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Review

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The pivotal role of copper in neurodegeneration: a new strategy for the therapy of neurodegenerative disorders

Roberta Giampietro,[a1] Francesco Spinelli,[a1] Marialessandra Contino,[a] Nicola Antonio Colabufo[a,b]*

[a]Dipartimento di Farmacia-Scienze del Farmaco, Università degli Studi di Bari Aldo Moro, Via Orabona 4, 70125, Bari, Italy

[b]Biofordrug srl, Dipartimento di Farmacia-Scienze del Farmaco, Università degli Studi di Bari Aldo Moro, Via Orabona 4, 70125, Bari, Italy

ABSTRACT

Copper is an essential trace element for human body since it is a cofactor of several enzymes and proteins and plays a pivotal role in several biological functions (e.g., respiration, protection from oxidative damage, iron metabolism, etc.), also including the central nervous system development and functioning (e.g. synthesis of neurotransmitters, myelination, activation of neuropeptides, etc.). Therefore, copper dysmetabolism is associated with different toxic effects, mainly represented by oxidative stress, and it has been reported in many neurodegenerative disorders, such as Wilson's disease, Menkes disease, Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis. This paper shows a detailed report of how copper is involved in the pathophysiology of these diseases. Moreover, a hint on novel therapeutic approaches based on restoring copper homeostasis through metal chelators will be pointed out.

KEYWORDS: *copper, oxidative stress, neurodegenerative diseases, metal chelators, natural compounds.*

INTRODUCTION

Copper is an essential element responsible for different biological functions in human body.[1] In the human brain and in the liver copper tissue levels are 5 μg/g, while the cerebrospinal fluid (CSF) shows copper concentration 0.3-0.5 μM.[2-3] Copper is present throughout the brain, in particular it has high concentration in basal ganglia, hippocampus, cerebellum, numerous synaptic membranes, cell bodies of cortical pyramidal neurons, and cerebellar granular neurons.[4] This metal exerts an important role for the development and functioning of the central nervous system (CNS), indeed, CNS neurons possess the machinery to uptake copper and subsequently release it at the synaptic cleft.[5] For instance, advanced researches highlighted a role for copper in synaptic transmission (modulation of neurotransmitters biosynthesis, neurotransmitters receptors, synaptic vesicles trafficking, etc.), axonal targeting, neurite outgrowth, and modulation of signalling cascades induced by neurotrophic factors.[6,7] In detail, it has been observed that copper may modulate excitatory and inhibitory neurotransmission by blocking GABAergic and AMPAergic neurotransmission; and an important role in the production and maintenance of myelin has been also reported.[5]

At subcellular level, copper is required for many activities, such as respiration, erythrocyte formation, iron absorption and transport into the body, peptide amidation, pigment formation, and development and maintenance of connective tissues.[8] Copper is also an electron donor or acceptor, oscillating between the reduced (Cu^{+}, cuprous ion) and the oxidized (Cu^{2+}, cupric ion) states, and behaves as cofactor of several enzymes involved in diverse metabolic functions and redox reactions: superoxide dismutase 1 and 3 (SOD1 and SOD3) for the antioxidant activity, cytochrome-C oxidase for ATP production in mitochondria, lysyl oxidase for collagen maturation, tyrosinase for melanin synthesis, ceruloplasmin (CP) for iron metabolism, etc.[9]

As depicted in Figure 1, copper introduced with the diet, after its reduction to Cu^{+}, is taken up in the small intestine by the copper transporter receptor 1 (CTR1), a high affinity copper protein localised

on the apical site of enterocytes, and is subsequently delivered to the blood by the copper transporting ATPase (Cu-ATPase) ATP7A. Once in the blood, Cu^{+} is mainly bound to serum albumin and reaches the liver, where it is absorbed in the hepatocytes via CTR1 transport. In the hepatocytes, copper is mainly bound to CP, a ferroxidase protein involved in the regulation of the iron oxidative state, and the corresponding complex is then delivered in the bloodstream. The formation of the copper-CP complex takes place in the *trans*-Golgi network (TGN), where copper is supplied to nascent CP thanks to the active transport mediated by another Cu-ATPase, the ATP7B. When copper exceeds the needs of the cell, ATP7B moves toward the cell membrane, and, via hepatic lysosomes, copper is released in the bile canaliculus together with the degradation products of CP.[10,11] The absorption of copper from the bloodstream into the CNS is mediated by CTR1 and ATP7A, respectively localised on the apical and basolateral sites of the blood-brain barrier (BBB) endothelial cells. Then, copper is absorbed by brain cells through a transport mediated by CTR1 and by the prion protein (PrP), a membrane protein involved in copper homeostasis.[10]

Depending on tissues demand, copper is mobilized from hepatic stores, it enters the cells, and, to preserve a physiological concentration of free copper within the cell at around 10^{-18} M, it is immediately bound by different proteins, such as metallothioneins (MTs), glutathione (GSH), and a group of chaperons shuttle copper, specialized protein for delivering copper to copper-dependent enzymes. For instance, the copper chaperon for superoxide dismutase (CCS) delivers copper to SOD1, Cox17 catalyses the incorporation of copper into cytochrome c oxidase, the antioxidant protein 1 (ATOX1) mediates the delivery of copper to the copper-transporting ATPases ATP7A and ATP7B.[8]

Based on the above mentioned several physiological roles of copper, copper dyshomeostasis has been associated with diverse pathological conditions. For example, an excess of free copper could translate in an excessive production of reactive oxygen species (ROS) through a Fenton-like reaction, leading to oxidative damages of protein, lipids, and nucleic acids. Besides, free copper can

directly bind to these latter causing additional noxious transformations lethal to cells, such as enzymes' inactivation, disruption of mitochondrial protein complexes, modification of low-density lipoprotein, or DNA damage.[9] On the other hand, deficit of copper translates in the impairment of all copper-dependent enzymes and functions, causing toxicological manifestations.

In the following sections, we review the pathological involvement of copper in several neurodegenerative diseases, such as Wilson's disease (WD), Menkes disease (MD), Alzheimer's disease (AD), Parkinson's disease (PD), and Amyotrophic Lateral Sclerosis (ALS). Moreover, a hint on novel therapeutic approaches based on restoring copper homeostasis will be also pointed out.

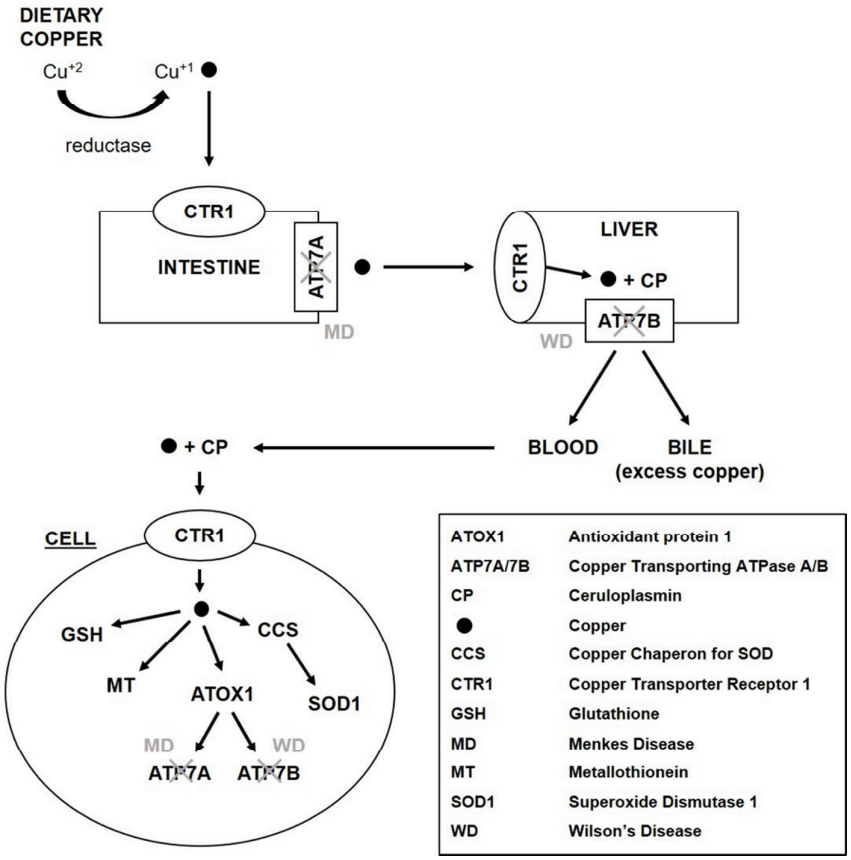


Figure 1. Schematic representation of copper homeostasis in physiological conditions and copper dyshomeostasis in Wilson's and Menkes diseases.

