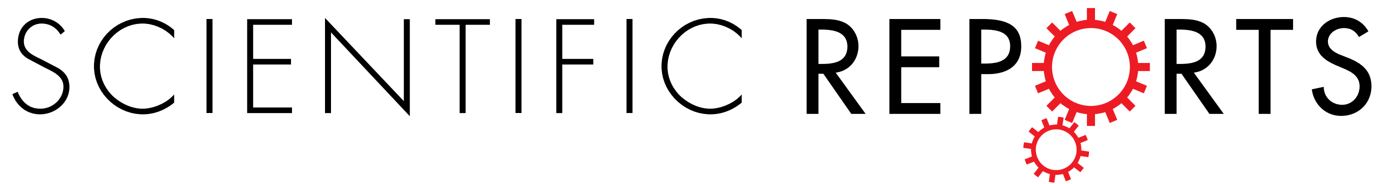

OPEN

Received: 27 October 2017
Accepted: 9 January 2018
Published online: 23 January 2018

Copper(I)-binding properties of de-coppering drugs for the treatment of Wilson disease. α-Lipoic acid as a potential anti-copper agent

Julia Smirnova[1], Ekaterina Kabin[1], Ivar Järving[1], Olga Bragina[1], Vello Tõugu[1], Thomas Plitz[2] & Peep Palumaa[1]

Wilson disease is an autosomal recessive genetic disorder caused by loss-of-function mutations in the P-type copper ATPase, *ATP7B*, which leads to toxic accumulation of copper mainly in the liver and brain. Wilson disease is treatable, primarily by copper-chelation therapy, which promotes copper excretion. Although several de-coppering drugs are currently available, their Cu(I)-binding affinities have not been quantitatively characterized. Here we determined the Cu(I)-binding affinities of five major de-coppering drugs – D-penicillamine, trientine, 2,3-dimercapto-1-propanol, *meso*-2,3-dimercaptosuccinate and tetrathiomolybdate – by exploring their ability to extract Cu(I) ions from two Cu(I)-binding proteins, the copper chaperone for cytochrome c oxidase, Cox17, and metallothionein. We report that the Cu(I)-binding affinity of these drugs varies by four orders of magnitude and correlates positively with the number of sulfur atoms in the drug molecule and negatively with the number of atoms separating two SH groups. Based on the analysis of structure-activity relationship and determined Cu(I)-binding affinity, we hypothesize that the endogenous biologically active substance, α-lipoic acid, may be suitable for the treatment of Wilson disease. Our hypothesis is supported by cell culture experiments where α-lipoic acid protected hepatic cells from copper toxicity. These results provide a basis for elaboration of new generation drugs that may provide better therapeutic outcomes.

Wilson disease is characterized by loss-of-function mutations in a P-type copper ATPase, ATP7B, which is expressed mostly in liver[1,2]. The WD protein has dual roles, it is functioning in the transport of copper into the trans-Golgi network, for incorporation into the plasma protein ceruloplasmin[3,4], and into the bile, for excretion of excess cellular copper[5]. Defective ATP7B functioning is causing reduced incorporation of copper into ceruloplasmin and copper accumulation primarily in liver and in brain, leading to liver disorders and/or neuropsychiatric symptoms[6].

Unlike many other genetic disorders, Wilson disease is treatable, primarily by copper-chelation therapy, which promotes copper excretion. Several de-coppering drugs have been used for the treatment of Wilson disease, two of which have also been approved by the FDA. D-penicillamine (PA), the first orally administered copper-chelating agent available, was approved for therapeutic use in 1956[7]. PA induces copper excretion into urine[8]; however, it also has many adverse effects[9]. The second oral copper chelating drug, trientine (TR), was approved in 1982. TR also acts by enhancing urinary excretion of copper, however, it is better tolerated than PA[9,10]. In addition to these two major drugs, two other copper-chelating compounds have been used for treatment of Wilson disease in the past: an injectable drug, British anti-Lewisite (BAL or dimercaptopropanol), which was used in the UK in 1951[11] and dimercaptosuccinate (DMS), which has been used in China for half a century already[12]. In Western medicine, BAL and DMS are used primarily for the treatment of arsenic, mercury, and lead poisoning[13,14]. The fifth de-coppering drug selected for our study is tetrathiomolybdate (TTM), which was introduced in 1984 and was used in a limited number of patients with Wilson disease. Initial studies with ammonium TTM[15] and a recently completed Phase II clinical study with bis-choline TTM[16], demonstrate that TTM acts

[1]Department of Chemistry and Biotechnology, Tallinn University of Technology, Akadeemia tee 15, 12618, Tallinn, Estonia. [2]Wilson Therapeutics AB, Västra Kungsgatan 3, S-111 43, Stockholm, Sweden. Correspondence and requests for materials should be addressed to P.P. (email: peep.palumaa@ttu.ee)

rapidly, resulting in improvements in copper control and this is accompanied by improved neurologic outcomes and stabilization of liver function, with a favorable safety profile[17].

De-coppering drugs should compete for copper ions with cellular Cu(I)-binding proteins, for which the Cu(I)-binding affinities are known[18]. However, the Cu(I)-binding affinities of de-coppering drugs, which are of fundamental importance for understanding their therapeutic action, are currently not known. In the current study, Cu(I)-binding affinities of de-coppering drugs were determined from their competition with two cellular Cu(I)-binding proteins, Cox17 and metallothionein (MT), which have different Cu(I)-binding affinities[18]. These proteins were reconstituted to form Cu_1Cox17 and $Cu_{10}MT$ metalloforms and incubated with increasing concentrations of the de-coppering drugs. Demetallation of the copper proteins was monitored by electrospray ionization mass spectrometry (ESI MS)[18] and the copper-binding affinities were determined from dose-dependent demetallation curves. The absolute values for dissociation constants (K_d) of Cu(I)-drug complexes were obtained by comparing demetallation potencies of de-coppering drugs with those of copper chelators with known copper-binding affinities such as dithiotreitol (DTT) and diethyl dithiocarbamate (DETC). Analysis of structure-activity relationships suggested that an endogenous biologically active substance, α-lipoic acid (LA), may serve as a potential de-coppering drug. LA was able to protect hepatic cells from copper toxicity *in vitro*, which further supports its potential as an anti-copper agent.

