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Review article

Chelating principles in Menkes and Wilson diseases
Choosing the right compounds in the right combinations at the right time

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ABSTRACT

Dysregulation of copper homeostasis in humans is primarily found in two genetic diseases of copper transport, Menkes and Wilson diseases, which show symptoms of copper deficiency or overload, respectively. However, both diseases are copper storage disorders despite completely opposite clinical pictures. Clinically, Menkes disease is characterized by copper deficiency secondary to poor loading of copper-requiring enzymes although sufficient body copper. Copper accumulates in non-hepatic tissues, but is deficient in blood, liver, and brain. In contrast, Wilson disease is characterized by symptoms of copper toxicity secondary to accumulation of copper in several organs most notably brain and liver, and a saturated blood copper pool. It is a challenge to correct copper dyshomeostasis in either disease though copper depletion in Menkes disease is most challenging. Both diseases are caused by defective copper export from distinct cells, and we seek to give new angles and guidelines to improve treatment of these two complementary diseases. Therapy of Menkes disease with copper-histidine, thiocarbamate, nitrilotriacetate or lipoic acid is discussed. In Wilson disease combination of a hydrophilic chelator e.g. trientine or dimercaptosuccinate with a brain shuttle e.g. thiomolybdate or lipoate, is discussed. New chelating principles for copper removal or delivery are outlined.

1. Introduction

Defects in the cellular copper pumps, ATP7A and ATP7B, lead to Menkes disease (OMIM #309400) and Wilson disease (OMIM #277900), respectively. Both diseases are characterized by accumulation of copper (Cu) in tissues though with disease specific differences leading to distinct and opposite clinical pictures. Menkes disease shows severe copper deficiency while Wilson disease is characterized by copper toxicity. Cerebral symptoms are prominent in both diseases. Characteristics of these two multi-systemic disorders are listed in Table 1.

Low serum copper initially suggested that Menkes disease was a simple copper deficiency syndrome secondary to intestinal malabsorption of copper [1]. However, copper accumulation in non-hepatic tissues pointed to a more intricate explanation for the observed copper deficiency [2–4]. Severe deficiency symptoms, mostly of cerebral origin, are combined with numerous systemic defects. In brain, several important copper-requiring enzymes are deficient because of poor copper uptake [3], and in classic Menkes disease the clinical picture is dominated by neurological symptoms [5,6]. Milder forms with partially functioning ATP7A may primarily have systemic affections dominated by connective tissue symptoms as clearest seen in Occipital Horn Syndrome (OHS) [OMIM #304150] [7]. Some mutations in ATP7A lead to adult-onset motor neuropathy [8] that is not in need of copper replacement therapy.

Deficient ATP7B primarily leads to copper retention in hepatocytes and cerebral tissue, though ATP7B is expressed in almost all the same tissues as ATP7A [Table 1]. Copper leakage from loaded and degenerating hepatocytes to blood is found in a low molecular weight compound that is filtered through glomeruli into urine, but is unavailable for re-uptake by SLC31A1/CTR1 [9]. The labile copper pool (see later) is high and constantly feeding tissues [10]. Special brain regions such as the lenticular nuclei including *putamen* and *globus pallidus* are in particular susceptible, explaining the previously used term, *degeneratio*

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Table 1
Characteristics of Menkes and Wilson diseases.

	Menkes (MD) [OMIM 309400]	Wilson (WD) [OMIM 277900]
Gene	*ATP7A* [*OMIM 300011*]	*ATP7B* [*OMIM 606882*]
	X-linked	Autosomal recessive
	Xq21.1	13q14.3
	> 500 different mutations[a]	> 500 different mutations[a]
	All known mutations but one have a low prevalence	All known mutations but a few have a low prevalence
	Incidence: 1/300,000–1/360,000	Incidence: 1/30.000; endemic in some populations
	Mutation rate: 6.7×10^{-6}/gamete/generation	Mutation rate: unknown
Protein	ATP7A	ATP7B
	Cu ATPase with six Cu MBD's and 8 TM domains	Cu ATPase with six Cu MBD's and 8 TM domains
	Needs ATOX1, Cu, ATP, and Ca for pump activation	Needs ATOX1, Cu, ATP, and Ca for pump activation
	Tissue-specific expression pattern: kidneys, placenta, lung, BBBs, brain, heart, muscle, pancreas, intestine, connective tissue, but not liver	Tissue-specific expression pattern: liver, kidneys, mammary glands, placenta, lung, BBBs, brain, heart, muscle, pancreas, and intestine
	Delivers Cu to AOC, LOX, DBH, PAM, TYR, SOD3, HEPH	Delivers Cu to CP, factors V and VIII
	Exports Cu from cells	Exports Cu from cells
Laboratory markers	Cu accumulation in intestine, kidneys, spleen, pancreas, BBB, lung, placenta. Low Cu in brain, liver	Cu accumulation in liver, brain, kidneys, heart, eyes and other organs
	S-Cu reduced	S-Cu reduced
	Free S-Cu reduced	Free S-Cu increased
	S-CP low - not diagnostic the first months of life	S-CP reduced
	P-catecholamines abnormal	P-catecholamines may be abnormal
	Urinary HVA/VA abnormal	Urinary HVA/VA may be abnormal
	Blood MT1/2 increased	Blood MT1/2 highly increased
	Urinary Cu low to normal	Urinary Cu increased
	Genetic screening	Genetic screening
Clinical features	Multiorgan affection: CNS (seizures, mental retardation), eyes (poor visual acuity, myopia, strabismus, retinal hypopigmentation), lung (emphysema), kidney (urogenital abnormalities like bladder diverticulae), heart (aortic aneurysm), muscle (hypotonia), endocrine organs (hormonal imbalances), bones and joints (deformities, lax and hypermobile joints, osteoporosis), circulation (tortous and fragile blood vessels), skin (scaly, lax, fragile, hypopigmented), hair (coarse, sparse, easily friable, pili torti), early death	Multiorgan affection: CNS (tremor, ataxia, dysartria), eyes (Kayser-Fleischer ring, sun flower cataract), liver (hepatitis, cirrhosis), kidney (hypercalciuria, nephrocalcinosis, nephrolithiasis#, and aminoaciduria), heart (cardiomyopathy, arrhythmias), endocrine organs (gigantism, hypoparathyroidism), bones and joints (osteoarthritis, pathological fractures) and pancreas (pancreatitis), near normal life span
Treatment	Main objective is to deliver Cu to brain through the BBB block. Oral administration is ineffective as Cu is trapped in the intestine. Systemic Cu accumulation especially in kidneys should be followed	Objective is to remove toxic Cu levels using chelators to facilitate Cu excretion and prevent Cu from re-accumulating by restricting Cu uptake from food. Symptoms, in particular neurological, may worsen initially. Cu depletion should be monitored closely to avoid deficiency
	- May extend life span	- Needs to be life-long
	- Early parenteral Cu-HIS before closure of BBB may modify neurologic symptoms and slow disease progression by providing extra Cu to tissues and Cu-dependent enzymes. LOX deficiency persist.	- Chelating drugs (e.g. penicillamine, trientine, tetrathiomolybdate) alone or in combination
	- New drugs are being applied to facilitate BBB and gut delivery. Compounds for Golgi Cu delivery are needed	- Zn induces metallothionein in gut and Cu-thionein is removed when gut epithelium is slaught of
	- Cu therapy in milder MD needs close monitoring to avoid systemic Cu accumulation; important to closely follow connective tissue problems	- Administration may be adjusted in relation to new knowledge about brain clearance
	- Administration may be adjusted in relation to new knowledge about nurture of the brain	- Certain drugs like imipramine may be contraindicated, due to instability of lysosomes; others may be beneficial
	- Functional classification of mutations may facilitate patient specific therapy	- Functional classification of mutations may facilitate patient specific therapy
Ethics	Cu-HIS therapy may ameliorate, but never cure. Palliative care including pain relief is an issue. Neonatal treatment is optimal, though prenatal start in familial cases need thorough discussion. Genetic counselling is important	Treatment is the only decent choice, and younger sibs should be tested. Adverse effects of some neuroleptic and antidepressant drugs should be of concern. Genetic counselling is important in endemic regions

[a] Data from: ATP7A mutation database: https://grenada.lumc.nl/LOVD2/MD/home.php?select_db=ATP7A, and ATP7B mutation database: http://www.wilsondisease.med.ualberta.ca/database.asp.

hepatolenticularis. The same regions also deposit iron. One theory claims that pathological distributions of copper and iron [11] aggravate oxidative damage in the lenticular nuclei and triggers dysfunction of the catecholaminergic system contributing to Parkinson-like symptoms including incoordination and involuntary movements. The process is likely initiated by toxic accumulations of copper in neuromelanins in dopaminergic and noradrenergic neurons [12,13]. Neuromelanins are a subset of melanins that are negatively charged, polymerized pigments that can work as capacitor to absorb energy. The neuropigments bind copper and iron strongly giving names to *substantia nigra* and *locus coeruleus* in *basal ganglia*. In case of copper overload constant electron input may result in outburst of reactive oxygen species (ROS) eventually destroying neurons.

Chelating agents in treatment of Wilson disease promote copper-chelate excretion in either urine or faeces. Chelating agents in Menkes disease promote copper delivery (drugs act as ionophores) to deficient tissues especially the brain, a critical organ of this copper deficiency syndrome. Ionophores mean “ion carriers” as these compounds catalyze ion transport across hydrophobic membranes. Some chelating agents possess ionophoric properties and can traverse biological membranes (e.g. disulfiram), whereas other chelators do not pass across plasma membranes or the blood-brain barrier (e.g. dimercaptosuccinate).

Copper is an essential yet potentially toxic metal needed as cofactor for numerous vital enzymes throughout the body [Table 2]. Copper is absorbed from food in the upper intestine and transported as Cu(II) via portal blood to the liver for regulation of body copper levels [14].

