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Quest for a stable Cu-ligand complex with a high catalytic activity to produce ROS

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ABSTRACT: Metal ion catalysed overproduction of reactive oxygen species (ROS) are believed to contribute significantly to oxidative stress and be involved in several biological processes, from immune defence to development of diseases. Among the essential metal ions, copper is one of the most efficient catalysts in ROS production in the presence of O₂ and a physiological reducing agent such as ascorbate. To control this chemistry, Cu ions are always tightly coordinated to biomolecules. Free or loosely bound Cu ions are generally avoided to prevent toxicity, a process however occurring in the Cu-overload Wilson's disease.

In the present report, we aim to find stable Cu-ligand complexes (Cu-L) that can catalyse efficiently the production of ROS in presence of ascorbate under aerobic conditions. Stability would be needed to avoid dissociation in biological environment and high ROS catalysis is of interest for applications as in antimicrobial or anticancer agents. A series of known tripodal and tetradentate ligands with reported high affinities were synthesized. Two of them showed higher catalytic activity in oxidation of ascorbate and subsequent ROS production than "free" Cu, which is an unprecedented result. Despite these high catalytic activities, no increased antimicrobial activity towards *E. coli* or cytotoxicity against eukaryotic AGS cells related to Cu-L based ROS production could be observed. The potential reasons for discrepancy between in vitro and in cell data will be discussed.

INTRODUCTION

Ascorbate (AscH^-) is a water-soluble vitamin and hence essential for humans. It is one of the main water-soluble organic reducing agents in humans and a very abundant molecule in biology, reaching mM concentration in cells and hundreds of μM in the blood.¹ The fact that AscH^- is an efficient one electron donor, makes it also a potential prooxidant, because donating one electron to a non-radical molecule forms a radical. With most compounds this is not a favoured reaction.²

An important case is the reduction of dioxygen that can form superoxide, hydrogen peroxide and hydroxyl radical (Figure 1).³ These oxidizing molecules are members of the so-called reactive oxygen species (ROS). However, due to the spin conservation principle, direct reduction of O_2 by ascorbate is very sluggish, and indeed autooxidation of AscH^- at pH 7.4 under aerobic conditions is very slow.⁴

But these reactions can be accelerated in a catalytic fashion by metal ions, and from the essential metal ions in biology mainly by Cu and Fe.⁵⁻⁷ Thus, in biological systems, the Cu and Fe metabolisms are tightly controlled by coordinating these ions strongly. This minimises the aqua complexes (often called “free”) and loosely bound Cu or Fe, which could catalyse the production of ROS by oxidizing AscH^- and other reductants with O_2 leading to ROS. However, in certain cases, fast ROS production is wanted such as in defence against invaders. Macrophages pump Cu into the phagosome and increase the amount of ROS to attack bacteria.⁵ Hence the use of redox active Cu/Fe complexes is an attractive approach to kill bacteria⁶ or cancer cells⁷ via local over-production of ROS, inducing oxidative stress.

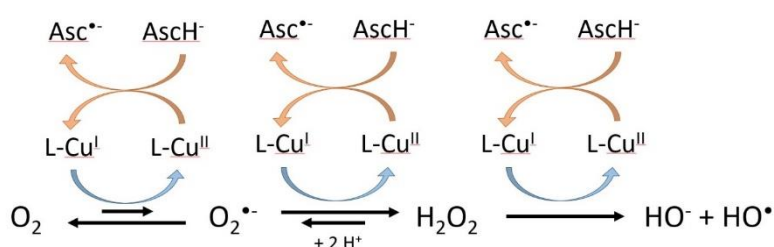


Figure 1: Schematic representation of the formation of reduced dioxygen species (members of the so-called reactive oxygen species (ROS)) by copper complexes in presence of ascorbate. Classically Cu cycles between Cu^{I} and Cu^{II} and donates one electron at a time.

However, a major issue is the stability of these Cu and Fe complexes in biological environments where strong Fe and Cu chelating proteins are present to avoid loosely-bound metal ions. In particular, proteins and small molecules that are involved in metal ion transport, exist in their non-metalated form, and can readily withdraw $\text{Cu}^{\text{I/II}}$ / $\text{Fe}^{\text{II/III}}$ ions from their complexes. Siderophores from bacteria or transferrin in humans ($\log K > 22$ for Fe^{III} at pH 7.4)⁸, Cu-chaperones ($\log K > 17$ for Cu^{I} at pH 7.4)^{9,10} or serum albumin for Cu^{II} ($\log K \sim 13$)¹¹ are examples of potent and abundant competitors that could compete with the Cu or Fe chelating ligands.