COPPER ROLE IN NEURODEGENERATIVE DISEASES

Wilson's disease (WD). WD is an autosomal-recessive disorder caused by mutations in the gene *ATP7B* encoding for the Cu-ATPase ATP7B (Figure 1), the transporter mainly responsible for copper incorporation into CP and for its biliary excretion. More than 500 homozygous or heterozygous mutations have been identified so far, including missense mutations, small deletions/insertions in the coding region, and splice junction mutations.[12] This loss-of-function of ATP7B causes an impaired incorporation of copper into CP and a defective bile excretion with consequent copper overload in the liver.[13] In fact, copper concentration in the liver of WD patients is 4.5-16.5-fold higher than in healthy individuals. This copper excess leads to an increased oxidative stress responsible for lipids, proteins, DNA, and RNA damages, which in turn causes apoptosis of hepatocytes and liver steatosis. Furthermore, when the liver is no longer able to store copper, free copper, nonceruloplasmin-bound, overflows into the bloodstream reaching other organs and causing extrahepatic copper toxicity. For instance, free copper could exert toxic effects on erythrocytes, leading to haemolysis and anaemia, or it could accumulate in skeletal muscle cells, in cardiomyocytes, in renal parenchyma, and in CNS, causing respectively rhabdomyolysis, cardiomyopathy, renal tubular dysfunction, and a wide variety of neurological and psychiatric symptoms (tremor, movement disorder and ataxia, which typically begin in the second or third decade).[12,14,15]

With the aim of reducing free copper in the blood to prevent its accumulation in tissues, three drugs (Figure 2), able to regulate copper bioavailability, have been approved by FDA for WD treatment: D-penicillamine, trientine, and zinc salts. The first two are chelating agents which bind copper and other metals in blood and tissues, facilitating their excretion in the urine; instead, zinc salts (e.g., zinc acetate) reduce copper blood levels interfering with its intestinal uptake. The general use of

these drugs depends on the disease progression: the chelating agents are recommended for patients with advanced symptomatology, whereas zinc salts are preferred for asymptomatic patients or patients in maintenance therapy.[12] Another chelating agent, the tetrathiomolybdate (TTM), has been extensively clinically tested and clinical trials are still ongoing for its use in WD.[12,16] TTM reduces free copper level in the serum through two different mechanism depending on whether it is administrated with meals or away from meals. In the former case, it reduces copper intestinal uptake making a tripartite complex with copper and food protein; in the latter case, it is well adsorbed in the blood flow where it forms a tripartite complex with copper and albumin.[16] The efficacy and safety of oral TTM (Coprexa^{TM}) in reducing serum free copper compared to trientine and zinc salts have been demonstrated in several clinical trials, but the FDA refused to evaluate its application.[16] A clinical trial involving a different TTM preparation (bis-choline tetrathiomolybdate, WTX101) is currently ongoing and the first report showed how the primary endpoints have been achieved (control of copper, reduction of serum free copper, significant improvements in neurological status.[17,18]

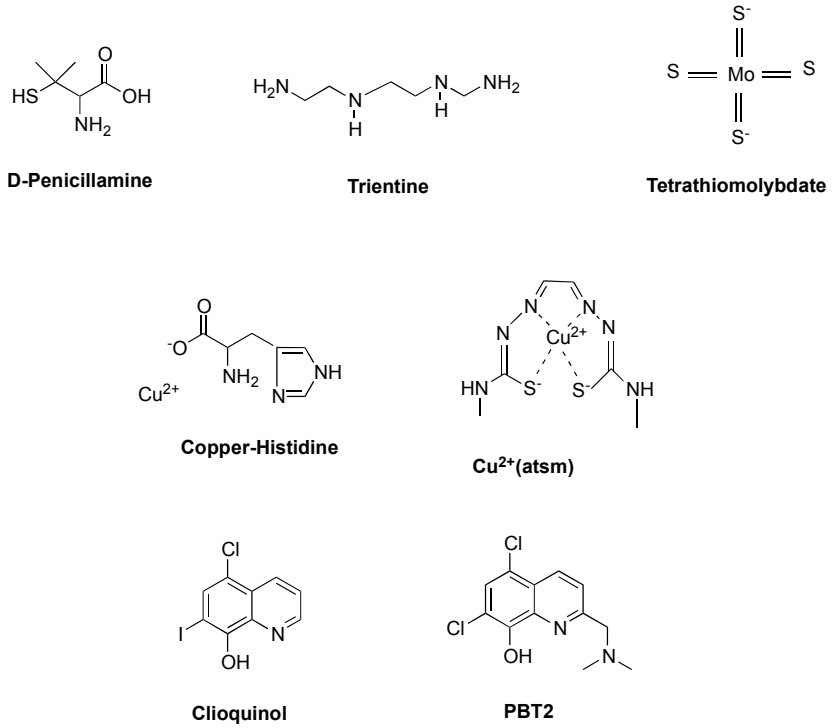


Figure 2. Copper chelators and copper delivery agents approved or (pre)clinically tested for the treatment of neurodegenerative diseases.

Menkes disease (MD). MD is a X-linked recessive disorder caused by mutations in the gene *ATP7A* encoding for the Cu-ATPase ATP7A (Figure 1), the transporter typically localised on the basolateral surface of the plasmatic membrane and responsible for the transfer of Cu^{+} from the enterocytes to the circulation.[19] ATP7A is also localised on the TGN where it supplies Cu^{+} to newly produced copper dependent enzymes.[19] To date, about 200 mutations on gene ATP7A causing loss of protein activity have been reported.[20] Inactivation of ATP7A results in alteration of copper homeostasis: Menkes patients have a very low copper plasma concentration, due to poor absorption of dietary copper, and an impaired tissues distribution with reduced copper level in brain, serum, and liver, but elevated one in kidney and intestine.[21] In brain of MD patients, copper deficiency impairs the physiological activity of copper-dependent enzymes and consequently leads to altered myelination, energy metabolism, catecholamine balance, and mRNA translation.[22] However, it is not yet clear whether and how ATP7A inactivation and copper dyshomeostasis act at subcellular

level. In vitro studies have shown that inactivation of this ATPase alters copper transport from the cytosol into the secretory pathway, and copper concentration results altered in nuclei, cytosol, and mitochondria, leading to different effects on the redox environment of these intracellular compartments. For instance, excess of copper in mitochondria causes high glutathione oxidation and thus an increase in the amount of peroxide. Therefore, it has been observed a damaged mitochondrial redox balance.[21] This impaired copper metabolism in MD patients causes neurodegenerative symptoms and connective tissue manifestation with early growth retardation, peculiar hair, vascular complications, and death in early childhood.[12]

A possible treatment for MD, aiming to restore the reduced copper concentration in blood and several tissues, has been object of clinical trial.[22,23] The trials consist of subcutaneous injection of copper-histidine (Figure 2), which provides copper directly through the blood stream to tissues, bypassing the malfunctioning mechanism of physiological copper absorption through the gastrointestinal tract. The studies so far performed have demonstrated that the treatment with copper-histidine is strictly related to the severity and the stage of the disease. Patients with mutations, which allow to retain some capacity for copper transport, generally have a favorable prospect to benefit from the treatment. Moreover, the treatment generally leads to better neurological outcomes in asymptomatic patients rather than in symptomatic ones.[24]

Alzheimer's Disease (AD). AD is one of the most common progressive neurodegenerative disease characterized by profound dementia, aphasia, disorientation, depression, and irreversible memory loss. The cause and progression of AD are not yet well understood. However, the main pathological hallmarks of AD are senile plaques deposits around neurons and neurofibrillary tangles inside neurons, respectively due to extracellular aggregation of amyloid-β (Aβ) peptides and intracellular aggregates of tau protein.[25] Aβ is generated by cleavage of amyloid precursor protein (APP), a neuronal receptor involved in neurite growth, neuronal adhesion, axon genesis, axonal transport,

and oxidative stress through copper reduction. APP can be cut through three proteases: α-secretase, β-secretase, and γ-secretase. α-Secretase and γ-secretase cleavage APP to obtain an harmless product, called P3; otherwise β-secretase, also named beta-site APP-cleaving enzyme (BACE), and γ-secretase realize a different cut producing Aβ40 and Aβ42, the main components of the plaques found in brain of AD patients (Figure 3).[26] These plaques, accumulated around cerebral blood vessels and in brain parenchyma, interrupt synaptic transmissions between neurons and lead to progressive damages, as neuroinflammation, neuronal dysfunction, neurotransmitter deficits, and neuronal death.