In blood, copper is carried and buffered by a group of molecules with imidazole motifs including histidine. Imidazoles have a high though not specific Cu(II) binding and will attract other metals like Zn (II). About 10% of blood copper bound to albumin (ALB) and alpha 2 macroglobulin (A2M) is easily exchangeable and usually referred to as the labile or “free” copper pool [10]. Copper bound in ceruloplasmin (CP) represents the major pool, but is tightly bound and not easily exchangeable with tissue pools. ALB, A2M, and CP are synthesized in liver. Copper expelled from tissues and organs is excreted via the liver, and only in case of high toxic levels via kidneys [9].

Table 2
Copper-requiring enzymes.

Cofactor group	Enzyme	Function	Menkes disease (MD)	Wilson disease (WD)	Other information
Copper-dependent Ferroxidases Uses ascorbate as cofactor	**Ceruloplasmin (CP) [EC 1.16.3.1]** **Cu loaded by ATP7B**	Cu-dependent oxidase important for oxidation of Fe^{2+} into Fe^{3+}. CP binds Cu tightly and cannot work as a tissue exchange transporter. Cu oxidizing capacity for CP has been suggested. The apoform is unstable and cannot be Cu loaded after biosynthesis.	Lower-than-normal due to hepatic Cu deficiency. Replacement therapy results in normal hepatic production of the holoenzyme.	Lower-than-normal.	Glycosylphosphatidyl-inositol-linked isoform (GPI-CP) attached to cell membranes is the major pool in CNS, leaving the interstitial fluid almost devoid of free CP
	Factor V + VIII **Cu loaded by ATP7B**	Factor V + VIII are clotting factors. Possess similar Cu binding sites as CP and cannot exchange or be Cu loaded after biosynthesis.	Reduced clotting activity due to hepatic Cu deficiency. Replacement therapy results in normal clotting activity.	Reduced clotting activity.	
	Hephaistin (HEPH) [EC 1.16.3.1] **Cu loaded by ATP7A**	The Cu site has a similar architecture as CP and cannot be Cu loaded after biosynthesis. Main role is in the transfer of Fe across membrane barriers like enterocytes and BBB. Cu oxidizing capacity has been suggested.	Copper replacement therapy may have limited effect in MD	Likely no defect	CP and HEPH may potentially substitute for each other
Cytochrom C oxidase (COX) Uses heme as cofactor	**Cytochrom C Oxidase (COX) [EC 1.9.3.1]** **Cu loaded by COX11 and COX17**	COX is the last enzyme in the respiratory electron transport chain located in the inner mitochondrial membrane. Cu insertion after biosynthesis is unlikely. COX is Cu loaded within mitochondria and no defect in Cu chaperoning in either MD or WD exists. In principle Cu can be delivered to the two active centres CuA and CuB	Numerous deficiency signs. Extra copper may activate COX indicated by normalization of brain function and muscle tone. See also lipoic acid activated enzymes	Not known.	
Quinone activated enzymes Contain an internal quinone cofactor	**Lysyl Oxidase Family (LOX) [EC 1.4.3.1]** Cu loaded by ATP7A LOX LOXL1 LOXL2 LOXL3 LOXL4	Important in connective tissue stabilization and substrates containing a collagen-like stretch. Substrates for different enzymes are not yet clearly defined. Comprise lysyl oxidase (LOX) and lysyl oxidase-like (LOXL) enzymes. The cofactor is an internal lysyl-tyrosyl quinone (LTQ) that needs Cu at the right time for biosynthesis. Cu is also required for the enzymatic process itself.	Limited improvement of connective tissue symptoms in MD by parenteral copper supplementation.	Chelation therapy in WD may potentially result in copper depletion of LOX. Defective Complement q1 (Cq1) a substrate, with a collagen stretch, may give rise to autoimmune symptoms like lupus.	
	Copper-containing Amine Oxidases (AOC) [EC 1.4.3.21] Cu loaded by ATP7A AOC1 AOC2 AOC3	AOCs participate in catabolic regulation of body polyamine levels. Histamine (monoamine), putrescine (diamine), spermidine (triamine) and spermine (tetramine) are ubiquitous and essential polyamines involved in cell proliferation, differentiation, inflammation, apoptosis, and modulation of neurotransmitter receptors. Cu has a dual function in catalysis and is required during the biogenesis of the internal TOPA quinone cofactor (TPQ). AOCs are sensitive to semicarbazide indicating that Cu may be exchangeable. Histamine is a neurotransmitter primarily found in hypothalamus. AOC1 regulates histamine, AOC2 has a high retinal expression, and AOC3 play a role in renal and vascular disease.	Abnormal spermidine catabolism may result in skin and hair problems (alopecia of the scalp, eyebrows, and eyelashes) and eye problems (photophobia, blepharitis/ conjunctivitis) typical symptoms in MD. Faulty metabolism of polyamines may participate in persistent diarrhoea.	WD may experience induction and over activation of AOC catabolic enzymes and over stimulation of polyamine functions when treated with the polyamine analog trientine. Polyamines play important roles in regulation of membrane potentials in rapidly dividing and excitable tissues. Chelation therapy may potentially result in copper depletion of AOC and possibly diarrhoea.	The copper containing amine oxidases (AOCs) should not be confused with the flavin containing monoamine oxidases MAO A and B, that work in a copper independent manner to modulate monoamine levels. Regulation of dietary intake of polyamines may be beneficial.
Formylglycine activated enzymes Contain attached FG cofactor	**Formylglycine-generating Enzyme (FGE) [EC 1.8.3.7]** Cu loaded by ATP7A	Sulfatases are activated by FGE (~SUMF1) catalyzed oxidation of a cysteine to formylglycine. Cu activation may occur after biosynthesis [149]. Sulfatases are responsible for degradation and recycling of complex sulfate-containing sugars from mucolipids and mucopolysaccharides.	Deficient sulfatase activity causes symptoms reminiscent of muco-lipidoses and muco-polysaccharidoses giving widespread effects in CNS (leucodystrophy), bone (malformation), and skin (erythroderma). All symptoms observed in MD	Not known.	Sulfatase deficiencies are lysosomal storage disorders and may lead to secondarily disturbed metal handling.
Lipoic acid (LA) activated enzymes Contain attached LA cofactor	**Lipoic Acid (LA) Requiring Enzymes [EC 6.3.1.20]** Cu loaded by ATP7A	LA is a sulfur-containing covalently bound cofactor required for the function of several multi-enzyme complexes located within mitochondrial matrix. The disulfide redox site is during the enzymatic reactions reduced to dihydrolipoic acid (DHLA) that is reoxidized by dihydrolipoamide dehydrogenase [EC 1.8.1.4] by a Cu and flavoprotein (FAD) dependent process	Defects in the lipoylation pathway lead to multiple mitochondrial dysfunctions including hypotonia, respiratory insufficiency, lactic acidosis, neurologic deterioration, severe encephalopathy with seizures,	Not known	The lipoylation is an elaborate, complicated, and energy requiring process that involves several enzymatic steps. Mutations in the biosynthetic genes lead to distinct disease states [151].

(*continued on next page*)

Table 2 (*continued*)

Cofactor group	Enzyme	Function	Menkes disease (MD)	Wilson disease (WD)	Other information
		[59,63]. Initial amidation of an epsilon amino group in conserved lysine residues is essential for attaching the cofactor to the enzyme [150], and this step likely requires Cu similar to epsilon lysyl amidation in LOX cross-linking. LA shows strong Cu-binding.	myopathy, poor growth, and developmental delay [151]. All these symptoms are seen in MD.		
Copper-dependent sulfhydryl oxidase (SOX) Cofactor unclear	**Copper-dependent Sulfhydryl Oxidase (SOX)**	SOXs stabilize protein structure by catalyzing formation of intra- and intermolecular disulfide (S-S) cross-links. SOXs are a very heterogeneous group of enzymes working on a broad variety of proteins using numerous different cofactors [152]. Keratinization requires S-S cross-linking for stabilization of hair, skin and nails. Mitochondrial SOX is involved in iron-sulfur cluster formation, and an apparent link between FGE/SUMF1 and Fe-S biogenesis may be the missing link [153].	Signs of defective keratinization (monilethrix, hair fragility, alopecia, twisted and beaded hair). Copper supplementation can normalize hair structure.	Not known.	
Copper-dependent monooxygenases Uses ascorbate as cofactor.	**Dopamine Beta Hydroxylase (DBH) [EC 1.14.17.1]** Cu loaded by ATP7A	Cu is needed during formation of DBH though likely both Cu and ascorbate can be added afterwards. DBH is expressed in vesicles in noradrenergic nerve terminals of the central and peripheral nervous systems, as well as in chromaffin cells of adrenal medulla. In analogy with PAM trafficking problems of the unloaded enzyme may exist (see below).	Early DBH complications are vomiting, dehydration, hypotension, muscle hypotonia, hypothermia, and hypoglycemia. Brain copper replacement may be beneficial. Orthostatic problems persist in milder MD treated with Cu-HIS. LOX deficiency may aggravate low BP.	Not known.	Imbalance in the dopaminergic system can be measured in blood or urine. Abnormal neurochemical pattern is an early diagnostic marker in MD [119]. Excretion of vanillic/homovanillic acid may prove useful as a non-invasive test in following treatment progress [154].
	Petidyl Alpha-amidating Enzyme (PAM) [EC 1.14.17.3] Cu loaded by ATP7A	PAM is a bifunctional enzyme (peptidylglycine monooxygenase + peptidylamidoglycolate lyase) required for activation of neuropeptides including ACTH, gastrin, cholecystokinin, vasoactive intestinal peptide, corticotropin-releasing hormone, thyrotropin-releasing hormone, calcitonin, oxytocin, vasopressin, and melanocyte-stimulating hormone [119]. PAM is present in secretory vesicles of the pituitary gland. Cu is not needed during biosynthesis and may activate the apoenzyme, but trafficking problems may affect correct vesicular location [119].	PAM deficient mice show CNS problems e.g. impaired vasoconstriction and thermoregulation, increased seizure susceptibility, anxiety, and increased response to noise (Mo^{Br} mice, $PAM^{+/-}$ mice).	Not known.	DBH and PAM share structural similarities. Functionally they are biologically overlapping.
	Tyrosinase (TYR) [EC 1.14.18.1] Cu loaded by ATP7A	TYR catalyzes the rate-limiting steps in mammalian melanogenesis from tyrosine to DOPA and dopaquinone. In skin melanins play a role in protection against harmful effects of UV sun radiation. Complex melanin pigments (neuromelanins) are also found in catecholaminergic neurons in the brain [11]. Synthesis and regulation of neuromelanins is still poorly understood [155].	Depigmented skin and hair is common. Copper replacement therapy leads to darkening of hair and skin.	Increased copper binding in melanosomes and neuromelanosomes may result in oxidative stress burden and irreparable damages of central nuclei.	Melanins are negatively charged, and can absorb energy and dissipate it again e.g. as heat. In case of too high energy absorption, the output can occur as activated chemical species (ROS) that can damage cellular components and result in cell death.
Copper/zinc-containing superoxide dismutases	Superoxide Dismutase 1 (SOD1) [EC 1.15.1.1] Cu loaded by CCS	SOD1 catalyzes dismutation of superoxide radicals (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). SOD1 may be loaded after biosynthesis and can act as a storage protein for copper. Folding and activation of SOD1 occurs through direct interaction with CCS. CCS guides the chaperone-enzyme complex in a Cu dependent manner to the right location in cytosolic vesicles, peroxisomes, and nucleus	There is no apparent defect in the SOD1 copper loading pathway as it uses the copper chaperone CCS, but copper deficiency in brain tissue will result in increased ROS.	Increased brain copper result in increased ROS and SOD1 and SOD3 may not be able to completely dissipate the increased load.	SOD2 in mitochondrial matrix is manganese dependent.
	Extracellular Superoxide Dismutase (EC-SOD/SOD3) [EC 1.15.1.1] Cu loaded by ATP7A	SOD3 also dissipates superoxides. SOD3 shows close structural relationship with SOD1 and in analogy it may be Cu loaded after biosynthesis.	Copper deficiency in brain tissue result in increased ROS secondary to low SOD3, and copper therapy should be beneficial.	See above	The copper donor for post biosynthetic delivery cannot be ATP7A but other molecules in blood and CSF may be potential donors [156].