Amongst bioavailable metals, Cu is the most efficient in catalytic ROS production in presence of AscH^- ^{12,13} and considering the strong competition for chelation of both Cu^{I} and Cu^{II} by native proteins, we aim to select Cu-complexes with a strong affinity for both Cu^{I} and Cu^{II} . This is necessary, because for AscH^- oxidation by O_2 , the Cu ion has to redox cycle (Figure 1) and hence Cu^{I} or Cu^{II} might dissociate and be transferred to competitors. Moreover, to allow rapid

redox cycling, both redox states should be accessible and the barrier between them small. Indeed, chelators stabilizing too much only one redox state, are generally little redox active.¹⁴ Indeed, Cu-L are often less active than “free” Cu in buffer.^{15–17} We selected Cu complexes containing tripodal tripyridine type ligands (Figure 2) because: (i) they are well-described in terms of structure and redox potential from the literature;^{18–24} (ii) they are tetradentate ligands, and with four donor atoms they have a high Cu affinity due to the chelate effect;^{20,21} (iii) the Cu complexes have one additional binding site available to bind substrates and the reduced forms are able to activate O₂, i.e. reduce O₂ to form ROS; (iv) and the complexes have geometries that can accommodate Cu^I and Cu^{II}.^{20,23,25} There is a broad interest in identifying efficient Cu-based anticancer agents, however only few cellular studies have been realized.^{26–28} Various cytotoxicities of different tripodal Cu complexes have been observed on human cancer cell lines, however the properties required to explain the antitumor activity could not be highlighted. To evaluate how the redox properties impact the AscH⁻ oxidation by O₂, we investigated a series of four tripodal tripyridine ligands (here called L₀ to L₃) known from the literature.^{21,29} These ligands L have the same coordination bonds and differ only in the number (0 to 3) of ethylene groups, which has an impact on the redox properties.²¹ It has been shown that the redox potential increases steadily from Cu-L₀ to Cu-L₃, in DMF, which is in line with the fact that Cu^{II} stability decreases from L₀ to L₃, whereas Cu^I stability increases.¹⁸ In this paper, we aimed at identifying the optimal complex in this series as catalyst for AscH⁻ oxidation by O₂ and at evaluating the cytotoxicity of these Cu-L complexes in presence and absence of AscH⁻. To do this, we first recharacterized the complexes under the conditions used in our assays.

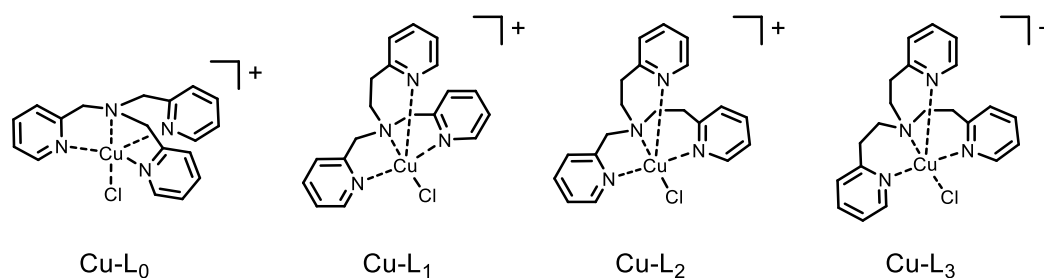


Figure 2: Scheme of the studied series of copper complexes Cu^{II}-L₀₋₃. Cristal structures showed penta-coordinated Cu^{II}-L₀₋₃ with a Cl⁻ bound.²⁹ For Cu^I complexes (not shown) with the ligands L₁₋₃, Cu^I ions were tetra-coordinated with no ion or solvent bound.¹⁸ Cu^I-L₀ was penta-coordinated with a supplementary monodentate ligand being a solvent molecule.