Copper plays a pivotal role in AD pathogenesis, since it interacts with key components, such as Aβ, APP, tau and, as a result, it enhances Aβ aggregation and neurotoxicity.[27] In detail, studies have shown that copper in excess can compromise the physiological brain ability to remove Aβ through the Low-density Lipoprotein Receptor-Related Protein 1 (LRP1), a protein expressed in brain endothelium.[27] Indeed, an excess of copper in brain capillaries causes the nitrotyrosination of LRP1 and induces its proteasomal-dependent degradation, thus interrupting Aβ excretion and leading to an increase of brain Aβ deposits.[27] Furthermore, copper turned out to be even more damaging because, not only it downregulates LRP1, but it also binds directly to Aβ peptide (affinity constant in the range 10^8-10^{10} M^{-1}), enhancing further Aβ aggregation and stimulating a strong oxidative stress damage.[27,28,29] The effects of copper interaction with the Aβ peptide and the redox properties of the corresponding Cu-Aβ complex have been recently reviewed by Cheignon and colleagues.[29] Cu^{+} and Cu^{2+} coordination spheres are very different in term of both amino acids involved and geometries (linear and square planar respectively). In addition, a further modification of this binding mode and the existence of a *catalytic in-between state* have been suggested to describe the ability of copper to cycle between the two oxidative states when bound to Aβ, leading to a strong ROS overproduction.[29]

In order to reduce free copper concentration and to minimize its role in AD pathogenesis, diverse copper chelators have been synthetized and studied in vitro and in vivo showing important beneficial effects in AD mouse models, such as inhibition of Aβ aggregation, antioxidant effects, and amelioration of the symptomatology. For instance, preclinical studies demonstrated that clioquinol (5-chloro-8-hydroxy-7-iodoquinoline, Figure 2), a non-specific copper-zinc chelator, is able to decrease Aβ deposits and to improve memory and learning capacities in an APP transgenic mouse model of AD.[30] PBT2 (5,7-dichloro-2-(dimethylaminomethyl)quinolin-8-ol, Figure 2), a clioquinol derivative, showed good tolerability and efficacy in a phase IIa clinical trial.[31] Despite these encouraging results, clioquinol showed severe neurotoxic side effects,[32,33] while PBT2 did not hit the prefixed end points and the clinical trial was stopped.[34] In addition, pharmacokinetics issues are also frequent, given that copper chelators for the treatment of AD must act in the CNS and the ability to cross the BBB is strictly required. D-Penicillamine, for example, could not be used in AD cause to its incapacity to cross the BBB and to reach therapeutic concentrations in the CNS.[35]

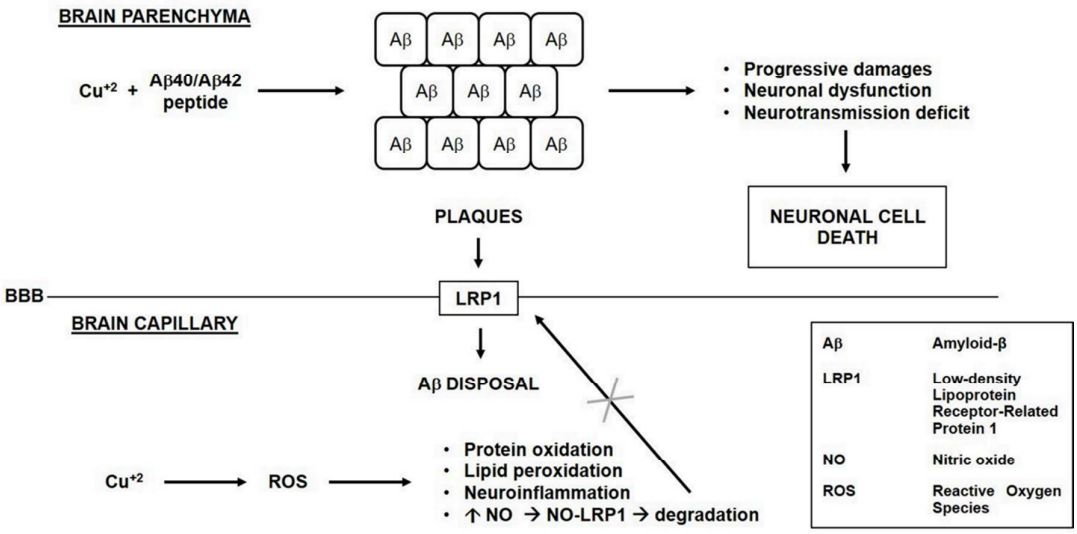


Figure 3. Schematic representation of copper-Aβ peptides interaction in Alzheimer's disease.

Parkinson's disease (PD). PD is the second most common neurodegenerative disorder characterized by tremor at rest, muscle rigidity, bradykinesia, postural instability, and movement impairments. PD pathogenesis is not clearly understood, although studies define PD as a multifactor disease caused by environmental and genetic factors. The main pathological hallmarks of PD are the loss of dopaminergic neurons and the presence of intracellular proteinaceous inclusions, named Lewy bodies, consisting mostly of aggregation of misfolded form of α-synuclein (αSyn) accumulated in brainstem, spinal cord, and cortex. Indeed, some PD cases have been associated with autosomal dominant mutations of αSyn gene, *SNCA* (e.g., A53T, H50Q, E46K, A53E, G51D, A30P), which lead to αSyn overexpression, accumulation, and aggregation.[36-38] αSyn is a highly soluble and "intrinsically unfolded" protein localised in the cytosol, in the presynaptic terminals close to synaptic vesicles, or associated with the mitochondrial membrane. Its specific role is not yet completely known; however, it has shown the ability of interaction with lipid membranes, synaptic vesicle recycling, dopamine metabolism, and it also has a high affinity toward metals, such as Cu^{2+}.[39,40] As shown in Figure 4, copper-αSyn interaction have been associated with an increase of the oxidative stress and with the production of toxic oligomers which, in vitro, form pore-like structures in the membrane bilayer changing conductance activity and, in vivo, break the membranes leading to cell death.[41] In addition, the specific combination of H50Q αSyn mutation and the presence of Cu^{2+} has been associated with a further enhancement of αSyn fibrillation, leading to stronger neuronal damages.[41] Finally, copper can perform a cooperative binding with dopamine (DA) to αSyn, enhancing to a greater extent the fibrillation of αSyn and the production of ROS.[40]

PD patients reveal a dysregulation of copper homeostasis and recent studies showed a decreased CTR1 expression and a reduced total tissue copper in the substantia nigra (SN).[41] Contrariwise, high levels of unbound copper have been found in the CSF, probably responsible of motor impairments. Finally, copper dyshomeostasis in PD patients has been also associated with genetic mutations of

ATP7B.[41] On these bases, the treatment aimed to regulate copper dyshomeostasis in PD has been widely assessed. Preclinical studies in a PD animal model investigated clioquinol (Figure 2), the previously described copper chelator, for its potential use in therapy and showed a SN neuron survival.[42] Furthermore, the in vivo assessment of the copper complex Cu^{2+}-diacetylbis(4-methylthiosemicarbazone) (Cu^{2+}(atsm)) in four different PD animal models showed neuroprotection, improvement of cognitive performance, and restoration of motor function. The proposed mechanism of action of Cu^{2+}(atsm) derives from the in vitro ability to inhibit peroxynitrite-mediated formation of α-synuclein oligomers; however, this does not exclude a neuroprotective mechanism linked to copper release from the complex.[43,44] Based on these findings, a clinical trial of Cu^{2+}(atsm) is currently recruiting patients with early idiopathic PD.[45]