Methods

Reagents. Chemical reagents: PA, TR, BAL, DMS, LA, ammonium TTM, diethylammonium DETC were purchased from Sigma-Aldrich, DTT (ultrapure) from USB Corporation, DLA from Santa Cruz Biotechnology. Bischoline TTM (WTX101) was provided by Wilson Therapeutics AB. Rabbit apo-MT2A was purchased from Bestenbalt LLC and apo-$Cox17_{3S\text{-}S}$ was produced as described previously[19]. Recombinant human Cu,Zn-SOD was purchased from Biovision Inc. All solutions were prepared immediately before the experiments in 20 mM ammonium acetate, pH 7.3 buffer in the absence of organic solvents. To avoid oxidation of DTT and proteins, the buffer was saturated with argon.

Reconstitution of proteins with Cu(I) ions. Lyophilized apo-Cox17 was dissolved in 20 mM ammonium acetate, pH 7.3 containing 50 μM DTT and metallated at 1 μM concentration with one equivalent of Cu(I)–DTT complex in the presence of 50 μM DTT. $Cu_{10}MT2$ was reconstituted in the presence of 10 mM DTT from 3 μM apo-MT2 dissolved in 20 mM ammonium acetate, pH 7.3 by addition of 10-fold excess of Cu(I)–DTT. The stock solution of Cu(I)–DTT complex was prepared by dissolving Cu(II)-acetate at 1.3 mM concentration in argon-saturated 20 mM ammonium acetate containing 10 mM DTT at pH 7.3.

ESI-MS settings and determination of copper-binding affinities. DTT is suitable for applications in ESI MS as being a nonionic compound it does not suppress the ionization efficiency of proteins during ESI-MS substantially, which enables detection of protein peaks even if ligand is present in millimolar concentrations. The Cu(I)-binding affinity of DDT has been studied extensively[18,20], and was therefore used as a standard for determination of Cu(I)-binding affinities of DETC, PA, TR, TTM, BAL and DMS. It should be mentioned that DTT is air sensitive and its Cu(I)-binding affinity depends on pH of solution[20]. Therefore all experiments with DTT have been conducted in oxygen-free argon-saturated solutions at pH = 7.3, where the Cu(I)-binding affinity has reliably been determined ($K_d = 5.01 \times 10^{-16}$ M)[20]. Since DETC, PA, TR TTM and DMS are ionic compounds, they lead to ionic suppression of protein peaks in ESI MS spectra. However, ionic suppression is low at submillimolar concentrations and presumably similar for all protein peaks. In a standard experiment, the reconstituted protein was incubated with increasing concentrations of copper chelators for 1 min and samples were injected into the electrospray ion source of an Agilent Technology 6540 UHD Accurate-Mass Q-TOF MS instrument (Agilent) by a syringe pump at 7 μL/min. ESI-MS spectra were recorded for 10 min in the m/z region from 100 to 3,000 Da using the following instrument parameters: capillary voltage = 3500 V, drying gas = 4 l/min, drying gas temperature 100 °C, nebulizer 15 psig, skimmer voltage = 65 V. ESI MS spectral composition was stabilized after 5 min of monitoring time and spectra between 8–10 min were averaged for further analysis. Obtained ESI MS spectra were deconvoluted using the Mass Hunter software (Agilent). The fractional content of metallated protein forms was calculated from the integrated peak areas for metallated and nonmetallated protein forms. C_{50} values for compounds have been calculated by fitting the dose-dependent demetallation curve to the simple 1:1 binding isotherm (in case of Cu_1Cox17) or Hill equation (in case of $Cu_{10}MT$). K_d values for the Cu(I)-protein complexes at pH 7.3, 20 mM ammonium acetate, and 25 °C were determined using previously determined K_d values for protein-Cu(I) complexes[18] and the re-estimated apparent K_d for the Cu(I)–DTT complex of 5.01×10^{-16} M[20]. K_d values for PA, TR, BAL, DMS and TTM, were determined by the ability of these ligands to extract Cu(I) ions from Cu_1Cox17 or $Cu_{10}MT$ in comparison with DTT (in case of Cu_1Cox17) or DETC (in case of $Cu_{10}MT$) respectively as described above.

Demetallation of Cu,Zn-SOD with TTM. Commercial lyophilized Cu,Zn-SOD was dissolved in argon-saturated 20 mM ammonium acetate pH 7.3 to the final concentration of 5 μM, and injected at different time points into an Agilent Technology 6540 UHD Accurate-Mass Q-TOF MS instrument by a syringe pump at 7 μL/min. ESI-MS spectra were recorded for 10 min in the m/z region from 500 to 3,000 Da using the instrument parameters presented above. ESI MS spectrum of the sample exposed two peaks: Cu,Zn-SOD and partially demetallated Zn-SOD (Fig. S9A). Following incubation of the sample with 10 μM ammonium TTM, there was a time-dependent shift in intensities of the peaks, whereas peak of Cu,Zn-SOD disappeared and peak of apo-SOD appeared in the spectrum (Fig. S9A). Spectral changes could be interpreted with extraction of Cu(I) and Zn(II) ions from Cu,Zn-SOD and Zn-SOD and formation of Cu-SOD and apo-SOD. The demetallation process occurred with a half-life of 23 ± 14 min (Fig. S9B).