RESULTS AND DISCUSSION

UV-visible absorbance, EPR characterization and electrochemical properties. First, the four copper complexes Cu-L₀₋₃ were recharacterized by electronic absorbance spectroscopy (Figure S1 & S2), EPR (Figure S3 & S4) and cyclic voltammetry (Figure S5) in HEPES buffered water (100 mM, pH 7.4) to confirm Cu^{II}-coordination according to the literature.^{18,30} The results are summarized in Table 1 and details are given in the SI (Figures S1-6). Cyclic voltammetry (CV) results were generally in agreement with the reports in the literature, although they were not performed under the very same aqueous conditions. The with mid-potential increased steadily in the series from Cu-L₀ to Cu-L₃.^{18,20,21,24,31,32}

Table 1: Summary of the data obtained by cyclic voltammetry, EPR and UV-Vis absorption: Redox potentials ($E_{1/2}$) and peak-to-peak separation (ΔE) of the copper complexes and anodic peak potential ($E_{p,a}$) of ascorbate in absence and in presence of a copper complex.^a EPR parameters obtained through simulations where g strain parameter was used to account for experimental line broadening

Compound	Electrochemistry			EPR			Absorption
	$E_{1/2}$ (V) ^b	ΔE (mV)	$E_{p,a}$ (V) Asc ^c	$g_{\parallel} \pm 0.002$	$g_{\perp} \pm 0.005^d$	$A_{\parallel} (\times 10^{-4} \text{ cm}^{-1})$	λ_{max} (nm)
Cu-L ₀	- 0.12	85	+ 0.71	2.002	2.195	65	860
Cu-L ₁	+ 0.00	94	+ 0.43	2.249	2.061	175	660
Cu-L ₂	+ 0.08	119	+ 0.42	2.247	2.060	171	630
Cu-L ₃	+ 0.39	90	+ 0.55	2.256	2.062	167	655
Ascorbate			+ 0.71				

^a Potentials are referred to the SHE reference electrode after correction at a scan rate of 0.05 V.s⁻¹ in HEPES buffer (pH 7.4).

^b $E_{1/2} = 1/2(E_{p,c} + E_{p,a})$.

^c 10 equivalents of ascorbate (10 mM) are added to the copper complexes.

^d g_{\perp} are given on an indicative basis as being mostly unresolved experimentally.

The correlation between the mid-potential ($E_{1/2}$) and the coordination chemistry has been explained in the literature with the difference in stabilization of Cu^{II} compared to Cu^I complexes. Generally, the stabilities of the Cu^I complexes are more similar and spanning over a range of 3 log only. Cu^{II}-complexes stability decrease more drastically (8 log units) from L₀ to L₃.³³ The longer linker between the central amine and the pyridine destabilizes Cu^{II} and hence leads to redox potential increase.

CV of L₀₋₃ with ascorbate. Cyclic voltammetry measurements showed no effect on the oxidation of AscH⁻ in presence of Cu-L₀, while in presence of Cu-L₁, Cu-L₂ and Cu-L₃ the oxidation of AscH⁻ occurred at lower potential (see Figure S6 and Table 1), suggesting that these three complexes catalyze the oxidation of AscH⁻. We observed, however, that Cu-L₃ is less efficient than Cu-L₁ and Cu-L₂.

Ascorbate oxidation assays. Kinetic measurements were performed to compare the ability of Cu^{II}-L₀₋₃ complexes to catalyse the ascorbate oxidation by O₂. This is a classical assay that has been used in the past for Cu-complexes and has shown to produce oxidized ascorbate and ROS (Figure 1).³⁴ During the catalysis reaction, Cu cycles between Cu^I and Cu^{II} oxidation states. Ascorbate acts as a reductant giving one electron to Cu^{II} to form Cu^I. Cu^I is then able to give one electron to dioxygen to produce ROS (Figure 1). Thus, monitoring ascorbate consumption by its absorbance at 265 nm is an indirect method to show ROS production by the copper complexes.³⁴

Ascorbate oxidation was measured with a 100 μ M ascorbate solution under aerobic conditions at pH 7.4 with a slight excess of L (1.2 times) compared to Cu to avoid contribution of “free” Cu in the buffer. Three Cu-L concentrations were used, 5 μ M, 1 μ M and 0.3 μ M. The initial

reaction rates were determined and are shown in Figure 3 (for kinetic traces see Figures S7). From our experiences with Cu-peptide and Cu-amino acid complexes, free Cu in the buffer was the most active in this type of test.³⁵ In this study, at 5 μM Cu concentration, ascorbate oxidation with free Cu was quite fast (8 $\mu\text{M}/\text{min}$) in the buffer, in line with the literature.⁹

The complex Cu-L₀ showed basically no catalytic activity whereas Cu-L₃ was almost three times slower than free Cu. On the other hand, Cu-L₁ and Cu-L₂ were slightly faster catalysts than Cu alone. To have a better measure and explore the biological relevant higher nM range, the Cu concentration was decreased to 1 and 0.3 μM (Figure 4 (solid lines) and Figure S7). As expected, the rates became slower. Both Cu-L₁ and Cu-L₂ complexes were more active than Cu in buffer but now the difference between Cu-L₁ and Cu-L₂ became more significant. Cu-L₂ was the most active of all at 0.3 μM , 1.5 times as active as Cu-L₁ and more than 5 times faster than free Cu in buffer.