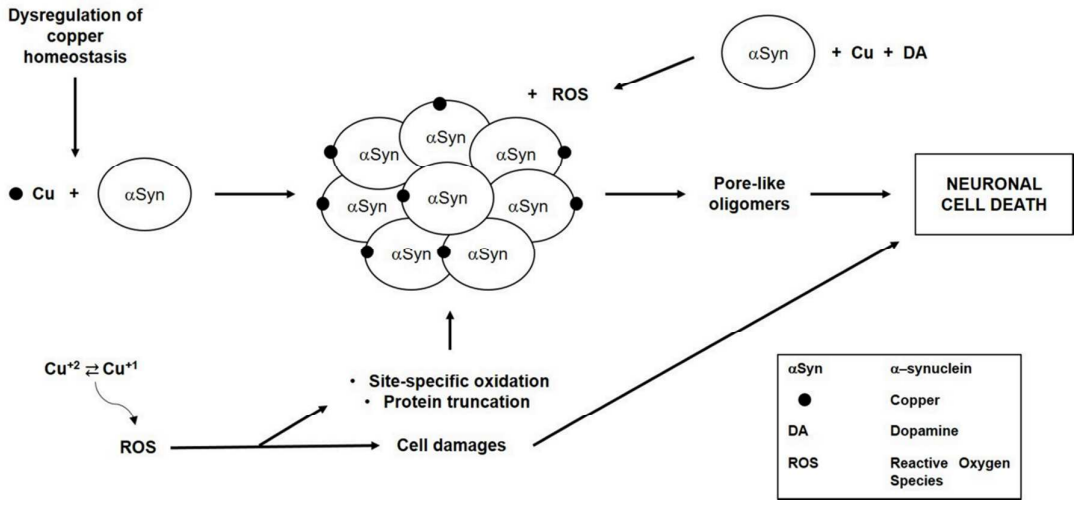


Figure 4. Schematic representation of copper-α-Syn interaction in Parkinson's disease.

Amyotrophic Lateral Sclerosis (ALS). ALS is a progressive neurodegenerative disease characterized by loss of motor neurons in motor cortex, brainstem, and spinal cord. Patients affected by this disease undergo muscle weakness, atrophy, spasticity, paralysis, and consequently death within 3-5 years after diagnosis. Compared to healthy individuals, misfolded SOD1 aggregates contained a lower level of copper has been found in spinal cord motor neurons of ALS patients.[46]

One of the first described cause of ALS has a genetic origin: 5-10% of patients have a family history of ALS associated with point mutations to the gene *Sod1*, encoding for the enzyme SOD1. SOD1 is an antioxidant protein, expressed in all cell types, which reduces superoxide ion (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) in a two steps reaction. The mechanism by which mutant SOD1 cause the ALS is still far to be completely understood, but it is widely accepted that a pro-oxidant gain of function plays a more crucial role than the loss of the SOD1 physiological function.

SOD1 is a metalloenzymatic homodimer in which each monomer binds one copper ion and one zinc ion important respectively for protein activity and stability.[47] The physiological activation pathway of SOD1 is depicted in Figure 5: SOD1 monomer in a metal-deficient state (apo-SOD1), after zinc binding and dimerization through a disulphide bond, receives copper ions from CCSs, one for each monomer, and reaches the fully metalated state (Holo-SOD1), which is the active enzymatic form.[10] Nowadays 170 mutations have been found and classified into two classes: 1) "wild type-like" mutations (A4V, G37R, G39A, etc.), which lead to a protein with similar properties to wild type SOD1, and 2) "metal binding region" mutations (G85R, H46R, H80R, D125H, etc.), which lead to a protein characterized by an altered metal binding ability and a reduced enzymatic activity.[46] For instance, it has been observed that H80R mutation, found in the zinc-binding region, leads to a reduction of zinc binding and consequently of copper binding.[48] Instead, other mutations can prevent the mutant SOD1 monomer from interacting with CCS and thus to achieve two copper ions and to reach the fully metalated state.[10] In general, the mutant SOD1, in a copper-deficient state, accumulates and forms aggregates of misfolded protein, which induce the toxic gain of function through which SOD1-induced motor neurons death occurs.[10] Furthermore, in this situation, copper remains bound to CCS and it is not delivered to other copper-dependent enzymes, impairing their function and leading to additional toxic effects (e.g., decrease of mitochondrial respiration).[10]

Interestingly, Weihl and Lopate in 2006 reported about three patients out of seven in which the clinical symptoms associated with copper deficiency were similar to those of amyotrophic lateral sclerosis (ALS).[49] This might suggest a direct role of copper deficiency in the aetiology of the ALS independent from SOD1 mutations (sporadic ALS). Indeed, several studies have shown how wild-type SOD1 may become unstable and misfold in certain condition, including perhaps a copper-deficient state, to form aggregates that are selectively toxic to motor neurons.[50,51]

Although copper role in the ALS pathophysiology is various, interestingly both copper chelators and copper delivery agents have shown beneficial effects in different mouse model of the disease. Backman et al. demonstrated the clinical efficacy of the Cu^{2+} delivery agent before mentioned, Cu^{2+}(atsm) (Figure 2). Its oral administration in the SOD1G37R mouse model of ALS, indeed, showed improved locomotor function and prolonged survival compared to untreated mice. The underlying proposed mechanism of action consists in the delivery of copper from Cu^{2+}(atsm) to the metal-deficient mutant apo-SOD1 form, allowing its conversion in the more stable and less toxic fully metalated holo-SOD1 form.[52] Two clinical trials of Cu^{2+}(atsm) are currently recruiting ALS patients.[53,54] On the other hand, many studies showed the neuroprotective effects of copper chelators, such as D-penicillamine and TTM, in the SODG93A mouse model of ALS. The mechanism proposed for these therapeutic benefits consists in the mitigation of the copper-dependent strong peroxidase activity associated with the mutant SOD1.[55,56]

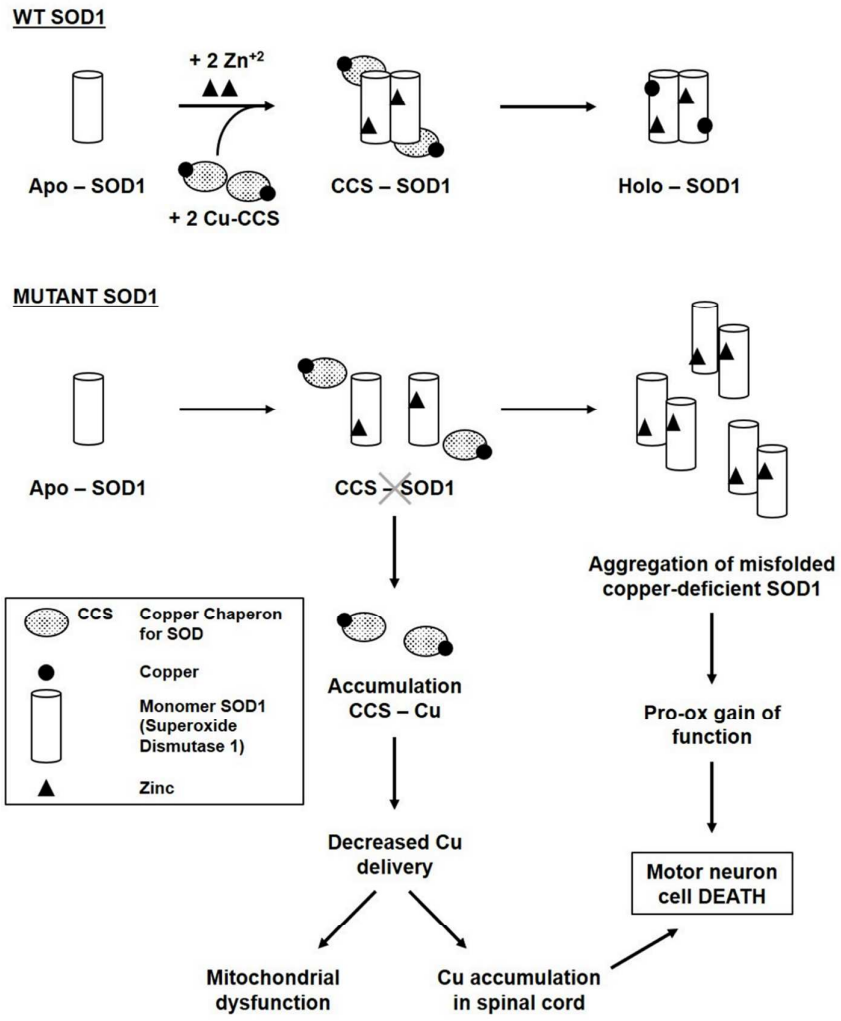


Figure 5. Schematic representation of copper-SOD1 interaction trough CCSs in wild-type or mutant cases associated with amyotrophic lateral sclerosis.