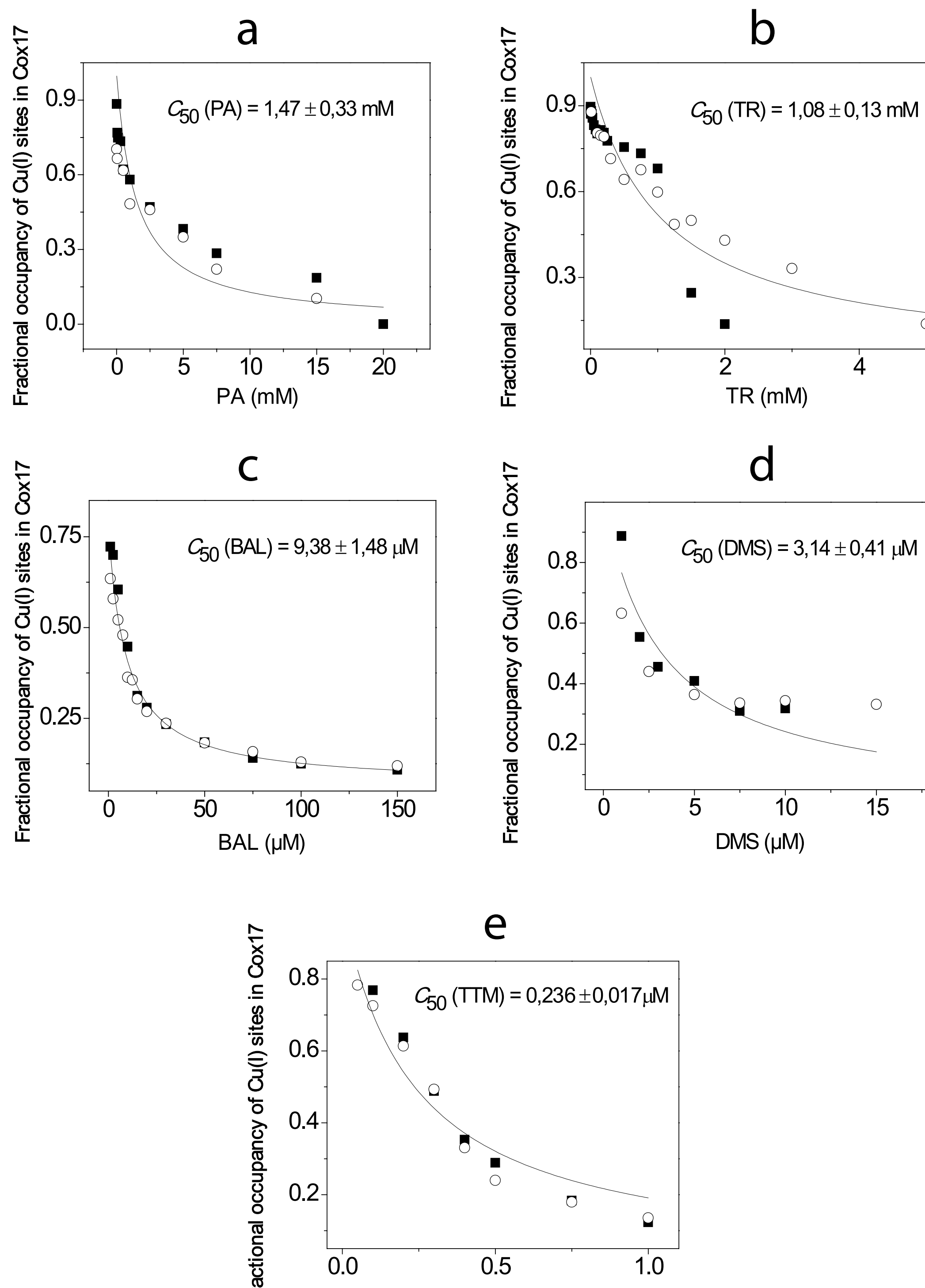

Figure 1. Determination of the relative Cu(I)-binding affinity of de-coppering drugs in competition with Cu_1Cox17. Fractional occupancy of Cu(I)-binding sites in Cox17 at different concentrations of PA (**a**), TR (**b**), BAL (**c**), DMS (**d**) and TTM (**e**) in a metal competition experiment. Conditions: Cox17 1 μM; 20 mM ammonium acetate, pH = 7.3, DTT 50 μM; T = 25 °C. Results of duplicate experiments are presented with different symbols. The solid line shows the fitting curve with hyperbolic equation ($y = P1*(1 - [x/(P2 + x)]) + P3$), where $P2 = C_{50}$.

Cell culture. Huh7 hepatocyte derived cellular carcinoma cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Gibco) and 50 U/ml penicillin, 50 μg/ml streptomycin solution (PAA) in an incubator at 37 °C and 5% CO_2. The medium was changed every 2–3 days and cells were split using Trypsin-EDTA solution (Gibco).

Ligand	C_{50} (mM) for Cu_1Cox17	K_d (M)
DTT	3.10 ± 0.26	$5.01 \times 10^{-16*}$
DETC	0.00528 ± 0.00032	8.53×10^{-19}
PA	1.47 ± 0.33	2.38×10^{-16}
TR	1.08 ± 0.13	1.74×10^{-16}
BAL	0.00938 ± 0.00148	1.52×10^{-18}
DMS	0.00314 ± 0.00041	5.07×10^{-19}
TTM	<0.001	$<1.6 \times 10^{-19}$
DLA	0.498 ± 0.098	8.05×10^{-17}
Ligand	**C_{50} (mM) for $Cu_{10}MT$**	**K_d (M)**
DETC	0.772 ± 0.087	8.53×10^{-19}
TTM	0.0211 ± 0.0014	2.32×10^{-20}

Table 1. Determination of K_d values for de-coppering drugs. The efficiency of de-coppering drugs to demetallate Cu_1Cox17 and/or $Cu_{10}MT$ (C_{50}) was in linear correlation with K_d values for Cu(I)-binding complexes (conditions: 20 mM ammonium acetate, pH 7.3, 25 °C). *Taken from reference[20].