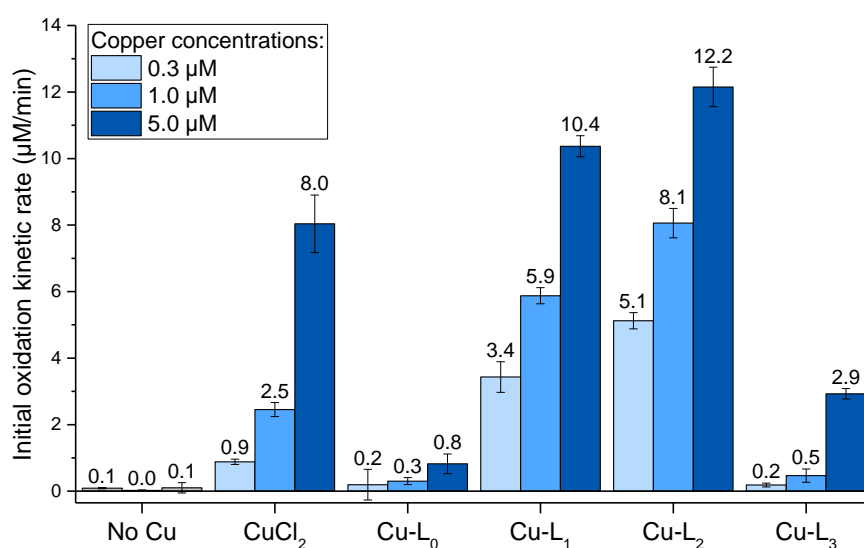


Figure 3: Initial reaction rates of ascorbate oxidation by copper complexes at following concentrations: 0.3 μM , 1 μM , 5 μM , with a 1:1.2 Cu:L ratio, 100 μM ascorbate in HEPES buffer, pH 7.4. (Average of three independent experiments)

A feature that was observed for 0.3 μM Cu-L₁₋₃ but not Cu in buffer was a sudden slow-down of the ascorbate oxidation after about five minutes corresponding to about 15-30% ascorbate consumption (*i.e.* 15-30 μM), which correlates to about 100 – 200 turnovers of the Cu-L (Figure 4). This slow-down was also observed in a less marked manner at 1 μM but not at 5 μM Cu-L. We suspected degradation of the ligand as a potential explanation. The hydroxyl radical is the most reactive species of the reduced dioxygen species, and hence most likely the species responsible for degradation. Therefore, we tested the same reaction in the presence of catalase, that degrades H₂O₂, the precursor of HO[•] (Figure 4).

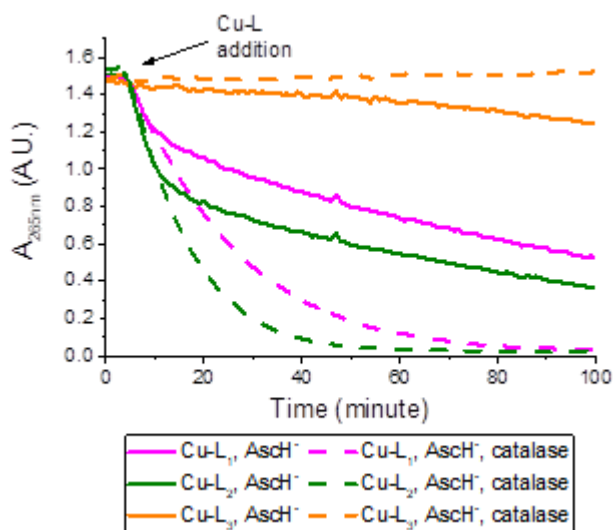


Figure 4: Kinetics of ascorbate oxidation catalyzed by the complexes Cu-L₁₋₃ in absence (solid line) and in presence (dotted line) of catalase monitored by ascorbate absorbance at 265 nm). Copper 0.3 μM ; tripodal ligand 0.36 μM ; catalase 15 mg/L; ascorbate 100 μM ; HEPES buffer 100 mM; pH 7.4; 25°C. (Representative experiment of at least three repetitions)