Within cells, copper delivery for enzyme integration is tightly regulated to secure sufficient metal at the right place at the right time without buildup of toxic levels [15].

Copper crossing of cellular membranes requires specific transporters, and two medically important are ATP7A and ATP7B. These two proteins are homologous membrane bound transporters that actively export copper from cells utilizing ATP to drive the process [16]. Both ATPases are located in the secretory pathway, albeit with tissue specific differences, where they in the Trans Golgi Network (TGN) deliver the metal to copper requiring enzymes or for export.

ATP7A is highly expressed in transport epithelia with a primary role in transfer of copper across the barrier to another tissue compartment e.g. copper crossing gut mucosa to portal blood. Several other cell types express ATP7A. An important function of ATP7A is copper delivery to numerous secreted copper enzymes, and a large pool comprise cross-linking enzymes needed for the extracellular matrix.

An important expression site for the homologous pump, ATP7B is liver cells, where it controls secretion of copper into bile for elimination from the body, and copper loading of the plasma protein ceruloplasmin. Several other organs express ATP7B e.g. brain barriers, kidneys, spleen, heart, lungs, and pancreas [Table 1]. In case of high liver copper, ATP7B is relocated to an excretory lysosomal pool by a copper sensitive motif [14]. In contrast, ATP7A normally integrates in the basolateral membrane.

2. Mutations and treatment potential

Menkes disease (including OHS) and Wilson disease are caused by mutations in *ATP7A* (OMIM #300011) and *ATP7B* (OMIM #606882), respectively.

2.1. ATP7A

De novo mutations in the Menkes gene, *ATP7A* on the X-chromosome (Xq21.1) theoretically accounts for a third of the cases, while two thirds are transmitted through female carriers. The mutation rate is 6.7×10^{-6}/gamete/generation [17,18]. The incidence is low (1/300,000–1/360,000) [18,19] grouping Menkes disease as an ultra-rare orphan disease. > 500 different *ATP7A* mutations have been observed and are unique in most families [20]. However, occurrence of the G727R mutation [21] in several unrelated Menkes patients [22] points to a mutation hot-spot at the gene level. Most mutations cause severe phenotypes, but some lead to a residual pump activity supporting a better although suboptimal loading of copper requiring enzymes [23,24].

Due to considerable clinical heterogeneity, treatment of Menkes disease has been difficult to predict [25]. Early molecular diagnosis and evaluation of genotype-phenotype relationships may help evaluate the prognosis for a newborn Menkes baby and help predict the treatment potential [24,26]. Small amounts of functional ATP7A protein may permit a milder Menkes phenotype [24] and a better treatment prognosis, reflecting almost sufficient loading of copper requiring enzymes [Table 2].

2.2. ATP7B

ATP7B is located on an autosome (13q14.3) requiring two mutated alleles for manifestation of Wilson disease. > 500 different *ATP7A* mutations have been recorded and all known mutations but a few have a low prevalence [27]. The incidence is about 1/30.000 grouping the disease as a rare orphan disease, though founder effect may account for a higher prevalence of single *ATP7B* mutations in certain more isolated areas. H1069Q mutation is more prevalent in Wilson disease patients of European origin while a *ATP7B* promotor mutation is endemic in Sardinia. The prevalence of Wilson disease in China is higher than in Western countries, and Arg778Leu is the most frequent mutation in the East [28].

Partial activity of certain mutant ATP7B may underlie the disparate clinical outcomes observed. The most consistent genotype–phenotype correlation in Wilson disease is that the most severe, early-onset phenotype with predominant hepatic presentation is associated with mutations causing low ATPase activity [28]. Other genetic factors may influence the disease phenotype by affecting ATP7B protein structure and function [28]. Investigation of genotype-phenotype correlation in Wilson disease patients has not led to any clear association between genotype and clinical presentation, although several attempts have been made. In contrast to Menkes disease, where only one mutation is identified in each patient, most Wilson patients are compound heterozygotes for two different mutations, one on each allele. This complicates substantially genotype-phenotype prediction in Wilson disease. A study on a Danish Wilson cohort [29], inspired by a successful genotype-phenotype correlation in patients with phenylalanine hydroxylase deficiency [30] suggested that the least severe ATP7B mutation could be functional dominant. A partial correlation was obtained between severity of the least severe ATP7B mutation and age of onset. However, no correlation was observed between the least severe mutation and the clinical presentation (hepatic versus neurological) [29]. It is reasonable to assume that experience gained from ATP7A e.g. effect of similar missense mutations in a homologous area may predict a similar affection in ATP7B. Alignment similarities have been established for ATP7A and ATP7B, which may help predict the severity of each ATP7B variant. Due to fundamental difference in inheritance, X-linked with a single pathogen variant versus autosomal recessive inheritance with two different pathogenic variants, prediction of clinical outcome is however still difficult.

3. Chelation therapy

Metal chelation refers to how potentially toxic metal ions are shielded to prevent biological damage. Several specific copper chaperones and carriers are natural chelating agents in the body [14]. Within cells copper is found as Cu(I) strongly attached to sulfur groups while extracellular copper is usually bound as Cu(II) to nitrogen groups. Binding constants for intracellular Cu(I) and extracellular Cu(II) differ by a magnitude of about a million [14]. To deliver extra or remove surplus copper, binding principles mimicking biological carriers are ideal to avoid that mobilized copper redistributes or deficiencies arise.

Traditionally chelation therapy refers to removal of excess copper from the body, but a subset of copper chelators has good metal delivering potential and can operate as ionophores. Medical copper chelators ideally form metal complexes that mimic endogenous compounds and use their exchange transporters for cellular influx and efflux (e.g. NTA and trientine mimic polyamines) circumventing the block caused by either copper pump. Copper “hitchhikes” with chelators around the body and across cell membranes.

A major hurdle to effective treatment of CNS disorders is blood-brain-barriers (BBBs) that prevent entry of many drugs into the brain [31]. The barriers secure controlled uptake, distribution and elimination from the brain. The two most significant brain barriers are the blood endothelial barrier (BEB) and the blood-cerebrospinal fluid barrier (BCB). BEB consists of endothelial cells located around all blood vessels in the brain and completely enclose the small vessels. It is a quantitatively important barrier comprising numerous tight junctions between endothelial cells and astroglia. BCB is an internal barrier system that separates copper from brain tissue and ensures vectorial exchange between brain tissue and the cerebrospinal fluid (CSF). Cerebrospinal fluid itself (CSF) has a dual function and serves as a sink and a hub for exchange of waste and nutrition [14].

ATP7A and ATP7B participate in regulation of cerebral copper and are found in the two main barriers, BEB and BCB [13]. ATP7A seems rate-limiting in copper uptake across BEB as well as BCB into the brain and CSF, and ATP7B appears rate-limiting in extrusion at BCB and BEB

[14]. Brain tissue does not contain a proper lymphatic system but extravascular liquid flow is regulated by variations in pressure secondary to circadian rhythms. Brain clearance is highest at low pressure during night, and the brain is more permeable at night [32].

3.1. Treatment of Menkes disease

Due to blocks in all transcellular transport tissues, Menkes disease can be very hard to treat and outcome is often poor. Copper needs delivery after the block (e.g. parenteral administration to circumvent the gut block) and the blood labile copper pool should be saturated to allow constant availability to cells. An example of positive balance is in utero Menkes disease where constant copper supply is sufficient to bring the affected baby-boy to birth [2]. Transport into brain is compromised, and once copper is delivered intracellularly, loading of enzymes is still an obstacle. Lack of copper may compromise trafficking of copper-requiring enzymes, e.g. peptidyl alpha amidating enzyme (PAM) [Table 2]. Several enzymes and enzyme complexes require copper during biosynthesis of an internal cofactor that cannot be formed afterwards. Quinone requiring lysyl oxidases (LOX, LOXL) [33] and amine oxidases (AOC) are examples explaining prevalence of connective tissue problems and widespread excitability of CNS. Activation of lysosomal sulfatases has recently been demonstrated to be dependent on copper catalyzed formation of the cofactor formylglycine [34]. Lipoic acid appears to require copper for activation of numerous mitochondrial enzyme complexes (see Section 3.1.4).