COPPER CHELATORS

The key role played by copper and other metals in the pathogenesis of several diseases has prompted many researchers to develop copper chelators as new potential therapeutic approach and some comprehensive reviews having appeared in the literature.[25,57] Firstly, once chelated, copper is no longer available for the Fenton reaction and a decrease of ROS production could be observed. Furthermore, copper chelation could decrease all the toxic effects associated with, for example, Cu-Aβ interaction in AD and Cu-αSyn interaction in PD, leading to additional therapeutic effects.

As for the structural requirements of copper chelators, important features that must be taken into account are the chelate denticity, the donor binding groups, and the cavity size. Cu^{+} is a soft metal ion, it prefers soft donors, mainly *S*-atoms (thioethers, thiolates, tiocarbonyls) but also *N*-atoms (nitriles, cyanide), and it adopts tetrahedral, trigonal, or linear geometries. Cu^{2+} is a borderline hard-soft metal ion, it binds to borderline donors such as *N*- and *O*-atoms (amines, imines, carbonyls, and alchols), and it usually acquires square-planar, distorted square-planar, trigonal-pyramidal, or square-pyramidal geometric conformations.[55] In general, Cu^{2+}-chelators are the most developed so far, given that Cu^{2+} is more stable than Cu^{+} in aqueous solution, and *N*,*N*-donor groups confer the highest stability to Cu^{2+}-complexes, followed by *N*,*O*- and *O*,*O*-donor atoms.[25]

In the following sections, we report about natural and synthetic compounds that showed copper chelating ability and that have been recently used as starting point to design and develop new compounds with potential therapeutic outcomes based on the copper-chelation.

Natural compounds and their corresponding synthetic derivatives

Several dietary factors have been suggested as modifying agents in different neurodegenerative diseases because of the ameliorating effects on the onset of these pathologies due to their ability to chelate metal ions, such as copper, iron, and zinc. Among them, the antioxidant agents L-ascorbic acid and α-tocopherol, some flavonoids (e.g., (-)-epigallocatechin gallate), gallic acid, propyl gallate, resveratrol, curcumin, caffeine, and caffeic acid were investigated for their metal chelating and neuroprotective activities (Figure 6).[58] (-)-Epigallocatechin gallate, gallic acid, and curcumin showed multifunctional neuroprotective activities, such as: *i*) good chelating ability toward metal ions (Cu^{2+}, Fe^{2+}, and Zn^{2+}); *ii*) free radicals scavenger properties; *iii*) inhibition of Aβ fibrils deposition. However, the poor brain uptake limited their therapeutic actions. On the other hand, the potent antioxidants L-ascorbic acid and α-tocopherol, displaying a better brain uptake profile, were poor metal chelators and did not inhibit Aβ fibrillation.[58]

In the following part of this review we summarize the best synthetic derivatives of some natural compounds highlighting their metal chelating ability, antioxidant properties, and Aβ fibrillation inhibitory activity.

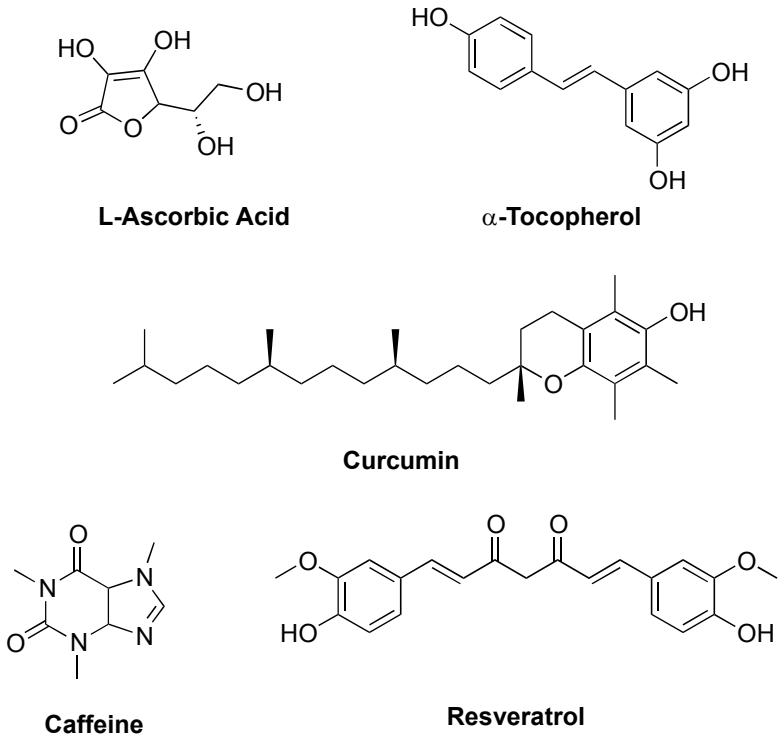


Figure 6. Some natural compounds that showed beneficial effects on the onset of some neurodegenerative diseases.

Curcumin and derivatives. Curcumin ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione, Figure 6) is a bright yellow compound produced by some plants (mainly turmeric) and characterized by various beneficial properties, such as antioxidant and anti-inflammatory effects, inhibition of Aβ aggregation (43.1 % inhibition of self-induced Aβ aggregation and 64.0 % inhibition of Cu^{2+}-induced Aβ aggregation at 25 μM),[59] and metal chelation.[60,61] In vivo assessment of curcumin showed inhibition of Aβ oligomerization, Aβ deposition, and tau phosphorylation in the brain of AD animal models, and also improvements in behavioural impairment.[61] In addition, Cu^{2+} chelation was proposed as one of the mechanism through which curcumin might exert the

aforementioned neuroprotective effects against Aβ-toxicity in AD animal models.[60] Furthermore, in vitro experiments showed that curcumin binds to reduced wild-type SOD1, allowing the formation of less toxic SOD1 aggregates and leading to a decreased cytotoxicity.[62]

Given all these findings, curcumin has been extensively clinically tested and many clinical trials are still ongoing.[63] Among the completed trials performed in AD patients, only one, which recruited only three single cases, showed improvement in behavioural symptoms and quality of life, on the contrary, no significative differences between curcumin and placebo groups were found in the other two. As for the clinical trials currently ongoing, two of them involve patients with mild cognitive impairments and aim respectively at studying the effects of the dietary supplement curcumin on age-related cognitive impairment[64] and at evaluating the clinical benefits of curcumin alone or in combination with physical exercise.[65]

From a structure-activity relationship point of view, it has been shown that curcumin phenolic groups (aromatic rings and polar substituents) are responsible for the binding to the Aβ peptide through π-stacking and hydrogen bond interaction, while the β-diketone function, which can also exist in the tautomeric enolic form, is involved in the formation of stable complexes with copper.[66] Considering this, Chen et al. designed different curcumin analogues aiming to the development of a new class of metal chelators and Aβ aggregation inhibitors.[66] In order to define the structural requirements for the Aβ aggregation inhibitory activity, the phenolic hydroxy groups of the lead compound curcumin were substituted with *N*-methylpiperazine moieties, and the flexibility of the linker between the two aromatic rings was differently modified. However, aiming at preserving the metal chelating ability, a carbonyl group in the linker was kept in all the derivatives designed. Among all tested compounds, compound **1**, depicted in Figure 6, emerged as the most valuable in term of Aβ aggregation inhibitory activities (assessed through the thioflavin T – ThT – fluorescence assay) and antioxidant properties (assessed through the oxygen radical absorbance capacity – ORAC – assay). Compared to curcumin, it showed a ~5-fold higher self-induced Aβ aggregation

inhibition (IC_{50} = 2.5 μM), a strong inhibition of the Cu^{2+}-induced Aβ aggregation, and a 2-fold increase of the antioxidant activity. In addition, UV/Vis spectrometry was used to demonstrate the metal chelating ability of **1** toward Cu^{2+} and Fe^{2+}, but not Zn^{2+}.[66]