Cell viability measured by WST-1. The effects of $CuCl_2$ on the cells were determined using the cell viability assay WST-1 (Roche). WST-1 assay allows colorimetric measurement of cell viability due to reduction of tetrazolium salts to water-soluble formazan by viable cells. 105 cells were seeded in triplicates in a 96 well plate and cultivated for 24 h. Various concentration (0–100 µM) of $CuCl_2$ were added and the measurements were performed 24 hours after cells treatment. The experiments with no $CuCl_2$ added were used as a negative control. 5 µl/well of WST-1 reagent was added to 100 µl of cell culture medium, incubated at 37 °C for 2 h and absorbance was measured at 450 nm using TECAN Genios Pro microplate reader. For LA testing, cells in triplicates were preincubated with various concentrations (0–100 µM) of LA (dissolved in ethanol) for 24 h and medium was changed to fresh medium, containing 50 µM of $CuCl_2$ and various concentrations (0–100 µM) of LA. Cell viability was determined after 24 h incubation with WST-1 test. Experiments were performed twice.

Results and Discussion

Demetallation of Cu_1Cox17 by the de-coppering drugs. All de-coppering drugs were able to demetallate Cu_1Cox17; however, it occurred at different concentrations with different drugs. Demetallation potency of compounds was defined as the concentration where 50% of Cu_1Cox17 was demetallated and was denoted as C_{50}. PA and TR extracted Cu(I) from Cu_1Cox17 at millimolar concentrations with the corresponding C_{50}(PA) = 1.47 mM (Figs 1A and S1) and C_{50}(TR) = 1.08 mM (Figs 1B and S1). BAL extracted Cu(I) from Cu_1Cox17 at micromolar concentrations with C_{50}(BAL) = 9.38 µM (Figs 1C and S3). DMS and TTM showed very low C_{50} values: C_{50}(DMS) = 3.14 µM (Figs 1E and S4) and the C_{50}(TTM) was less than 1 µM (Figs 1F and S5). C_{50} values were determined also for reference compounds, DTT (C_{50}[DTT] = 3.10 mM) (Fig. S6) and DETC (C_{50}[DETC] = 5.28 µM) (Fig. S7). Based on the linear relationship between C_{50} and K_d values and the known K_d value for DTT[20], we calculated the K_d values for studied compounds and the results are presented in Table 1. As an example we present here the mathematics for calculation of K_d value for DETC. DETC was 587 times more effective Cu(I) chelator than DTT (C_{50}[DTT] / C_{50}[DETC] = 587), which is in line with earlier results obtained with a different ESI-MS setup[18]. Consequently the K_d for Cu(I)-DETC complex should also be 587 times lower as that for DTT and therefore K_d(DETC) = 5.01×10^{-16} / 587 = 8.53×10^{-19} M.

Demetallation of $Cu_{10}MT$ by the de-coppering drugs. In the second series of experiments, we explored the ability of different de-coppering drugs to extract copper from $Cu_{10}MT$, which has 42 times higher Cu(I)-binding affinity as compared to Cu_1Cox17[18]. TTM demetallated $Cu_{10}MT$ at micromolar concentrations through a stepwise process. First, a complex $Cu_{10}MT$-TTM was formed with 1:1 stoichiometry (Fig. 2A). Multiple peaks appeared at higher concentrations of TTM, indicative of the binding of a second, third and fourth TTM molecule to the complex and the simultaneous build-up of apo-MT (Fig. 2A). The binding of multiple TTM ions appeared to lead to the opening of Cu(I)-thiolate clusters and dissociation of Cu(I) ions from the protein. As dissociation of Cu(I)-thiolate clusters of $Cu_{10}MT$ occurred cooperatively we did not observe hyperbolic demetallation curves like in case of Cu_1Cox17. By this reason fitting of the $Cu_{10}MT$ demetallation curves by the influence of TTM was performed by using the Hill equation[18]. A C_{50}(TTM) value of 21.1 µM was obtained by taking into account all CuMT-TTM complexes (Fig. 2B). Both salts of TTM, ammonium and bis-choline TTM, behaved similarly in ESI-MS experiments. Earlier HPLC/ICP experiments have also demonstrated the binding of TTM to CuMT[21]; however, the exact composition of ternary complexes remained unclear.

DETC also extracted Cu(I) from $Cu_{10}MT$ (Fig. 2C) at millimolar concentrations and the corresponding C_{50}(DETC) was equal to 0.77 mM (Fig. 2D). Other de-coppering drugs were unable to demetallate $Cu_{10}MT$ up to millimolar concentrations. Based on the linear relationship between C_{50} and K_d values and the known K_d value for DETC (see Table 1), the K_d value for TTM was calculated ($K_d = 2.32 \times 10^{-20}$ M) and inserted into Table 1. The absolute value of K_d for $Cu_{10}MT$ of 4.1×10^{-16} M was first determined in 2010[18] based on the absolute value of available K_d for the Cu(I)-DTT complex known at that time. Subsequently, the K_d for the Cu(I)-DTT complex has been corrected from 7.94×10^{-12} M[22] to 5.01×10^{-16} M (pH 7.3)[20]. Based on the corrected absolute dissociation constant for Cu(I)-DTT complex, the corrected K_d for $Cu_{10}MT$ was calculated to be 2.59×10^{-20} M.