Copper build up in several tissues of untreated Menkes disease and OHS patients and total body copper content is near normal [2,4]. Copper treatment may lead to toxic accumulations in kidneys, and clearance of copper may be of concern [3,35].

3.1.1. Copper-histidine

Copper-histidine (Copper(II)–Bis(L-histidinato) complex) [IUPAC (2*S*)-2-amino-3-(1H-imidazol-5-yl)propanoic acid)] (Fig. 1) exchanges copper easily with other imidazole compounds and is a natural source for extra copper in circulation and the only clinically available treatment option for Menkes disease (NIH trial programme) [26,36].

Chemical information about the Copper(II)–Bis(L-histidinato) complex is reported in SI 1. Copper-histidine needs to be given parenterally, but will not pass BBB as a complex. Copper-histidine delivers the metal to receptors on cell surfaces, and as a block occurs within cells extra copper may not be sufficient to correct the brain block [23,26,37] and activate enzymes. The exchangeable blood copper pool needs to be saturated to secure sufficient supply to the brain.

Use of copper-histidine is based on the discovery that this biological complex is a natural transport form in human blood [38,39]. The first Menkes patient to benefit was a Canadian patient who started a promising treatment in 1976. Later several sporadic case stories have been published [40–42] and long-term use has been followed in a few cases [35,41,43–45]. Currently clinical outcomes are being evaluated at NIH [36] for a cohort of both classical and milder Menkes patients defining necessary molecular genetic requirements for successful treatment. Other groups have reported on treatment with copper-histidine [39,44]. Copper-histidine therapy ameliorates many Menkes disease symptoms while others are refractory. Although the amount of functional ATP7A is an important factor for success, the outcome is highly dependent on early treatment [24,44], and delay may result in irreparable developmental changes. Successful outcome is observed in about 20% of Menkes disease patients [46].

A need for copper compounds that easily can transverse BBB is obvious.

It should be kept in mind that all copper delivering drugs potentially give rise to ROS. This is the sought for feature in cancer treatment and a reason why copper modulating drugs have been given such tremendous interest the last two decades.

3.1.2. Dithiocarbamate derivatives

Dithiocarbamates belong to a class of sulfur-containing bidentate chelating agents with a well-known ability to complex a range of transition metal ions including Cu(II) [47].

A) Disulfiram and Copper Diethyldithiocarbamate

Disulfiram (Antabuse; tetraethylthiuram disulfide) (DEDTC) [IUPAC diethylcarbamothioylsulfanyl *N*,*N*-diethylcarbamodithioate] is metabolized by glutathione reductase to diethyldithiocarbamate (DETC) [IUPAC *N*,*N*-diethylcarbamodithioate], another potent copper chelating agent [48], and copper-chelating activity of disulfiram is likely due to diethyldithiocarbamate (Fig. 2). Chemical information about the Copper(II)–diethyldithiocarbamate complex is reported in SI 2.

The Cu-DETC complex is more acid-stable, neutral and hydrophobic than disulfiram facilitating absorption into bloodstream and brain. Disulfiram [Table 3] has shown a treatment potential in Menkes disease [49,50].

B) Thiuram and Copper Dimethyldithiocarbamate

The simplest dithiocarbamate, dimethyldithiocarbamate [IUPAC *N*,*N*-dimethylcarbamo-dithioate] and its oxidized dimer thiuram (DMDTC) (tetramethylthiuram disulfide) [IUPAC dimethylcarbamothioylsulfanyl *N*,*N*-dimethylcarbamo-dithioate] can potentially deliver copper to intracellular sites (Fig. 2). Chemical information about the Copper(II)–dimethyldithiocarbamate complex is reported in SI 3. Prenatal treatment with thiuram in combination with copper of mosaic mice, a model for Menkes disease, has shown increase in brain copper and reduction in kidney copper, leading to normalization in these tissues. It was concluded that Cu-DMDTC should be considered as treatment for Menkes patients [51]. Of interest here is also the recent studies using oral thiosemicarbazone derivatives to deliver copper to brain in a mouse model of Menkes disease, since oral administration of these copper complexes extended the lifespan of the mice [52]. Chemical information on these complexes is reported in SI 4. These thiosemicarbazone complexes can deliver the metal across the blood brain barrier and into the intracellular space, and appear promising also

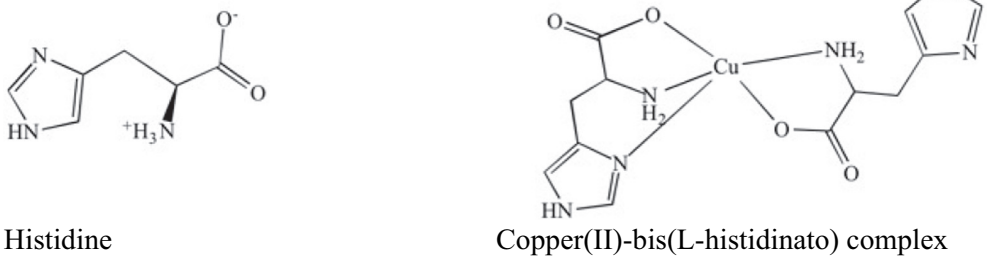


Fig. 1. Formulas of histidine and of Cu(II)-bis(L-histidinato) complex (X-ray structure in SI 1).

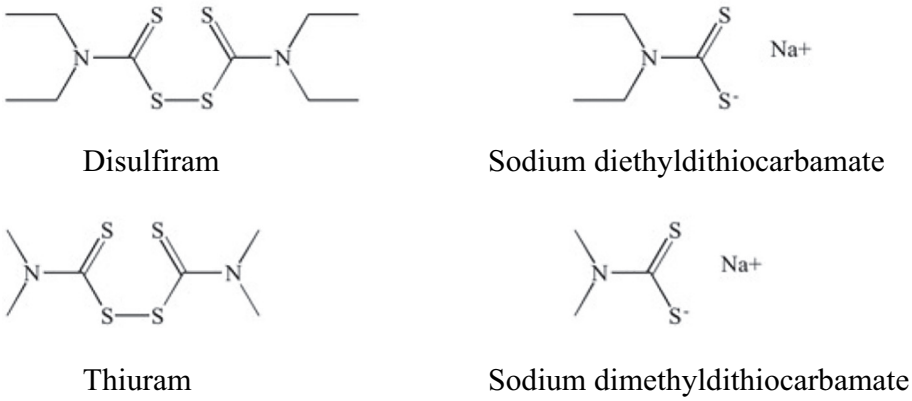


Fig. 2. Formulas of disulfiram, sodium diethyldithiocarbamate, thiuram and sodium dimethyldithiocarbamate.

when treating animal models of Alzheimer and other neurodegenerative diseases [53]. However, to the knowledge of the authors, clinical evaluation of the copper-thiocarbamate complexes as well as of the copper-thiosemicarbazone remains to be done.

3.1.3. Cu/Zn NITRILOTRIACETATE

Nitrilotriacetic acid (NTA) [IUPAC 2-[bis(carboxymethyl)amino] acetic acid)] is a polyamine carboxylate derivative of acetic acid [54] (Fig. 3).

It will chelate Cr^{2+}, Ca^{2+}, Cu^{2+} and Fe^{3+} and form stable complexes with Zn^{2+}. NTA is chemically related to EDTA but has less chelating ability, and has the advantage of being easily biodegradable and rapidly catabolized in the body [55]. Chemical information about the Copper(II)–nitrilotriacetic acid complexes is reported in SI 5. Cu-NTA is absorbed very efficiently by the intestinal tract, taken up by tissues and pass BBB. Zinc is bound tighter than copper and may be eliminated mainly bound to nitrilotriacetic acid [55,58]. Therefore, the drug is usually administered as Cu/Zn-NTA [56].

Nitrilotriacetate is commercially available as free acid and as sodium salt, and it has been used for treatment of Menkes disease on an experimental basis using a magistral ordination. It was originally in 1980ties suggested as a suitable copper source for treatment of copper deficiency syndromes [57] but received little attention [55], likely, because the International Agency for Research on Cancer (IARC) required it labeled as a possibly carcinogenic substance [54]. Although this label may be questioned, as the carcinogenicity for copper ionophores depends on dose and clinical use, no drug approval exists as yet, neither in Europe nor in United States for NTA.

3.1.4. Lipoic acid

Alpha-lipoic acid (LA) (thioctic acid or 1,2-dithiolane-3-pentanoic acid) [IUPAC (*R*)-5-(1,2-dithiolan-3-yl) pentanoic acid] is a compound with a high copper delivery potential (Fig. 4).

The redox couple lipoic acid/dihydrolipoic acid is a biologically important vitamin-like cofactor for numerous mitochondrial enzymes, e.g. the pyruvate dehydrogenase complex [59,60]. Lipoic acid is synthesized in the mitochondrial matrix, and covalently attached to several enzyme complexes. It has profound antioxidant activity due to high binding of copper [61,62]. Possibly copper is a natural cofactor when linking lipoic acid to enzymes through an epsilon amino group of a lysyl residue, and reconstitution of the reduced form to the oxidized form also appears as a copper dependent enzyme reaction [63]. Chemical information on the Copper(II) complexes with lipoic and dihydrolipoic acids is reported in SI 6.