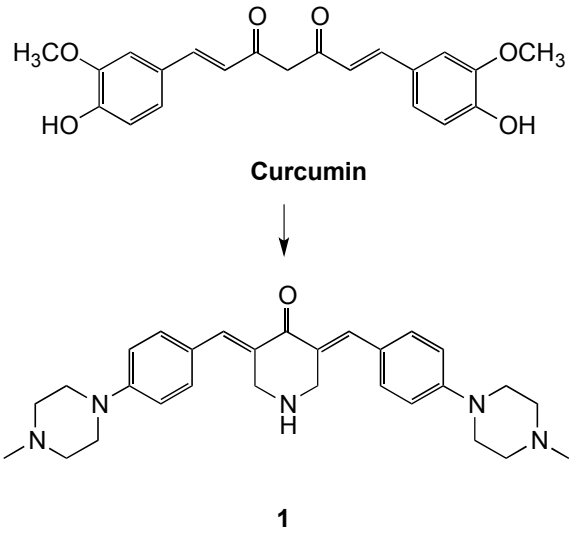


Figure 6. Structures of curcumin and compound 1.

Resveratrol and derivatives. Resveratrol (trans-3,4',5-trihydroxylstilbene, Figure 7) is a polyphenol present in different plants (e.g., grapes, berries, peanuts) and red wine. It is a well-described antioxidant and beneficial effects have been reported in diverse diseases, such as cancer, cerebrovascular illnesses, diabetes, arthritis, aging-related disorders, depression, and neurodegenerative diseases (e.g., AD, PD, ALS).[67-70] As reviewed by Tellone et al., resveratrol is a multitarget compound which exerts its neuroprotective effects not only through the strong and efficient antioxidant activity, but also improving mitochondrial function and efficacy, mediating an anti-apoptotic effect, and reducing Aβ and SOD1 aggregation.[70]

As for the curcumin, the important therapeutic outcomes of resveratrol found in preclinical studies prompted many clinical trials. In three of these clinical trials were recruited patients with AD[71-73] but, to the best of our knowledge, the results are available only for one, which showed no

significative improvement of the cognitive score.[71] Furthermore, one of the clinical trial is currently recruiting patients with mild cognitive impairment and aims at demonstrating safety and efficacy of the resveratrol preparation object of study.[74]

Considering the antioxidant properties and the ability to inhibit Aβ aggregation, conferred by its stilbene structure,[75,76] resveratrol has been also used as lead compound in the design and synthesis of a series of imine resveratrol derivatives, aiming to a bifunctional compound able to simultaneously chelate copper and inhibit Aβ aggregation.[77] The metal chelation ability has been introduced in resveratrol scaffold changing the position of the phenolic hydroxyl groups and adding a Schiff base, as shown in clioquinol structure, a classical metal chelator (Figure 7).[77]

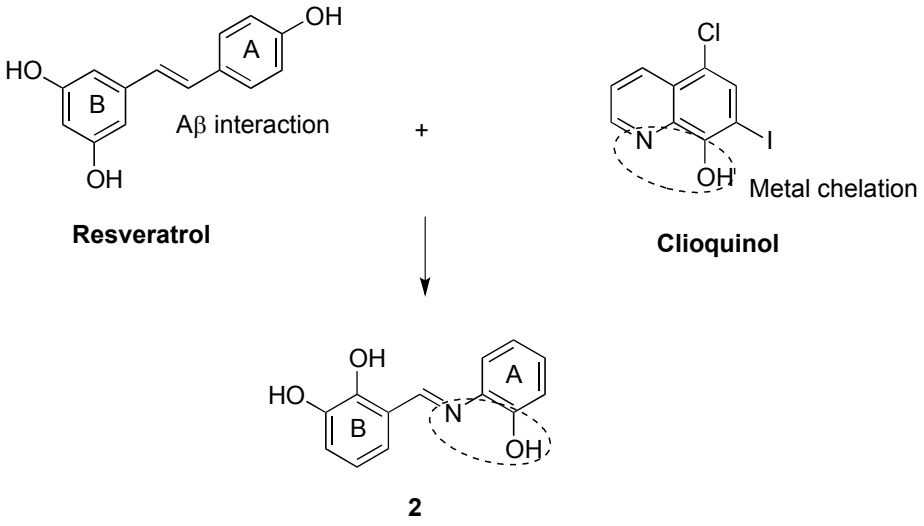
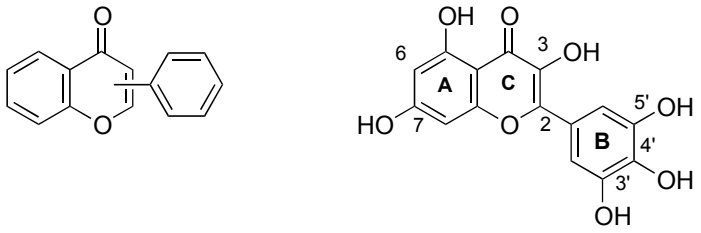


Figure 7. Compound 2 obtained from resveratrol and clioquinol.

Among all synthetized compounds, compound **2** (Figure 7) gave the best results in terms of antioxidant activity, inhibition of Aβ aggregation, and metal chelating ability. First of all, the ability of **2** to chelate Cu^{2+} was confirmed through UV/Vis spectrometry. In addition, DPPH (diphenyl-1-picryhydrazyl) free radical-scavenging assay demonstrated that **2** has a 7.7-fold higher antioxidant activity (IC_{50} = 14.1 μM) than the lead compound resveratrol (IC_{50} = 108.6 μM). Finally, the

fluorometric ThT binding assay showed only a slight increase of the inhibitory activity on self-induced (64.6 %) and Cu^{2+}-induced (68.1 %) Aβ aggregation than the lead compound resveratrol (64.2 % and 63.4 % respectively).[77]

Flavonoids and derivatives. Flavonoids are natural compounds belonging to the family of polyphenols present in fruits and vegetables. They are structurally characterized by a common phenylchromanone nucleus as reported in Figure 9 and, on the base of the different substitutions and oxidation status of the C-ring, they can be classified into flavones, isoflavones, flavanones, flavonols, flavanols and chalcones.[78] They have different beneficial effects such as: 1) antioxidant activity; 2) anti-cancer activity; 3) anti-inflammatory and neuroprotective capacities; and 4) metal chelating properties. For example, among the flavonoids, myricetin (Figure 9) was found able to strongly decrease the metal-induced Aβ deposition.[79,80]



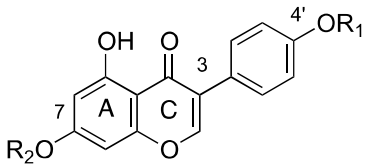
Phenylchromanone nucleus **Myricetin**

Figure 9. Phenylchromanone nucleus and the flavonoid myricetin.

