibition of MAO-B [139]. Pioglitazone showed a potent affinity in vitro towards human recombinant MAO-B (IC₅₀ = 220 nM) and its inhibitory efficacy was proved also in ex vivo studies on male C57BL6/J mice. The synergy of MAO-B inhibitory property and the ability to modulate inflammatory responses in neurodegenerative processes through the activation of PPARγ [140] increases the interest of this compound as a potential therapeutic agent in NDs.

**CONCLUSIONS**

The rational design, synthesis and bio-pharmacological evaluation of multipotent ligands addressing MAO inhibition among other targeted pharmacological activities in NDs has been a challenging research activity that led to the discovery of new interesting bioactive compounds that deserve further development towards pre-clinical and clinical trials. Although strong efforts have been produced by several research groups in the rational molecular design and in the explorative screening of large molecular libraries to discover new promising multi-targeting agents for NDs, few works succeeded in finding compounds with comparable potencies or adequate potency ratio towards the addressed targets. These difficulties arose mainly from the complexity of modulating appropriately and in a simultaneous way two or more different targets with a single molecule.

Unfortunately, the structure-based design of a multipotent ligand is possible only in limited cases, since the 3D structures of the targeted biomolecules are not always available. The lack of information about the binding mode of a multipotent ligand into the active sites of the diverse biological targets may force the researcher to a sort of “blind design” characterized by a long sequence of trials and errors. In these cases, indirect ligand-design methods may be profitably applied through the derivation of QSAR, [141] 3D-QSAR [142] and pharmacophore models [143] that may allow a more rational and successful design of a multipotent ligand. A more detailed knowledge of the main binding interactions occurring with the different biological targets is instead possible through structure-based computational methods which may lead to an unequivocal identification of the molecular determinants responsible for the activity at each single target and an easier development of a multi-target pharmacophore model.

![Fig. (19). “Repurposed drugs” inhibiting MAO-B with a potential in NDs [134, 139].](image-url)
In some examples reported herein, additional activities combined with a principal one, may be considered more properly side-actions or additional effects that require a too high dose to achieve the therapeutic threshold, thus increasing the risks of severe toxic side-effects. For the sake of completeness, we decided to report also those projects that surely need a further and sometime long optimization process to obtain a truly promising lead compound. Taking into consideration the elegance of the design strategy and the biological potential of these ligands, we reasoned that all the reported projects were worth of mention representing pioneering works in a fast developing field that may lay the groundwork for future investigations.

Regarding the multi-target ligands examined in the present review, a few compounds have been already submitted to deeper pharmacological profiling and showed promising features but many others need further and extensive development to improve their biopharmacological profile prior to any clinical trial. Above all, the main limitation of the drug candidates emerging from a multi-target ligand design approach examined herein may mainly derive from the lack of knowledge about the optimal balance among different pharmacological activities that could promote the desired therapeutic effect and limit eventual side effects. This important pharmaco-therapeutic aspect requires parallel pre-clinical studies on structurally close active molecules showing different potencies and potency ratios of the targeted biopharmacological activities in vitro. This comparative analysis could allow the final selection of the best clinical candidate exhibiting the most appropriate multi-targeting profile, with ideal mechanisms of action, potencies and potency ratio versus the different targets. It is worth noting that these pre-clinical studies are much less demanding than those necessary to develop any single component of a drug cocktail for NDs. In fact, once the selection of the most promising candidate has been made, the clinical study will be limited only to one compound. This opportunity represents an undoubted advantage of a MTDL approach compared to a polypharmacology strategy.

**ACKNOWLEDGEMENTS**

The financial support by MIUR-Rome (PRIN project 20085HR5JK) is gratefully acknowledged.

**ABBREVIATIONS**

Aβ = β-Amyloid  
AChe = Acetylcholinesterase  
AChe-I = Acetylcholinesterase inhibitor  
AD = Alzheimer’s disease  
ADME = Adsorption distribution metabolism excretion  
ALS = Amyotrophic lateral sclerosis  
APO E4 = Apolipoprotein E4  
APP = Amyloid precursor protein  
Bad = Bcl-2-associated death promoter  
Bax = Bcl-2–associated X protein  
BChE = Butyrylcholinesterase  
Bcl-2 = B-cell lymphoma 2  
Bcl-xL = B-cell lymphoma-extra large  
BH4 = Tetrahydrobiopterin  
CAT = Catalase  
CYP = Cytochrome P450  
COX-2 = Cyclooxygenase-2  
CSC = (E)-8-(3-Chlorostyryl)-caffeine  
DA = Dopamine  
DNA = Deoxyribonucleic acid  
DMPO = 5,5´-Dimethyl-1-pyrroline-N-oxide  
3D-QSAR = Three-dimensional quantitative structure-activity relationship  
EPR = Electron paramagnetic resonance  
eNOS = Endothelial NOS  
ERK = Extracellular-signal-regulated kinase  
FAD = Flavin adenine dinucleotide  
FDA = Food and drug administration  
GRD = Glutathione reductase  
GSH = Glutathione  
GPX = Glutathione peroxidase  
5-HT = Serotonin  
εNOS = Inducible NOS  
LA = Linoleic acid  
LBs = Lewy bodies  
LPO = Lipid peroxidation  
MAO-I = MAO inhibitor  
MAO-AI = Monoamine oxidase A inhibitor  
MAO-BI = Monoamine oxidase B inhibitor  
MAP = Mitogen-activated protein  
MAPK = Mitogen-activated protein kinase  
MEK = Mitogen-activated protein kinase  
MMTP = 1-Methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine  
MMDP+ = 1-Methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropiridinium  
MPP+ = 1-Methyl-4-phenylpyridinium  
MPTP = 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine  
MTDL = Multi-target directed ligand  
NA = Noradrenaline  
ND = Neurodegenerative disorder/disease  
NFT = Neurofibrillary tangles  
7-NI = 7-Nitroindazole  
NMDA = N-Methyl-D-aspartate  
nNOS = Neuronal NOS  
NO = Nitric oxide  
NOS = Nitric oxide synthase  
NOS-I = Nitric oxide synthase inhibitor  
NSAID = Non-steroidal anti-inflammatory drug  
6-OHDA = 6-Hydroxydopamine  
PAS = Peripheral anionic subsite  
PD = Parkinson’s disease  
PEA = β-Phenethylamine  
QSAR = Quantitative structure-activity relationship  
RAG = Rhizoma acori graminei  
RIMA = Reversible MAO-A inhibitors  
RNS = Reactive nitrogen species
ROS = Reactive oxygen species
PPARγ = Peroxisome proliferator-activated receptor-γ
PKCα = Protein kinase C α
PKCδ = Protein kinase C δ
sAPP = Soluble amyloid precursor protein
SARs = Structure-affinity relationships
SET = Single electron transfer
SIN-1 = 3-Morpholinosydnonimine
SOD = Superoxide dismutase
SSRs = Structure-selectivity relationships
SSRLs = Selective serotonin reuptake inhibitors
UPS = Ubiquitine-proteasome system

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Targeting Monoamine Oxidases with Multipotent Ligands

Current Medicinal Chemistry, 2011 Vol. 18, No. 30 4585