Lipoic acid is both water and fat soluble, readily taken up by SLC5A6 [64] and transported to various tissues including brain [65]. Lipoic acid promotes absorption of copper across the intestinal mucosa. Elimination occurs through biliary fatty acid degradation. Lipoic acid and its reduced form has a binding constant compatible with intracellular copper chaperones [66] and has been suggested as useful in promoting excretion of surplus copper in Wilson disease [66–68]. Clinical use in mercury toxicity shows risk of redistribution of the metal [67]. Analogously, lipoic acid will likely be useful in redistributing copper in Menkes disease. Several symptoms related to dysfunctional mitochondria are present in Menkes patients including deficiency of lipoic acid requiring enzymes [69]. Extra cofactor may theoretically improve enzymatic functions and may prove worth as supplementary treatment.

Lipoic acid is commercially available as a racemic form and has been used as dietary antioxidant supplement for > 50 years [61]. In humans, a number of clinical trials have assessed adverse health effects and lipoic acid shows a promising safety profile [65]. Lipoic acid is in Germany available by prescription to treat diabetic neuropathy and in many countries as an oral over-the-counter nutritional supplement. It can be administered parenterally and orally as liquid or tablets. Due to its strong copper chelation capability a copper salt should be added simultaneously. A suitable source is copper gluconate that is an orally bioavailable copper salt with fewer emetic side effects. A need for new Cu-lipoic acid formulations exists, and the use of the racemic form may need to be revised.

3.2. Treatment of Wilson disease

Wilson disease shows a much better prognosis following chelation therapy. The rationale is to minimize toxic symptoms of copper by limiting copper uptake from food and regulate copper content in the body using chelator-facilitated excretion through urine or bile.

Wilson disease gives rise to hepatic symptoms, usually before cerebral affection with basal ganglia symptoms. At initial stages, copper accumulates in the liver due to defective biliary copper excretion. At later stages, the saturated free fraction of copper (non-ceruloplasmin bound) may lead to depositions in brain and other tissues [70]. An important therapeutic challenge is adequate clearance of brain copper.

3.2.1. Zinc salts

High turnover of gut epithelia provides an attractive treatment option in Wilson disease. Zinc supplements induce transcription of the efficient chelator, metallothionein (MT), and subsequently zinc is displaced by copper due to its much higher avidity, and copper is lost in faeces when enterocytes are shed during physiological cell turnover. High oral zinc prevents efficiently copper uptake from food, and reduced absorption and faecal loss gradually result in a negative copper balance. In addition, zinc induced hepatic MT synthesis reduces copper damage on hepatocytes. Dyspepsia may be an annoying side effect, but choosing appropriate zinc salts may alleviate symptoms [71].

Zinc as monotherapy or combined with a traditional chelator, has

Table 3
Compounds used to deliver or remove copper in Menkes and Wilson diseases.

Compound	Disease	Administration and mode of action	Elimination and biological fate	Toxicity	Essential metal binding
Cu-histidine	Menkes	Parenteral; natural biological Cu carrier in blood; delivers Cu to SLC31A1 at the cell surface	Metabolized; copper through bile	No toxic effects; Cu accumulation in MD kidney may cause nephrotoxicity	Cu^{2+}
Disulfiram (DEDTC)	Menkes	Peroral; absorbed slowly probably due to decomposition; pass BBB	Rapidly catabolized to DETC urine	Generally well-tolerated; produce CS_2 in vivo that is neurotoxic; inhibits proteasome activity	Cu^{2+}, Zn^{2+}, Fe^{3+}
Diethyldithiocarbamate (DETC)	Menkes	Peroral; pass BBB	Urine	Neurotoxicity; produce CS_2 in vivo	$Cu^{2+} > Zn^{2+}$, Fe^{3+}
Thiuram (DMDTC)	Menkes	Peroral; pass BBB	Rapidly catabolized to DMTC urine	Generally well-tolerated; produce neurotoxic CS_2 in vivo	$Cu^{2+} > Zn^{2+}$, Fe^{3+}
Dimethyldithiocarbamate (DMTC)	Menkes	Peroral; pass BBB	Urine	Neurotoxicity; produce CS_2 in vivo	Cu^{2+}, Zn^{2+}, Fe^{3+}
Nitrilotriacetate (NTA)	Menkes	Peroral via SLC-transporter; pass BBB	Urine polyamine structure easily and rapidly degradable by AOC, possibly acetylated	Generally well-tolerated as Cu salt; Zn forms stable complex	Zn^{2+},Cu^{2+}, Fe^{3+}, Ca^{2+}
α-Lipoic acid (LA)/dihydro lipoic acid (DHLA) redox pair	Menkes, Wilson	Peroral via SLC5A6; natural biological compound; easy absorbable; pass BBB	Lipoic acid itself is rapidly eliminated by biliary fatty acid degradation; mobilizes Cu stores	Well tolerated low toxicity	Cu^{+}, Fe^{2+}, Zn^{2+}
D-Penicillamine (DPA)	Wilson	Peroral via SLCO1B1; rapid but incomplete uptake pass BBB	Excretion mainly renal and mainly as disulfides; forms complexes with cystine and prevent kidney stones	Numerous adverse effects including neuro- and hepatic toxicity; and nephrotoxicity. L-form shows strong inhibition of pyridoxine	Cu^{2+}/Cu^{+}, Fe^{3+}, Zn^{2+}
Trientine (TETA)	Wilson	Peroral via SLC3A2; low oral bioavailability of 8–30%; reduce Cu absorption; gastrointestinal side effects	Urine; rapid clearance; metabolized mainly via acetylation and AOC	Low liver toxicity; may interact with polyamine metabolism; telomerase inhibition	$Cu^{2+} > Zn^{2+}$
Ammonium tetrathio molybdate (ATTM)	Wilson	Peroral; reduce Cu absorption; pass BBB forms stable complexes with proteins	Cu-chelate-ALB complexes are glutathionylated and excreted via bile: mobilizes MT Cu stores	Generally well-tolerated; if GSH is depleted Cu will be released and cause liver toxicity; raised S-aminotransferase activities may occur;	Cu^{2+}
Bis-choline-tetrathio molybdate (BCTTM)	Wilson	Peroral; reduce Cu absorption; pass BBB forms stable complexes with proteins	Cu-chelate-ALB complexes are glutathionylated and excreted via bile; mobilizes MT Cu stores	Generally well-tolerated; if GSH is depleted Cu will be released and cause liver toxicity; hepatic overload of complex may redistribute Cu to the brain	Cu^{2+}
Dimercaptosuccinate (DMSA)	Wilson	Peroral via SLC3A3; about 60% of the drug is absorbed; does not pass BBB	Urine copper complex cannot use SLC3A3	Low toxicity	Cu^{2+}, Cu^{+}, Zn^{2+}
Dimercaptopropane sulphonate (DMPS)	Wilson	Peroral via SLC3A3 or parenteral	Urine copper complex cannot use SLC3A3	Low toxicity	Cu^{2+}, Cu^{+}

Drug information: PubChem Compound: www.ncbi.nlm.nih.gov/pccompound; DrugBank Database: www.drugbank.ca/; www.drugs.com/; Drug and drug target database: www.uniprot.org/database/.

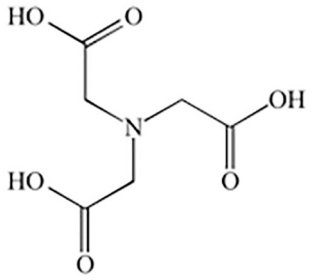


Fig. 3. Formula of nitrilotriacetic acid.

been studied both in US and Europe. In particular, zinc is considered applicable as maintenance therapy after an initial chelator treatment period [72], and has proved effective after treatment with trientine [73]. Furthermore, zinc supplementation has been effective in mild liver disease [74], though zinc monotherapy may not be sufficient in patients with more severe liver or cerebral disease [75]. Pre-symptomatic Wilson patients may successfully be managed by zinc monotherapy [76]. To help compliance it is important to choose a zinc salt with low risk of emetic problems, e.g. acetate, citrate or gluconate.

3.2.2. D-Penicillamine

D-Penicillamine (DPA) (Cuprimine; β,β-dimethylcysteine) [IUPAC (2*S*)-2-amino-3-methyl-3-sulfanylbutanoic acid] was introduced in the racemic form (PA) for treatment of Wilson disease by the classical work of John Walshe [77]. Penicillamine is a dimethylated cysteine with the thiol-group surrounded by two bulky methyl groups, making the molecule more resistant to in vivo interactions (Fig. 5).

D-penicillamine has less side reactions than the L-form and is the currently used therapeutic form [78,79]. The primary distribution volume of penicillamine is the extracellular space, though membrane transfer of the chelator and its Cu-chelates may occur via SLCO1B1 [80].

A mixed valence copper-penicillamine complex is primarily formed in blood plasma through chelation of the free fraction of copper [81]. This chelate complex is presumably responsible for urinary elimination [82]. Chemical information on this complex is reported in SI 7.

Unfortunately, penicillamine may give rise to serious side effects (see Section 3.4). To avoid early side effects, including worsening of neurological symptoms and copper redistribution to brain, a low initial dose during the first week has been recommended. The drug is best taken 1 h before or 2 h after food. Absorption might be reduced 50% if taken with a meal.

Close monitoring of blood count, liver function tests and urinary protein is recommended, because of possible adverse effects occurring in about 20% of the patients, and these can lead to treatment being stopped. In absence of adverse effects, the dose may be increased after the first week [83]. The initial chelation induced 24-h cupruresis is over 2 mg.

The clinical benefits of penicillamine in Wilson disease are well documented, but adverse effects may require changed antidotal regimen. In recent years other agents, in particular trientine, has been recommended as alternative drug [83].

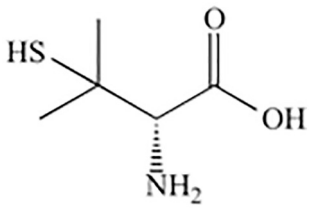


Fig. 5. Formula of D-penicillamine.

3.2.3. Trientine

Trientine or triethylene tetramine (TETA) [*N′*-[2-(2-aminoethylamino)ethyl] ethane-1,2-diamine;hydrochloride] was early used as an alternative chelator in patients with poor penicillamine tolerance [84]. As evidence has grown for the effectiveness of trientine, with apparently fewer side effects than penicillamine, several authors have regarded it as a first-line drug for treatment of Wilson disease [85,86].