And indeed, for Cu-L₁ and Cu-L₂, addition of catalase abolished the break point of the curve, and an exponential decrease was observed (Figure 4). No impact on the initial rate was observed (Figures 4 and 5). Further confirmation came from the observation that adding DMSO, a HO[•] scavenger. DMSO did not impact the initial rate but the slow-down became less marked. (see Figure S8).

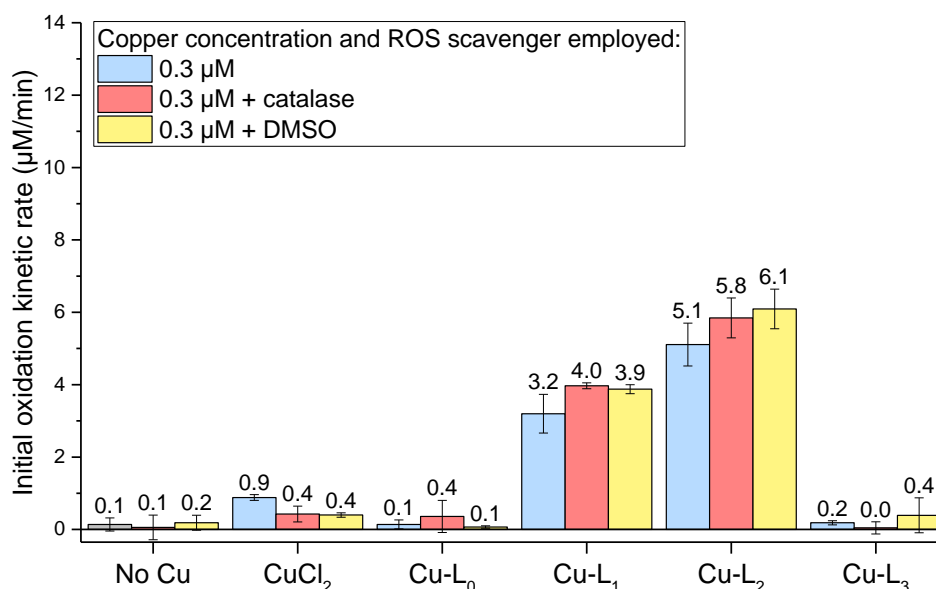


Figure 5: Initial reaction rates of ascorbate oxidation by copper complexes at 0.3 μM upon different conditions: complex alone, with catalase (15 mg.L⁻¹) or with DMSO (5% v/v). HEPES buffer 100 mM; pH 7.4. (Average of three independent experiments)

Taken together, the Cu-L₁₋₂ complexes have higher catalytic activity for AsCH[•] oxidation than free Cu ions in the buffer, which in turn is more active than Cu-L₀ or Cu-L₃. The differences were more marked at low catalyst concentration. Moreover, the results with catalase suggest

that the highly reactive HO[•] can degrade Cu-L₁ and Cu-L₂ and hence slow-down the catalytic activity after about 100-200 turnovers. To corroborate the higher catalytic activity of ascorbate oxidation for Cu-L₁ and Cu-L₂, the production of H₂O₂ was compared for all Cu-L₀₋₃ (Figure S9). The results indeed confirmed the tendency, as a faster accumulation of H₂O₂ for Cu-L₁ and Cu-L₂ was observed compared to Cu-L₀ or Cu-L₃. One could expect a high efficiency of Cu-L₁ and Cu-L₂ in terms of ROS production with ascorbate in biological medium. Therefore, the activity of these Cu-L complexes on bacteria *E. coli* and AGS (cancer cell line) cells in culture were evaluated.

Antimicrobial activity of Cu-L₀₋₃ in *E. coli* cell lines. In order to measure the toxicity of the copper complexes Cu-L₀₋₃, the ligands L₀₋₃, as well as the influence of AscH⁻, the minimal inhibitory concentration (MIC) for *E. coli* cells were determined. The concentrations of Cu-L and L were limited to 100 μM, because we were interested in compounds with quite high activity. All ligands and their copper complexes, as well as CuCl₂ had a MIC above 100 μM, in absence and even in the presence of up to 3mM AscH⁻. Upon these surprising results, we verified if we could have Cu^{II} dissociation from the complexes Cu-L₀₋₃, as the medium contains high concentrations of biomolecules. Absorption spectroscopy did not give any indication for such a dissociation for Cu-L₀₋₃.