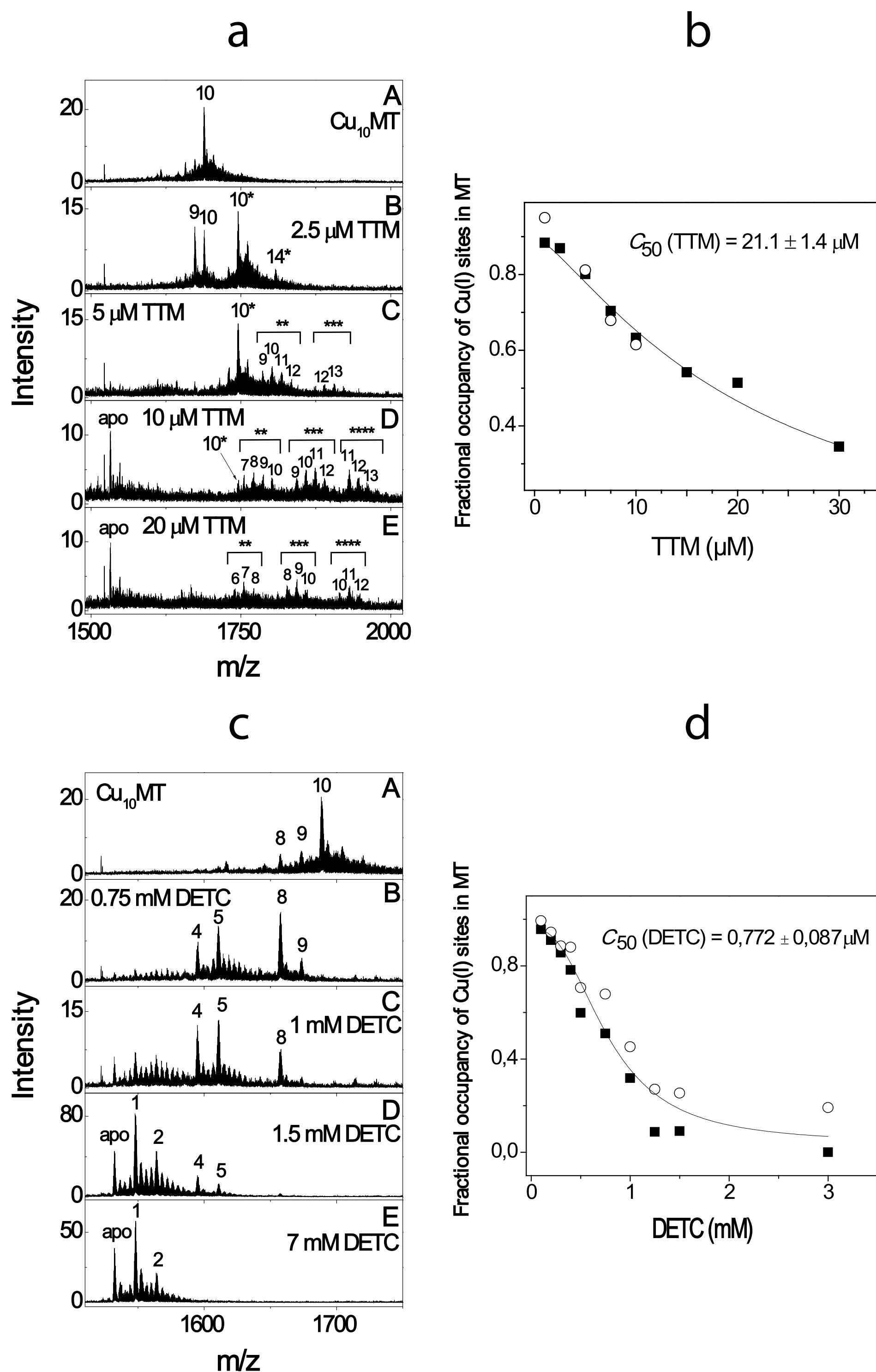

Figure 2. Determination of the relative Cu(I)-binding affinity of TTM and DETC in competition with Cu_{10}MT. ESI-MS spectra of Cu_{10}MT in the presence of 1 μM–20 μM TTM (**a**) and 0.1–7 mM DETC (**c**). Conditions: MT 3 μM; 20 mM ammonium acetate, pH = 7.3, DTT 10 mM; T = 25 °C. Ions with a charge state + 5 are shown; numbers on the peaks denote the metal stoichiometry of the complex. Number of asterisks denotes number of TTM molecules in the complex. Fractional occupancy of Cu(I)-binding sites in MT at different concentrations of TTM (**b**) and DETC (**d**) in a metal competition experiment. Results of duplicate experiments are presented with different symbols. The solid line shows the fitting curve with Hill equation (y = START + (END − START) * x^n / (K^n + x^n)), where $K = C_{50}$.