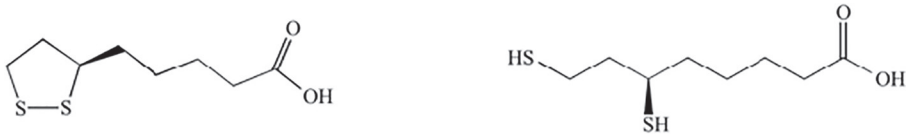
Trientine has a polyamine structure similar to biological amines (Fig. 6) and chelates copper by formation of stable complexes with four nitrogen atoms in a planar ring [Table 3] (see SI 8). Trientine uses natural polyamine transporters, however, without metal the drug is unstable and exhibits poor oral absorption with bioavailability of 8–30% [87] and requires up to four-times-a-day dosing. Timing of oral administration in relation to food is important. Trientine is distributed mainly in the extracellular phase, binds free copper in blood plasma, and may use the polyamine transporter SLC3A2 for membrane transfer. The formed chelate is excreted in urine, with only a minor fraction in faeces [88].

Neurological deterioration can still occur, though apparently not as frequently as with penicillamine [89]. Efficacy and side effects in patients with severe liver disease have been reported in several clinical trials. Askari et al. [73] studied nine adults with severe liver disease identified over a 10-year period, who received initial treatment with trientine plus zinc. Only one patient developed hepatic encephalopathy. One patient developed mild neurological symptoms and was switched to ammonium tetrathiomolybdate plus zinc already after 2 weeks on trientine plus zinc. In eight patients trientine and zinc was given for up to 4 months after which the regimen was changed to maintenance with zinc monotherapy. Over the first 12 months of treatment, prothrombin time as well as bilirubin and albumin concentrations returned to normal, and ascites disappeared. Treatment was maintained over 12 months to 14 years of follow-up with encouraging benefits [73].

3.2.4. The tetrathiomolybdates: ammonium tetrathiomolybdate and bis-choline tetrathiomolybdate

Ammonium tetrathiomolybdate (ATTM) ($[NH_4]_2MoS_4$) [IUPAC diazanium;bis(sulfanylidene)molybdenum; sulfanide] [Table 3] (Fig. 7), is suggested as a third alternative copper chelating drug [90] because it facilitates a different copper excretion mechanism, viz. via bile. Thiomolybdate compounds were first used for treatment and prevention of copper poisoning in sheep [91]. The ammonium drug has been under investigation in US, predominantly for treatment of patients with neurological Wilson disease [92]. Recently a promising new derivative, bis-choline-tetrathiomolybdate (BCTTM) [IUPAC bis(sulfanylidene)molybdenum;2-hydroxyethyl(trimethyl)azanium; sulfanide] (Fig. 7), has been applied for fast-track approval [93].

Taken together with meals, both derivatives will form complexes



Alpha-lipoic acid Dihydrolipoic acid

Fig. 4. Formulas of alpha lipoic acid and dihydrolipoic acid.

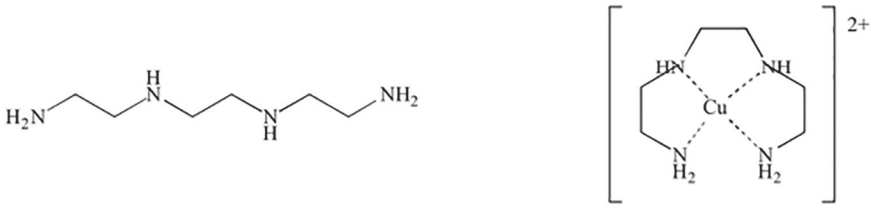


Fig. 6. Formula of trientine and of its Cu(II) complex.

with copper in food, preventing its absorption. Taken between meals, the drugs are to a significant extent absorbed, and bind circulating copper together with albumin in a tripartite complex, which is taken up by the liver for biliary excretion of the copper complex [94,96]. A randomized trial [89] compared the efficacy of ammonium tetrathiomolybdate with trientine in patients with neurological Wilson disease (both groups received zinc). In the ammonium tetrathiomolybdate group, one of 27 patients had neurological deterioration, compared with five of 27 patients in the trientine group. Anemia or leukopenia occurred in three patients in the ammonium tetrathiomolybdate group, and four patients got increased circulating levels of liver enzymes. Despite promising results as initial treatment of neuropsychiatric Wilson disease, the tetrathiomolybdates are still classified as investigational drugs in United States as well as in Europe, firstly because the ammonium formulation has proven unstable for routine clinical use, and secondly due to limited clinical experience with the bis-choline derivative.

3.2.5. Dimercaptosuccinic acid and dimercaptopropane sulfonic acid

In China, there is substantial experience with dimercaptosuccinic acid (DMSA) [2,3-bis[(2-amino-2-carboxyethyl) sulfanyl]butanedioic acid] [97–102] (Fig. 8), although this compound appears not to be sufficiently evaluated in Wilson patients in the Western world. Comparing long-term therapeutic effects in clinical trials, DMSA and penicillamine appear to have comparable therapeutic efficacies, and DMSA was reported to be superior to penicillamine due to lower side effect incidence [103]. The DMSA molecule has a dithiol structure [Table 3] implicating that it can bind not only Cu(II) but it can form stable chelates with the more toxic Cu(I). Unmetalated DMSA use SLC3A3 for uptake, but metalated DMSA cannot use the transporter and is excreted in urine [104]. DMSA does not easily pass BBB.

The related dithiol, sodium dimercaptopropane sulfonic acid (DMPS) [2,3-Bis(sulfanyl)propane-1-sulfonic acid] (Fig. 8), has low toxicity and can be given orally or intravenously, but appears to have little effect on severe Wilson disease [105].

3.3. Combination therapy

Although increased delivery of copper to the brain is of utmost importance in Menkes disease, a primary aim of combination therapy in this disease has been mobilization of copper accumulated in tissues to limit copper induced nephrotoxicity [106,107]. In this respect, penicillamine is the only drug examined [106], but other suitable chelating drugs like DMSA should be exploited in combination with copper-histidine.

Combination therapy is an important issue in Wilson disease [79] as

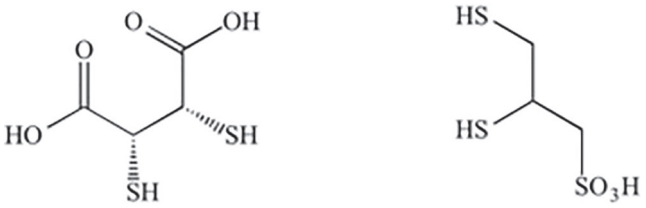


Fig. 8. Formulas of DMSA and DMPS.

it may keep the dose of each drug below the limit of adverse effects. The potential of combination therapy has not been fully explored. Combination of a zinc salt (e.g. zinc gluconate) with penicillamine is a recommended treatment in the initial phase today. In the maintenance phase, monotherapy with zinc may be sufficient.

Zinc is administrated orally to induce metallothionein (MT) in gut epithelium and hereby limit uptake of copper. MT is an important and very strong endogenous chelator ubiquitously expressed in humans. Besides MT is one of the most potent antioxidant systems in the body.

Therapy using tetrathiomolybdate and DMSA, given at low doses, might represent a promising new combination treatment of Wilson disease. Tetrathiomolybdate passes cellular membranes by a glutathione mediated carrier process and hereby functions as a brain-to-blood shuttle for brain copper mobilization [108], while the extracellularly distributed DMSA should promote urinary excretion [109]. However, this combination has not been evaluated clinically.

3.4. Side effects

Most chelators applied are not specific for copper, and may bind other metals some even with a higher avidity. Trientine presumably forms complexes with iron and may thus cause side effects like sideroblastic anemia. Zinc complexation may affect alkaline phosphatases and other zinc enzymes e.g. alcohol dehydrogenase [110].

Severe side effects have been observed in Wilson disease in relation to chelation therapy [111]. Besides neurological worsening, immunological side effects may result from penicillamine being bound to proteins or from inhibition of complement 1q secondary to deficient copper activation [112]. Among reported side effects with presumed autoimmune etiology are thrombocytopenia, granulocytopenia, pemphigus, nephrotic syndrome, and lupus-like syndromes [113–116]. Recurrent subcutaneous bleeding, dermatopathy, elastosis perforans serpiginosa, aphthous stomatitis, and undifferentiated connective tissue

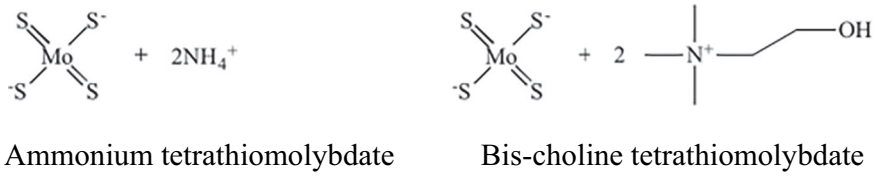


Fig. 7. Formulas of ammonium tetrathiomolybdate and bis-choline-tetrathiomolybdate.

disease may be attributed to drug-induced LOX deficiency [117]. The lysyl oxidase family is involved in cross-linking of elastin and collagen including the collagen stretch of complement 1q and is particularly vulnerable to lack of copper [118]. Copper containing amine oxidases (AOCs) are highly dependent on adequate copper. AOCs participate together with numerous other molecules in the polyamine pathways to precisely regulate the content of biogenic amines in CNS and periphery. Among functions regulated are wakefulness, inflammation, and neurotransmitter release [Table 2].

Side effects are not confined to penicillamine but may occur during therapy with trientine and thiomolybdates, which may potentially access intracellular sites by a carrier mediated process [Table 3] and remove copper and limit copper loading of vulnerable enzymes. Bone marrow depression, with resultant anemia and leukopenia, is presumably secondary to bone marrow copper depletion. Thiomolybdates showing risk of redistribution of copper may potentially precipitate CNS worsening if used too aggressively.