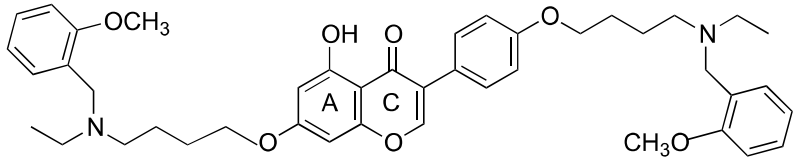
Structure-activity relationships assessment allowed to define the structure requirements for the antioxidant and the metal chelating activities. The antioxidant and radical scavenger activities have been linked to: 1) the catechol group in the B-ring and the 2,3-double bond conjugated with the 4-oxo group in the C-ring; 2) the hydroxyl groups in the 3-position of C-ring and in the 5,7-positions of A-ring.[25] Whereas the binding sites for metal ions have been identified in: 1) the catechol moiety

in the B-ring; 2) the 3-hydroxy and the 4-oxo groups in the C-ring; 3) the 4-oxo and the 5-hydroxyl groups between the C- and A-rings.[25]

Based on these findings, several synthetic flavonoid derivatives have been designed and, aiming to the development of a compound suitable for AD therapy, flavonoids have been also structurally combined with a pharmacophore able to affect at least another process or pathway involved in AD pathogenesis, such as the loss of cholinergic neurons, the production and the aggregation of Aβ, the ROS overproduction, etc. This strategy leads to the development of hybrid ligands, specifically named multitarget-directed ligands (MTDLs), analyzed through the spectrophotometric Ellman's method, ORAC assay, UV-vis spectrometry, and ThT fluorescence method in order to highlight AChE inhibitory activity, antioxidant properties, metal chelating ability, and inhibition of Aβ fibrillation activity, respectively. For example, **FLV1** (Figure 10) represents a series of genistein (4',5,7-trihydroxyisoflavone) derivatives functionalized in 4' and/or in 7 with *O*-alkylbenzylamine moieties, which is a structural requirement for adequate AChE inhibition.[25,59,81,82] In detail, compound **3** (Figure 10) emerged as a Cu^{2+} chelator and showed high AChE inhibitory activity ($IC_{50\ hAChE}$ = 0.35 μM), weaker antioxidant activity than genistein, and significative inhibition of Cu^{2+}-induced Aβ aggregation (77.8 % at 25 μM).[59]



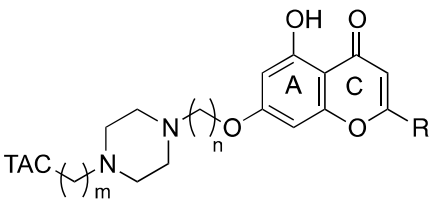
FLV1



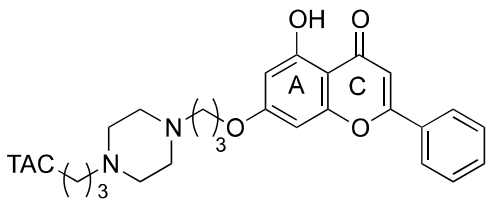
3

Figure 10. FLV1 series and compound 3.

Another important example of MTDLs based on flavonoids is **FLV2** series (Figure 11), where the AChE inhibitory activity was introduced by the combination with tacrine (TAC, Figure 11), a well-known AChE inhibitor (AChEi). In **FLV2** derivatives, the TAC moiety was introduced in the 7-position of flavonoid scaffold through a *N,N*-dialkylpiperazine linker.[83] In this series, the best pharmacological properties were detected for compound **5**, which showed copper chelating properties and the best balance between AChE inhibitory activity (IC_{50} = 133 nM, 2-fold more potent than TAC toward AChE) and inhibition of self-induced Aβ aggregation (79.1 % at 20 μM).[83]



FLV2



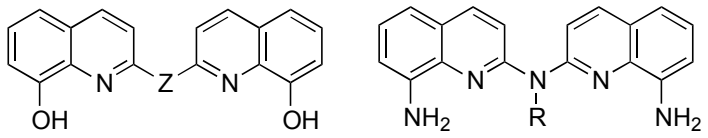
4

Figure 11. FLV2 series and compounds 4.

Synthetic Copper(II) Chelators

Among the copper(II) chelators developed in the past, clioquinol (Figure 2) has been broadly studied both in vitro and in vivo but, as mentioned in the previous sections, its neurotoxic side effects strongly limited its applicability in terapy.[32,33] Clioquinol is a bidentate ligand that forms 2:1 (ligand/metal ratio) complexes with Cu^{2+} (log*K* for $Cu^{II}L_2 \approx 10$,[84] pCu ≈ 15.5 at pH = 7.4[25]) and Zn^{2+} (log*K* for $Zn^{II}L_2 \approx 8.8$,[84] pZn ≈ 8.5 at pH = 7.4[25]) ions and structure-activity relationship assessment showed that the hydroxyl group and the nitrogen atom are essential for the metal binding.[32] Hence, several other copper chelators have been developed based on the 8-hydroxyquinoline scaffold of clioquinol. Among the changes performed, the dimerization of and the isosteric replacement of the 8-hydroxyl group with an amino group led to a general improvement of the copper chelating ability. The dimerization of two 8-hydroxyquinoline moieties by different linking unit (e.g., methyl, ethyl, carbonyl, etc.) led to tetradentate chelators (**5**, Figure 12, 1:1 ligand/metal ratio) displaying an affinity for Cu^{2+} 10^2-10^3-fold higher than the one for Zn^{2+} (logK_{aff} for $Cu^{II}L \approx 16$; logK_{aff} for $Zn^{II}L \approx 13$) and 10^5-fold higher than the one displayed by the *mono*-8-hydroxyquinoline derivative (logK_{aff} for $Cu^{II}L \approx 11$).[85] The replacement of the hydroxyl groups in the 8-position of these *bis*-8-hydroxyquinoline derivatives with amino groups and the insertion of a *N*-substituted-atom as linkage between the two monomers led to *bis*-8-aminoquinoline derivatives (**6**, logK_{aff} for $LCu^{II} \approx 18$, Figure 12) with high affinity for Cu^{2+} and negligible affinity toward Zn^{2+} and Fe^{3+}. This modification confirmed the aformentioned reported higher stability of Cu^{2+}-complexes with *N*,*N*-donors rather than *N*,*O*-donors.

The preclinical evaluation of one of these *bis*-8-aminoquinolines (PA1637, structure not disclosed) in an AD mouse model obtained by a single intracerebroventricular injection of short oligomers of Aβ42 showed better therapeutic effects and much less toxicity than clioquinol.[86]



5 **6**

Figure 12. *Bis*-8-hydroxyquinoline and *bis*-8-aminoquinoline derivatives.

The innovative drug design strategy already described for the flavonoids which aimed at the development of MTDLs has been extensively carried out also for synthetic metal chelators. One of the most assessed combination of metal chelators involves AChE inhibitors and leads to MTDLs that hit two of the main hallmarks of AD: the dysregulation of biometal ions and the loss of cholinergic neurons. Some examples of such MTDLs are depicted in Figure 13.[87] Worth noting is the presence of the TAC and the 8-hydroxyquinoline moieties as pharmacophores respectively of the cholinesterase inhibitor activity and the metal chelating activity.

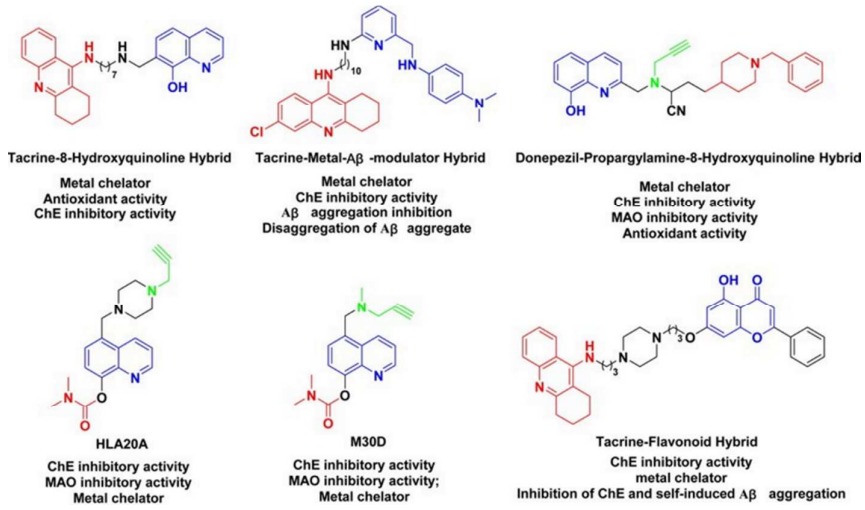


Figure 13. Representative MTDLs bearing AChE and metal-chelating properties. Red: the pharmacophores of AChE inhibition; blue: the pharmacophores of metal chelator; green: the pharmacophores of MAO inhibition. Adapted from ref. 87. Copyright © 2016 American Chemical Society.