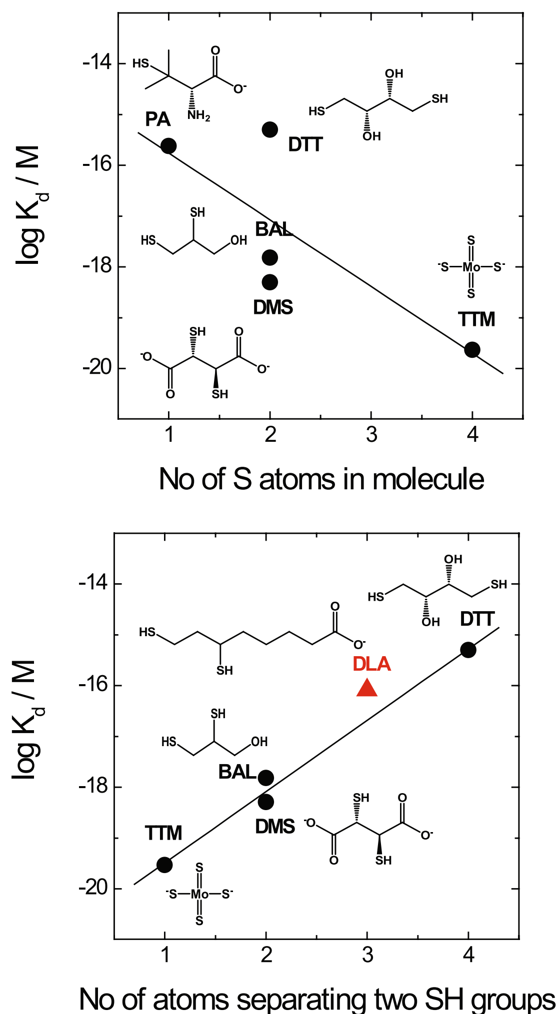

Figure 3. Structure-activity relationships. Correlation between the number of sulfur atoms in the molecule of copper chelators and their Cu(I)-binding affinity (**a**); correlation between the number of carbon atoms separating two SH groups in the molecule of copper chelators including DLA and their Cu(I)-binding affinity (**b**).

TTM had almost equal copper-binding affinity ($K_d = 2.32 \times 10^{-20}$ M) to that of MT. Indeed, TTM can form a 1:1 TTM-Cu_{10}MT complex at concentrations equimolar to MT, whereas at higher concentrations (from 5 to 30 μM) it can extract Cu(I) ions from Cu_{10}MT (Fig. 2A).

Clinical implications of demetallation by de-coppering drugs. The obtained information can be applied to the clinical context. For a sustainable treatment effect in Wilson disease, de-coppering drugs must extract copper from intracellular copper stores, which have, however, different Cu(I)-binding affinities. MT has a high affinity for Cu(I) ions and only one de-coppering drug studied, TTM, had the ability to demetallate Cu_{10}MT at low micromolar concentrations. For estimation of the physiological concentration of TTM, we used data from oncology patients where TTM 180 mg/day resulted in C_{max} of 5.7 μM[23]. At such concentrations, TTM can partially demetallate CuMT, which most probably forms the basis of its fast and efficient therapeutic action.

MT has one of the highest copper-binding affinities among cellular Cu(I) proteins[18] and TTM may therefore extract copper also from several other essential copper proteins. Other cellular copper chaperones and copper transporters have copper-binding affinities similar to that of Cox17[18] and most probably they can also be demetallated by TTM. Intracellular copper enzymes Cu,Zn-SOD (antioxidative defense) and mitochondrial cytochrome c oxidase (mitochondrial electron transfer) have copper-binding affinities comparable to that of MT[18]. However, the dissociation of metal ions from the active sites of these enzymes is very slow[18], which might protect them from fast demetallation by TTM. It has been shown that TTM does not inhibit mitochondrial cytochrome c oxidase activity in hepatic mitochondrial preparations at concentrations up to 100 μM even after 16-h incubation[24], strongly suggesting that TTM cannot demetallate cytochrome c oxidase. Nevertheless, in the same study, TTM inhibited Cu,Zn-SOD in a time-dependent manner[24]. Our data confirm that 10 μM TTM can extract copper from 5 μM Cu,Zn-SOD with a half-life of 23 min (Fig. S8). Thus, treatment with high doses of TTM may demetallate Cu,Zn-SOD, which should be taken into account in refinement of therapeutic doses for TTM.

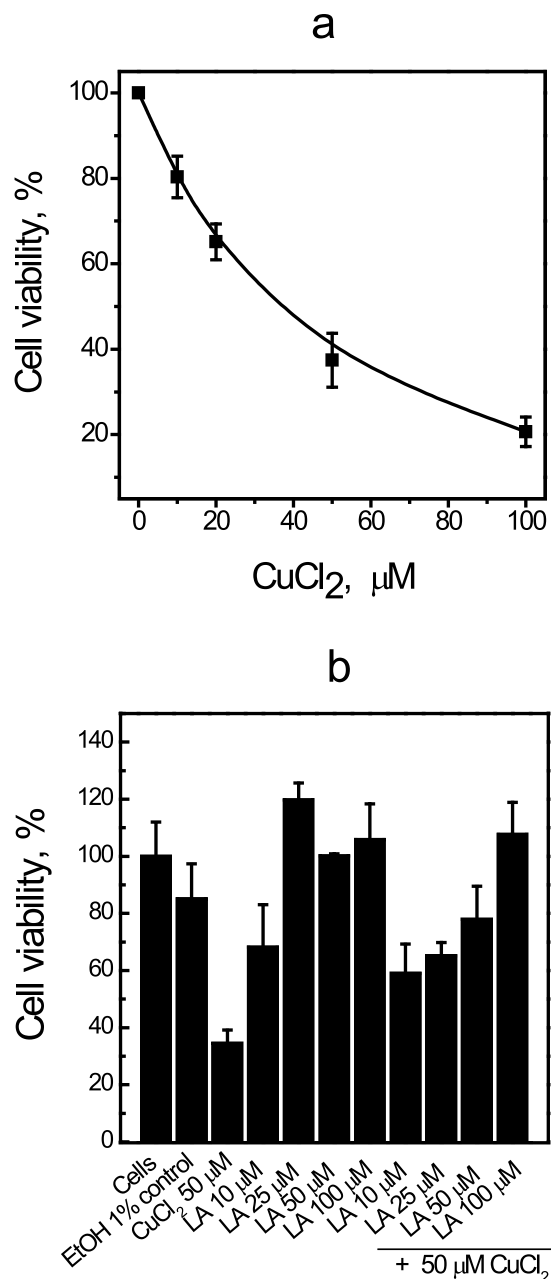

Figure 4. Effect of LA on cellular copper toxicity. Viability of Huh7 cells determined by WST-1 assay after 24 h of exposure to $CuCl_2$ (**a**). Viability of Huh7 cells after 24 h incubation with different concentrations of LA and additional treatment with 50 μM $CuCl_2$ for next 24 h, measured with WST-1 test. Data are shown as mean ± SD.