3.5. Biomarkers

Clinicians require valid biomarkers to guide therapy. Most commonly, copper is analysed in urine, whole blood and plasma, but an urgent need for more specific markers exists.

During treatment of Menkes disease, copper levels in serum and urine as well as ceruloplasmin levels should be monitored regularly, and should ideally be within normal range. Imbalance in the dopaminergic system can be measured in blood or urine, and an abnormal neurochemical pattern has been demonstrated to be an early diagnostic marker in Menkes disease [119]. The homovanillic-to-vanillic acid ratio in urine may be more convenient for monitoring in a clinical setting [120].

Menkes patients show low to normal copper urinary excretion. Most patients with Wilson disease have increased urinary copper [121,122], but values increase substantially during treatment with chelators.

After an initial three-month-period of copper chelation in Wilson disease, liver function tests including prothrombin time, bilirubin and albumin determinations are supplemented with determination of free copper in serum. This is measured as the difference between total S-Cu and CP-Cu, and since Menkes and Wilson patients have low CP-Cu, their total blood P-Cu will be low, and far below the physiological interval and difficult to evaluate correctly. Recently, a method to reliably deduce the exchangeable S-Cu fraction has been introduced [123–125].

A need for valid biomarkers for specific enzymes like LOX [126,127] is obviously needed in Menkes disease as well as in Wilson disease. Measurement of erythrocyte copper may serve to give an estimate of tissue copper storage in Cu/Zn-SOD enzymes [128]. Vesicular MT has been demonstrated in blood and could potentially provide information on copper overload in both Wilson disease and Menkes disease. Glutathione is involved in cellular copper buffering as well as in metal transport and excretion and may be a potent biomarker for copper overload.

Chelators applied in Menkes disease and Wilson disease may bind other metals and some with a higher avidity than copper. Information on the complexes formed by these chelators with trivalent iron and with zinc is reported in the SI. It is important to add markers for the most common, such as zinc and iron.

3.6. Ethical issues

Menkes disease and Wilson disease are designated 'orphan' diseases; they are both inborn errors of copper metabolism caused by defects in similar genes encoding energy requiring pumps involved in cellular and whole body copper homeostasis. Orphan diseases afflict a relatively small number of patients, ultra-rare or very rare diseases afflict < 1:50.000. By this definition Wilson disease is rare and Menkes disease is ultra-rare. So why develop treatment options for these rare diseases? However, if we can ameliorate the worst effects of living with a devastating condition and provide patients and their families with appropriate care and support at the same time gain new insight about copper homeostasis, the intervention may prove worth a lot of effort. Besides these diseases are of high impact in affected families given their inherited nature. Rare diseases like Menkes disease and Wilson disease are found in the cross-road of key metabolic pathways and may provide new knowledge about disturbed metal homeostasis found in more common neurodegenerative diseases like Alzheimer and Parkinson, as well as throwing light on particular challenges encountered in cancer therapy [129]. Orphan disease treatment options focus on management of disease symptoms as the underlying genetic defect will persist and therapies can rarely be curative. A range of medical and therapeutic issues is important such as treatment of symptoms resulting from a chronic and likely progressive course. In the majority of Menkes disease cases prognosis is poor, and for both diseases late diagnosis is a problem making treatment and rehabilitation delayed, difficult and less effective. Persistent disabilities create additional problems needing to be addressed.

Treatment for Menkes disease is primarily symptomatic and supportive. Presently only about 20% have true benefit from copper-histidine treatment. However, in rare patients with milder disease mutations and residual enzyme activity, early treatment may ameliorate symptoms substantially and slow down disease progression significantly. Treatment needs to be initiated before outcome prognosis is available, as delayed treatment is considerably less effective.

Wilson disease is fatal if left untreated and early recognition, prompt diagnosis and treatment are important. Liver transplant is lifesaving for WD patients with advanced liver disease refractory to medical therapy.

Younger sibs should be tested, and carriers in the family may be counselled before marriage and partners offered gene testing.

In Menkes disease with progressive neurodegeneration despite treatment, palliative care is an important issue. Copper chelation or ionophoric therapy may in no way be curative but could still alleviate pain and seizures. An antenatal treatment program for Menkes disease should be thoroughly discussed with parents [130] as accumulation of copper on one side of the placenta may limit the effectiveness of any in-utero treatment.

Families facing rare diseases may be willing to accept risks that are on the edge, if good and respectful counselling is lacking [131]. We need to be careful about inflicting false hope. Prenatal diagnosis for prevention of birth of affected boys may be another more appealing choice.

In both diseases, treatment needs to be life-long and may even in well treated patients still result in reduced life-span and require persistent treatment and life support. An emerging option is development of modern genetic techniques for evaluation of treatment efficacy before initiation. This could result in a more realistic evaluation of treatment benefits and provide a better ethical responsibility in counselling before decision making.

4. Discussion and conclusions

Traditionally, chelation therapy has been administered to obtain elimination of toxic metals from a system. In contrast, ionophores are applied for tissue copper delivery. In principle, both chelator types can remove or deliver copper to tissues and the grouping is somewhat arbitrary. In theory, chelators can be used for both purposes, but may be more suitable for one or the other due to metabolic fate or stability. Hydrophilic compounds are primarily mobilizing agents with a removal potential by rapid clearance through kidneys, while some thiophilic complexes facilitates intracellular copper exchange. However, it is a misconception that lipophilic agents traverse biological membranes more rapidly than hydrophilic. Biological access to cells is dependent on structural similarity with natural substrates and the use of

endogenous transport systems. Transporters used by copper chelators are currently identified [Table 3] [64,104,132].

Cancer therapy has shown great interest in removal of copper to limit angiogenesis, and for delivery of extra copper to enhance ROS production. So the two faces of copper therapy in these two inherited disorders, Menkes disease and Wilson disease have benefited from the cancer research field and vice versa. However, cancer therapy usually takes place within a limited time span; in contrast, treatment of inborn errors of copper metabolism is life-long. The latter treatment regimens should therefore aim to avoid depletion of copper from vital enzymes and to limit ROS production.

Better and more specific monitoring of efficient and sufficient treatment without development of copper deficiency in Wilson disease and toxic symptoms (nephrotoxicity) in Menkes disease is highly needed. It should be kept in mind that many chelators applied for copper chelation may bind other metals. For instance, chelator induced zinc disturbance may affect alkaline phosphatases and other zinc enzymes.

Chirality may prove important in drugs as enantiomers may act biologically very differently and this may apply to other drugs than penicillamine [78], DMSA [109] and lipoate [133]. The racemic form of lipoic acid appears safe, but the naturally occurring enantiomer may be more efficient in delivering copper, while probably less important when used for removing copper.

Crucial therapeutic principles, which are valid for Menkes and Wilson diseases, can be summarized as follows:

(1) Menkes disease and Wilson disease patients must ideally be identified early and copper therapy started before brain damage has occurred.
(2) Doses and circulating chelator levels must be controlled to enhance tissue copper delivery or removal.
(3) Copper must be made available within cells for cuproenzyme biosynthesis in Menkes disease, and should not be depleted from vital cuproenzymes in Wilson disease.
(4) Control of blood and tissue copper pools as well as copper enzyme activities is needed and new markers should be searched for.

Patients on chelation therapy need a close clinical follow-up and neurological assessment plus monitoring of kidney (Menkes) and liver (Wilson) function. During chelation therapy, labile S-Cu is estimated from total copper minus CP-Cu which is a rather crude measure. Newly a method to rapidly deduce the exchangeable S-Cu fraction has given hopes of a more reliable blood copper status [10,124,125]. New markers may be needed, e.g. monitoring of enzyme functions of LOX [126,127] and AOC. Collection of genetic information is highly requested in prospective clinical studies to establish valid genotype-phenotype information, and could probably be done retrospectively in some trials.

At present, parenterally given copper-histidine is the only clinically approved therapy for Menkes disease.

In Wilson disease, therapy is usually initiated with D-penicillamine or trientine, often followed by maintenance therapy with trientine or a zinc salt. However, there are still no controlled trials that have directly compared these agents and thus, recommendations are based mainly on observational data and clinical experience. Adverse effects of penicillamine are so common as to be therapy-limiting in several cases. This drug causes hypersensitivity reactions in > 25% of patients, in addition to proteinuria, myelosuppression, autoimmune reactions and an initial worsening of neurological symptoms. Thus, today many clinicians prefer to use trientine as first line therapy. Although trientine, often given in combination with zinc if hepatic failure is present at the time of diagnosis, causes side effects similar to those observed with penicillamine, these are less common [86]. Zinc monotherapy may be effective in delaying the onset of symptomatic disease if utilized early in the course of disease, and also as therapy in pregnant women and children. However, while efficacious as initial therapy, it acts more slowly than trientine or penicillamine, requiring up to six months to alleviate symptoms of Cu toxicity in Wilson disease. In order to be efficacious, zinc must be taken on an empty stomach (i.e., separated by at least 1–2 h from food or beverages other than the water taken to swallow the capsules). The rapid dissolution of zinc capsules in the stomach often results in gastrointestinal discomfort, the main adverse effect of zinc salts, which is observed in about 25% of the patients and results in non-compliance in about10% of patients [86].

5. Perspectives - future research directions

Currently available chelators are not as specific as endogenous copper chaperones, which should be aimed at when designing new metal chelators. Delivery to the secretory pathway (Golgi delivery) should be in focus for new chaperone-mimicking copper drugs [134,135], but targeting intracellular copper enzyme loading sites like endoplasmic reticulum (ER) is challenging. Most hydrophilic chelators in clinical use are less metal specific than endogenous circulating copper binding compounds. Development of more target specific drugs for copper delivery and mobilization represents a research priority.