Quintanova and co-workers recently developed a series of TAC hybrids bearing *S*-allyl-cysteine or *S*-propargyl-cysteine moieties which showed different pharmacological activities useful for the treatment of AD (e.g., inhibition of AChE, inhibition of Aβ aggregation, and anti-oxidant activity).[88] In order to assess whether these pharmacological effects could be also related to the metals chelation, the derivatives **7** and **8** depicted in Figure 14 were synthesized and evaluated in terms of their Cu^{2+} chelating capacity. Both compounds showed only a moderate chelating ability toward Cu^{2+} (pCu = 7.1-7.5 at pH = 7.4), as expected from the presence in the chelator of *N,S*-donors, which should prefer complex with Cu^{+} rather than Cu^{2+}. Despite this weak copper-chelation, these two compounds showed an higher inhibitory activity of the Cu^{2+}-induced Aβ aggregation (44.4-50.3 %) rather than the self-induced Aβ aggregation (10.9-13.0 %).[88]

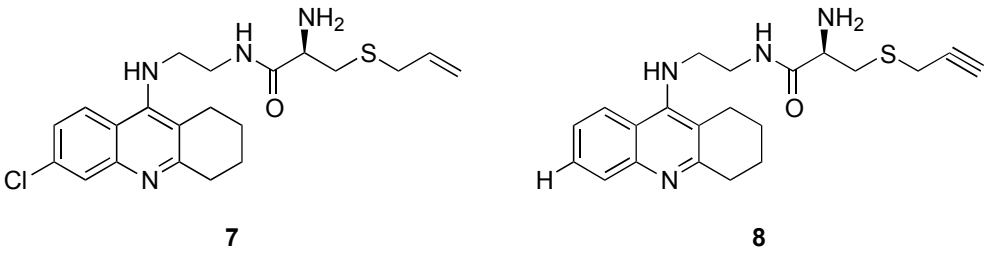
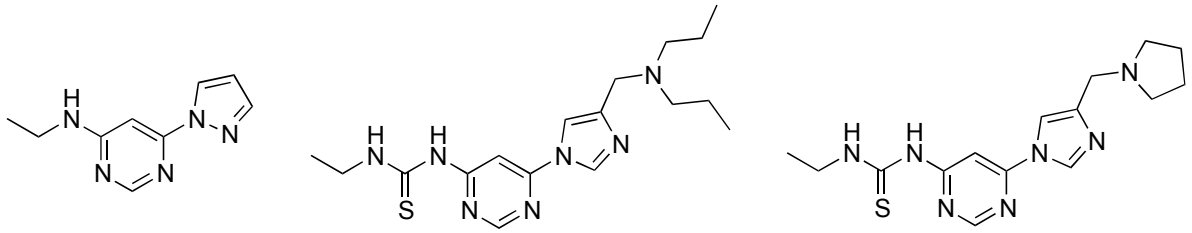


Figure 14. TAC hybrids bearing *S*-allyl-cysteine (7) or *S*-propargyl-cysteine (8) moieties

A different series of MTDLs was recently developed by Li and colleagues.[87] Starting from the screening of 630 small molecules (MW < 300 Da) in an AChE inhibition assay, a *N*-ethyl-6-(1*H*-pyrazol-1-yl)pyrimidin-4-amine derivative (**9**, Figure 15) was selected as hit structure. Structure-activity relationship modifications performed on **9** led to the development of strong AChE inhibitors with a 1-(6-(1*H*-imidazol-1-yl)pyrimidin-4-yl)-3-ethylthiourea general structure where the thiourea moiety resulted pivotal for both the AChE inhibitory activity and the metal chelating capacity. Compounds **10** and **11** (Figure 15) bearing a pyrimidinylthiourea scaffold were selected and were further assessed for their ability to chelate metals, inhibit Aβ aggregation and ROS

production, have either cytotoxic or neuroprotective effects.[87] Both the derivatives showed good Cu^{2+} chelating ability and good selectivity against Fe^{2+}, Fe^{3+} and Zn^{2+}. Furthermore, they did not show any effects on the self-induced Aβ aggregation, but they were able to significantly reduce the Cu^{2+}-induced Aβ aggregation. They also showed a complete inhibition of the Cu^{2+}-induced ROS production. As for the cytotoxic and neuroprotective properties, these two pyrimidinylthiourea derivatives showed very low cytotoxicity and a significant ability to increase the cell viability in presence of both Aβ and Cu^{2+}.[87]



9 **10** **11**

Figure 15. *N*-Ethyl-6-(1*H*-pyrazol-1-yl)pyrimidin-4-amine (9) and pyrimidylthiourea derivatives (10, 11) as MTDLs for the treatment of AD.

CONCLUSIONS

Currently, the therapeutic approaches for the treatment of neurodegenerative diseases show many limitations, in fact, they usually provide short-term amelioration of the symptoms, without exerting any effect on the causes of the neurodegeneration. For example, after the FDA-approval of memantine (NMDA receptor antagonist) in 2003,[25] many promising drugs have been developed, but none of them have reached the final approval for clinical use.[89] As reviewed in this paper, in the last years copper dyshomeostasis emerged as one of the main factor involved in the pathogenesis of some neurodegenerative diseases and the restoration of the physiological tissue concentrations by

the use of copper chelating agents have been proposed as new potential therapeutic strategy. Indeed, these chelators make ion copper no longer available preventing or decreasing the toxic effects associated with its excess.

AUTHOR INFORMATION

Corresponding author

*Phone: +39-080-5442747; e-mail: marialessandra.contino@uniba.it

[1]**These authors contributed equally**.

ABBREVIATIONS USED

AChE, acetylcholine esterase; AD, Alzheimer's disease; ALS, Amyotrophic Lateral Sclerosis; APP, amyloid precursor protein; ATOX1, antioxidant protein 1; Aβ, amyloid β-amyloid; αSyn, α–synuclein; BACE, Beta-Site APP-Cleaving Enzyme; BBB, blood-brain barrier; CCS, copper chaperon for superoxide dismutase; CNS, central nervous system; CP, ceruloplasmin; CSF, cerebrospinal fluid; CTR1, copper transporter receptor 1; Cu-ATPase, copper transporting ATPase; DA, dopamine; DPPH, diphenyl-1-picryhydrazyl; GSH, glutathione; H_2O_2, hydrogen peroxide; LRP1, low-density lipoprotein receptor-related protein 1; FDA, Food and Drug Administration; MD, Menkes disease; MTs, metallothioneins; MTDLs, multitarget-direct ligands; NMDA, *N*-methyl-*D*-aspartate; O_2, molecular oxygen; O_2^-, superoxide ion; ORAC, oxygen radical absorbance capacity; PD, Parkinson's disease; PrP, prion protein; ROS, reactive oxygen species; SN, substantia nigra; SOD1/3, superoxide dismutase 1/3; TAC, tacrine; TGN, trans-Golgi network; ThT, Thioflavin T; TTM, tetrathiomolybdate; WD, Wilson's disease.

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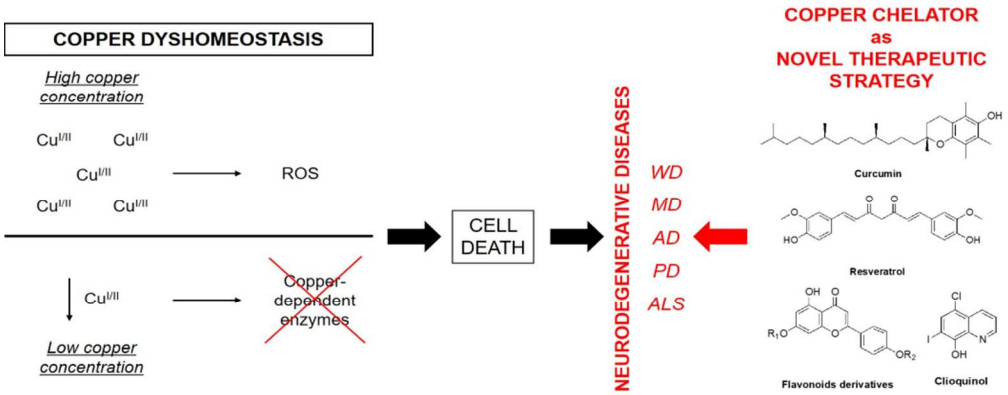

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