The K_d values for PA and TR were 2.38×10^{-16} M and 1.74×10^{-16} M, respectively. Therapeutic doses of PA and TR (750–1500 mg/day[17]) are substantially higher than that for TTM. The current study suggests that PA and TR cannot effectively compete with CuMT at therapeutic concentrations (approximately 50–100 μM); however, they can partially compete with copper chaperones. At therapeutic concentrations, BAL and DMS effectively demetallated copper chaperones and only partially CuMT; however, the action of DMS is restricted mainly to the extracellular space since it is unable to cross biological membranes.

Structure-affinity relationship analysis and identification of new copper-binding drugs. The Cu(I)-binding affinity of the de-coppering drugs studied varied by four orders of magnitude depending upon the molecular structure of the compounds. One can speculate that the number of sulfur-containing groups in the molecule, which are well adapted for the coordination of Cu(I) ions, is an important determinant of the binding affinity. Indeed, a positive correlation between the Cu(I)-binding affinities and the number of the S-atoms in the molecule (Fig. 3A) was observed: TTM, with four S-atoms, has the highest Cu(I)-binding affinity, and PA with only one S-atom has the lowest affinity. However, the Cu(I)-binding affinity of DTT is considerably different

from that of BAL and DMS, although all these compounds carry two SH groups. Thus, not only the number, but also the position of the sulphur-containing groups appears to affect the Cu(I)-binding affinity of compounds. Indeed, the Cu(I)-binding affinity decreased substantially with increasing number of atoms between the sulfhydryl groups: TTM > BAL, DMS > DTT (Fig. 3B). This structure-affinity relationship explains why TTM has the highest Cu(I) affinity of all de-coppering agents studied and opens new perspectives for the rational design of new copper-binding drugs.

In order to test the validity of this structure-activity relationship and its applicability for design of new copper-binding drugs, we looked for biomolecules with three intercalating atoms between two SH groups and found that dihydrolipoic acid (DLA) fits these criteria. The experimental Cu(I)-binding affinity of DLA was equal to $K_d = 8.05 \times 10^{-17}$ M (Fig. S9, Table 1), which fits well the predicted relationship between $\log K_d$ and the number of atoms between two SH groups, thus confirming the structure-activity relationship. The Cu(I)-binding affinity of DLA was 2 and 3 times higher than that of TR and PA respectively, however, the affinity was not high enough to directly decopper intracellular high-affinity Cu stores like Cu_{10}MT when tested experimentally (data not shown). *In vivo*, DLA is formed from α-lipoic acid (LA) by reduction in a cellular environment. LA is a well-tolerated food supplement that is able to cross biological membranes. Considering its higher Cu(I)-binding affinity than that of chelators approved for the treatment of Wilson disease (PA, TR), good membrane and blood brain barrier permeability[25] as well as very good tolerability[26], we suggest that LA might be a suitable de-coppering drug for treatment of Wilson disease. Some exploratory attempts to use LA in Wilson disease have been reported[27,28], however, the potency of LA in the treatment of Wilson disease has not been explored in cellular experiments.

Effect of LA on cellular copper toxicity. To further explore the potential of LA as a de-coppering drug we performed preliminary experiments with Huh7 hepatic cell lines to test the putative protective effect of LA against copper toxicity. Similar experimental setup has been used earlier to demonstrate the protective effect of PA on copper toxicity exerted on hepatic cell lines[29,30]. The results in Fig. 4 demonstrate that copper is toxic to Huh7 cells with LD_{50} of approximately 30 μM of $CuCl_2$ (Fig. 4a) and LA exerts a clear dose-dependent protective effect on toxicity of 50 μM $CuCl_2$ (Fig. 4b). The protective effect on cellular level supports the potential therapeutic role of LA in treatment of Wilson disease, however, the full spectrum of therapeutic activities of LA should be evaluated in further cellular and animal studies.