Optimal timing of treatment probably differs between Menkes disease and Wilson disease [136]. Most recommendations are in relation to food, but little attention has been given to chronobiology. New evidence indicates that BBBs are more permeable during night [32] and brain clearance appears to be highest during night while nurturing is highest during day [14]. Chronotherapy is soon becoming mainstream treatment for many drugs [136].

5.1. Future directions for treatment of Menkes disease

Among enzymes affected in Menkes disease, particularly in CNS, are superoxide dismutases (SOD1 and SOD3), cytochrome *c* oxidase, tyrosinase, peptidyl alpha amidating enzyme, and dopamine-β-hydroxylase, plus a number of previously unrecognized copper dependent processes like lysosomal sulfatases and lipoic acid dependent mitochondrial matrix enzyme complexes [Table 2]. The intent of treatment is that copper insertion into enzymes may be taken over by Cu-ionophores to circumvent the pump block.

In Menkes disease clinical experience with copper chelators is mainly based on sporadic case reports and experimental animal studies [35,137,138]. The use of the natural compound copper-histidine is an exception [26,139] and has previously been considered the therapy of choice in Menkes disease, although true benefits are observed only in about 20% of classical Menkes disease cases, most still having copper deficiency problems [140]. An obvious need for new drug formulations exist and recent research indicates that oral formulations will take over.

Cu-DEDTC or Cu-DMDTC may potentially furnish intracerebral chaperones and enzymes more efficiently than Cu-histidine with copper. Thiocarbamates display low acute toxicity but become neurotoxic after prolonged exposure due to release of neurotoxic CS_2 that destabilizes lysosomes. Hence, other drugs may be more appealing for clinical use. NTA has shown benefits [22] and may be a potential readily accessible drug. NTA is rapidly taken up and distributed in the body. The drug shows similarities with biologically active diamines and polyamines and share transporters [141] and catabolic mechanisms and is readily metabolized in the body. Limited experience with use of NTA in Menkes disease is available [56]. NTA will bind other metals and in Menkes disease [142], the drug is preferably administered as a combined copper-zinc salt.

Lipoic acid is appealing for copper delivery in Menkes disease. Lipoic acid exists as a redox pair and reduced and oxidized forms bind copper. It is naturally formed in mitochondria but can readily be taken up and transported to tissues including brain and it may potentially help in restoring enzymatic processes and bypass defective biosynthesis of the lipoic acid cofactor [143]. Lipoic acid supplementation show

limited effect in diseases caused by gene defects in the biosynthetic pathway [144]. However, in Menkes disease, no biosynthetic defect exists and the Cu-lipoic acid complex may potentially be beneficial. Apparently the biological R-form is more effective [133] than the synthetic S-form. Clinical trials using the racemic form show good tolerance [65].

Combination therapy in Menkes disease using copper-histidine and penicillamine has been exploited primarily with a view to mobilize copper accumulated in kidneys to limit copper-induced nephrotoxicity [106,145]. Cu-DMDTC normalizes kidney copper levels. Hopefully, lipoic acid with delivery and removal potential may provide buffering of copper to avoid nephrotoxic copper accumulations that may lead to discontinuation of copper therapy [145].

5.2. Future directions for treatment of Wilson disease

Penicillamine has long been first choice of treatment. Rapid provocation of side effects have suggested trientine as a better alternative [83], and in many countries, this is now considered the initial drug of choice. Clinical trials on thiomolybdate drugs are in progress. The ammonium formulation has proven unstable for routine clinical use [146], but a bis-choline formulation has recently been introduced [96]. In China DMSA has been extensively used while still experimental in Western countries.

After initial chelation therapy, and significant clinical improvement, the choice for maintenance therapy can be reduction of chelator dose or zinc monotherapy. However, the best therapeutic approach remains controversial, as no universally accepted regimen exists. Two aspects should be emphasized for obtaining optimal outcome: proper patient monitoring and support to ensure compliance.

Trientine has a polyamine structure and is presumed to interact with normal polyamine metabolism. High doses of trientine may influence the polyamine load. Polyamines regulate neuro-excitability through GABA excitatory systems and trientine may influence wakefulness.

Thiomolybdates are excreted in bile [95]. Given without food, thiomolybdates are absorbed into blood where they complexes copper with albumin, promoting biliary excretion of the metal. Excess hepatic Cu-MT is mobilized and complexed within a stable tripartite complex resulting in diminished urinary elimination and enhanced faecal excretion. Thiomolybdate removal of copper from metallothionein is however a warning sign. If a chelator can remove copper from MT that show a strong avidity for copper, it will potentially strip copper enzymes. It may be advisable to measure LOX activity [126,127].

Albumin is removed together with the thiomolybdate complex and may potentially aggravate hypoalbuminemia secondary to cirrhosis. Hypoalbuminemia increases the risk of hemolytic crisis in Wilson disease [147]. Thiomolybdate shows good treatment potential in neuropsychiatric Wilson disease, however if the excretory system is overloaded redistribution to brain may occur [148].

Strong copper-binding to lipoic acid has recently led to speculations about a possible de-coppering value in Wilson disease [66–68]. The compound is able to mobilize intracellular mercury but may redistribute the metal to the brain [67]. Still lipoic acid may prove efficient for removal of copper in combination with a hydrophilic second copper chelator. As both isomers of lipoic acid bind copper strongly and the safety profile is good, a racemic form of the compound may be suitable. Lipoic acid shows beneficial action on the cystin/cystein balance and may restore liver GSH biosynthesis and prevent formation of kidney stones [67].

All mentioned drugs have benefits and drawbacks and it is increasingly recognized that combination therapy may be the future treatment regimen. Combination therapy is an approach to enhance metal mobilization from the body while reducing individual doses of chelators, and thereby limit drug specific adverse reactions. Combination therapy using two or more drugs may prove more efficient and tolerable [79]. However, maximal chelation capacity should still be controlled to avoid copper deficiency symptoms. Chelation therapy with combination of a lipophilic and a hydrophilic chelator to obtain facilitated mobilization of metal deposits in tissues plus facilitated urinary excretion may improve the therapeutic value. Lipoic acid and tetrathiomolybdate can cross BBB and thus act as brain-to-blood shuttles. In blood a hydrophilic drug, e.g. penicillamine, trientine or DMSA, may efficiently serve to promote urinary excretion.

Conclusively, it should be emphasized that there is a lack of high-quality evidence to estimate the relative treatment effects of drugs used in Wilson disease, and multicenter randomized comparative trials are needed.

Acronyms and abbreviations

ALB	Albumin
A2M	Alpha-2-macroglobulin
AOC	Amine oxidase, copper containing
ATOX1	Antioxidant 1 copper chaperone
ATP	Adenosine triphosphate
ATP7A	Copper transporting ATPase A
ATP7B	Copper transporting ATPase B
ATTM	Ammonium Tetrathiomolybdate
BBB	Blood brain barrier
BCB	Blood–cerobrospinal fluid barrier
BCTTM	Bischolin-bisthiomolybdate
BEB	Blood–endothelial barrier
CCS	Copper chaperone for superoxide dismutase
CNS	Central nervous system
CTR1	Copper transporter 1
COX	Cytochrome c oxidase
COX11	Cytochrome c oxidase copper chaperone 11
COX17	Cytochrome c oxidase copper chaperone 17
COX19	Cytochrome c oxidase copper chaperone 19
COX23	Cytochrome c oxidase copper chaperone 23
CP	Ceruloplasmin
CS_2	Carbondisulfid
CSF	Cerebrospinal fluid
Cu	Copper
DBH	Dopamine beta hydroxylase
DETC	Diethyldithiocarbamate
DEDTC	Tetraethyldithiocarbamate
DMDTC	Tetramethyldithiocarbamate
DMTC	Dimethyldithiocarbamate
DMSA	Dimercaptosuccinic acid
DMPS	Dimercaptopropane sulphonate
DPA	D-penicillamine
ER	Endoplasmic reticulum
FAD	Flavoprotein
FDA	Food and drug administration
FG	Formylglycine
FGE	Formylglycine-generating Enzyme, see also SUMP1
GSH	Glutathione
HEPH	Hephaistin
HIS	Histidine
HVA/VA	Homovanillic acid/vanillic acid
IARC	International Agency for Research on Cancer Ionophor. From Greak meaning ion carrier.
IUPAC	The International Union of Pure and Applied Chemistry: http://www.chem.qmul.ac.uk/iupac/
LA	Lipoic Acid
L/D-form	Refers to the shape of a molecule; a mixture of both isomers is called racemic and each form is called an enantiomer; also see R/S-forms
LOX	Lysyl oxidase
LOXL	Lysyl oxidase like
LTQ	Lysyl-tyrosyl quinone

MBD	Metal binding domain
MT	Metallothionein
NIH	National Institute of Health
NTA	Nitrilotriacetic acid
OHS	Occipital horn syndrome
OMIM	Online Mendelian Inheritance in Man, URL: http://www.ncbi.nlm.nih.gov/omim/
P	Plasma
PA	Penicillinamine, racemic mixture
PAM	Peptidyl alpha amidating enzyme
R/S-form	Refers to the shape of a molecule; a mixture of both forms is called racemic and each form is called an enantiomer; L/D and R/S are not completely synonymous, but overall used for the same phenomenon
ROS	Reactive oxygen species
S	Serum
SLC	Solute carriers
SLCO1B1	Solute carrier organic anion transporter family member 1B1; also known as SLC21A6
SLC3A2	Solute carrier number 3A2
SLC5A6	Solute carrier number 5A6
SLC31A1	Solute carrier number 31A1; also known as CTR1
SOD1	Cu/Zn containing superoxide dismutase 1
SOD2	Mn containing superoxide dismutase
SOD3	Cu/Zn containing superoxide dismutase 3
SOX	Sulfhydryl Oxidase
SUMP1	Sulfatase-modifying factor 1, other name for FGE
TC	Thiocarbamate
TETA	Trientine
TGN	Trans Golgi network
TM	Transmembrane
TPQ	Topaquinone
TTM	Tetrathiomolybdate
TYR	Tyrosinase
Zn	Zinc

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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