References

1. Bull, P. C., Thomas, G. R., Rommens, J. M., Forbes, J. R. & Cox, D. W. The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nat Genet* **5**, 327–337, https://doi.org/10.1038/ng1293-327 (1993).
2. Tanzi, R. E. *et al*. The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. *Nat Genet* **5**, 344–350, https://doi.org/10.1038/ng1293-344 (1993).
3. Terada, K. *et al*. Copper incorporation into ceruloplasmin in rat livers. *Biochim Biophys Acta* **1270**, 58–62 (1995).
4. Terada, K. *et al*. Restoration of holoceruloplasmin synthesis in LEC rat after infusion of recombinant adenovirus bearing WND cDNA. *J Biol Chem* **273**, 1815–1820 (1998).
5. Terada, K. *et al*. Biliary excretion of copper in LEC rat after introduction of copper transporting P-type ATPase, ATP7B. *FEBS Lett* **448**, 53–56 (1999).
6. Patil, M., Sheth, K. A., Krishnamurthy, A. C. & Devarbhavi, H. A review and current perspective on Wilson disease. *J Clin Exp Hepatol* **3**, 321–336, https://doi.org/10.1016/j.jceh.2013.06.002 (2013).
7. Walshe, J. M. Penicillamine, a new oral therapy for Wilson's disease. *Am J Med* **21**, 487–495 (1956).
8. Walshe, J. M. Penicillamine: the treatment of first choice for patients with Wilson's disease. *Mov Disord* **14**, 545–550 (1999).
9. Roberts, E. A. & Schilsky, M. L. & American Association for Study of Liver Diseases. Diagnosis and treatment of Wilson disease: an update. *Hepatology* **47**, 2089–2111, https://doi.org/10.1002/hep.22261 (2008).
10. Walshe, J. M. Treatment of Wilson's disease with trientine (triethylene tetramine) dihydrochloride. *Lancet* **1**, 643–647 (1982).
11. Cumings, J. N. The effects of B.A.L. in hepatolenticular degeneration. *Brain* **74**, 10–22 (1951).
12. Li, W. J., Chen, C., You, Z. F., Yang, R. M. & Wang, X. P. Current Drug Managements of Wilson's Disease: From West to East. *Curr Neuropharmacol* **14**, 322–325 (2016).
13. Flora, S. J. & Pachauri, V. Chelation in metal intoxication. *Int J Environ Res Public Health* **7**, 2745–2788, https://doi.org/10.3390/ijerph7072745 (2010).
14. Rooney, J. P. The role of thiols, dithiols, nutritional factors and interacting ligands in the toxicology of mercury. *Toxicology* **234**, 145–156, https://doi.org/10.1016/j.tox.2007.02.016 (2007).
15. Brewer, G. J. *et al*. Treatment of Wilson disease with ammonium tetrathiomolybdate: III. Initial therapy in a total of 55 neurologically affected patients and follow-up with zinc therapy. *Arch Neurol* **60**, 379–385 (2003).
16. Weiss, K. H. *et al*. WTX101 in patients newly diagnosed with Wilson disease: final results of a global, prospective phase 2 trial. *J Hepatol* **66**, S88 (2017).
17. Rodriguez-Castro, K. I., Hevia-Urrutia, F. J. & Sturniolo, G. C. Wilson's disease: A review of what we have learned. *World J Hepatol* **7**, 2859–2870, https://doi.org/10.4254/wjh.v7.i29.2859 (2015).
18. Banci, L. *et al*. Affinity gradients drive copper to cellular destinations. *Nature* **465**, 645–648, https://doi.org/10.1038/nature09018 (2010).
19. Voronova, A. *et al*. Cox17, a copper chaperone for cytochrome c oxidase: expression, purification, and formation of mixed disulphide adducts with thiol reagents. *Protein Expr Purif* **53**, 138–144, https://doi.org/10.1016/j.pep.2006.11.014 (2007).
20. Xiao, Z. *et al*. Unification of the copper(I) binding affinities of the metallo-chaperones Atx1, Atox1, and related proteins: detection probes and affinity standards. *J Biol Chem* **286**, 11047–11055, https://doi.org/10.1074/jbc.M110.213074 (2011).
21. Suzuki, K. T. & Ogra, Y. Formation of copper-metallothionein/tetrathiomolybdate complex is the first step in removal of Cu from LEC rats. *Res Commun Mol Pathol Pharmacol* **88**, 187–195 (1995).
22. Krezel, A. *et al*. Coordination of heavy metals by dithiothreitol, a commonly used thiol group protectant. *Journal of Inorganic Biochemistry* **84**, 77–88, https://doi.org/10.1016/S0162-0134(00)00212-9 (2001).
23. Lowndes, S. A. *et al*. Phase I study of copper-binding agent ATN-224 in patients with advanced solid tumors. *Clin Cancer Res* **14**, 7526–7534, https://doi.org/10.1158/1078-0432.CCR-08-0315 (2008).
24. Juarez, J. C. *et al*. Copper binding by tetrathiomolybdate attenuates angiogenesis and tumor cell proliferation through the inhibition of superoxide dismutase 1. *Clin Cancer Res* **12**, 4974–4982, https://doi.org/10.1158/1078-0432.CCR-06-0171 (2006).
25. Packer, L., Tritschler, H. J. & Wessel, K. Neuroprotection by the metabolic antioxidant alpha-lipoic acid. *Free Radic Biol Med* **22**, 359–378 (1997).

26. Shay, K. P., Moreau, R. F., Smith, E. J., Smith, A. R. & Hagen, T. M. Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. *Biochim Biophys Acta* **1790**, 1149–1160, https://doi.org/10.1016/j.bbagen.2009.07.026 (2009).
27. da Costa, S. F., Rodrigues, E. M., da Silva, J. A., Relvas, M. E. & Halpern, M. J. Zur biochemischen Pathologie der Wilsonscher Krankheit. *Arzneimittel-Forschung* **11**, 438–444 (1961).
28. da Costa, S. F. Morbus Wilson und die mögliche therapeutische Bedeutung der Liponsäure. *Arzneimittel-Forschung* **20**, 1210–1213 (1970).
29. Chandhok, G. *et al*. The effect of zinc and D-penicillamine in a stable human hepatoma ATP7B knockout cell line. *PLoS One* **9**, e98809, https://doi.org/10.1371/journal.pone.0098809 (2014).
30. Chandhok, G. *et al*. Functional analysis and drug response to zinc and D-penicillamine in stable ATP7B mutant hepatic cell lines. *World J Gastroenterol* **22**, 4109–4119, https://doi.org/10.3748/wjg.v22.i16.4109 (2016).

Acknowledgements
This work was supported by the Estonian Ministry of Education and Research (grant IUT 19-8 and 19-9) and by a research grant from Wilson Therapeutics AB. The authors thank Richard Tamme and Emma Marshman for medical writing support.

Author Contributions
P.P. and T.P. conceived the study; J.S., E.K. I.J. and O.B. carried out the main experiments; J.S., E.K., V.T., O.B. and P.P. analyzed the data; P.P. wrote the main draft of the paper and all authors participated in revising the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-19873-2.

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018