Potential explanations for these results are either the amount of ROS produced is too low for toxicity as it occurs mainly extracellularly, or that the Cu complexes, the ligands and AscH⁻ cannot react due to different localization of at least two of the three.

To improve the activity, we decided to graft the most active Cu-L₂ to an antimicrobial peptide (AMP) H-WKWLKKWIK-NH₂, known to interact with the bacterial membrane.³⁶ This peptide was studied by us before and the MIC of 4 μM for *E. coli* was confirmed.³⁷ However, in this study, the ligand H-LKHLWKWLKKWIK-NH₂ obtained by addition of the Cu-binding motif LKH (known as the ATCUN (amino terminal copper and nickel) motif) to the AMP and the corresponding Cu-complex did not show any improvement of the antimicrobial activity. That has been tentatively assigned to the very low redox activity of the Cu-ATCUN complex, hence generating very low ROS production. Thus, we sought to redo the experiment with a highly redox competent Cu-binding motif, *i.e.* L₂. The ligand L₂ was bioconjugated to the AMP to generate L₂-WKWLKKWIK-NH₂. This bioconjugation did neither perturb the Cu-binding nor the catalytic activity of AscH⁻ oxidation by O₂ in HEPES buffer (see Fig. S10 and S11).

However, when measuring the MIC on *E. coli* cells, no improvement of the MIC by Cu-L₂-AMP compared to the AMP alone was observed, even in the presence of ascorbate. So, it seems that attaching a stable and redox competent Cu-L₂ complex to the AMP did not add any further activity. This raises questions about the strategy for using a Cu-complex bound to an AMP as a ROS producing catalytic center.

Indeed, in the test tube Cu-L₂ was about 10³ times faster in ascorbate oxidation compared to Cu-ATCUN, a Cu-binding motif often studied in this context and evaluated by us with the same AMP.³⁸ But this higher redox activity of Cu-L₂-AMP did not improve the antimicrobial activity as compared to Cu-ATCUN-AMP.

Cell toxicity of Cu-L₀₋₃ in AGS cancer cell line. The activities of the complexes Cu-L₀₋₃ on the cell line AGS (Human Caucasian gastric adenocarcinoma) were evaluated and compared to those of the ligands L₀₋₃ and in presence or absence of AscH⁻. AscH⁻ concentration was limited to 50 μM, a concentration in the range of AscH⁻ in the blood plasma and not toxic to AGS cells (see Table 2 and Fig. S12).

Table 2: 50% Inhibitory concentrations of L₀₋₃, CuCl₂ and Cu-L₀₋₃ on AGS cells:

Ligand	IC ₅₀ (μM)		Complex	IC ₅₀ (μM)		
	no Asc			no Asc	Asc 50 μM	
L ₀	5-10	4-6 ^a	Cu-L ₀	5-10	3-7 ^a	5-20
L ₁	10-20	20-31 ^a	Cu-L ₁	20	4-6 ^a	20-50
L ₂	> 100	>50 ^a	Cu-L ₂	20-50	2-4 ^a	20-50
L ₃	> 100	>50 ^a	Cu-L ₃	5-10	n.d. ^a	5-10
			CuCl ₂	> 100	n.d. ^a	> 100

^a: range of values obtained against four cancer cell lines (A375, 2008, A431, HCT-15) from reference³⁹

Among the ligands alone, L₀ showed the highest toxicity, followed by L₁, and no toxicity was observed for L₂ and L₃ up to 100 μM. Complex Cu-L₀ had very similar toxicity compared to the ligand L₀. Complex Cu-L₃ showed no toxicity up to 100 μM, as well as Cu without a ligand. A marked difference was observed for Cu-L₂, which showed a lower IC₅₀ value than L₂. Hence, this was the only case where the complex was more active than the ligand alone. Except for Cu-L₂, and to a lower extent Cu-L₁, these results are mostly in line with Jopp et al.³⁹ which measured the toxicity of the same compounds against different cancer cell lines (Table 2).

The cell viability experiments in presence of the complexes have been repeated with addition of AscH⁻. No significant impact of 50 μM AscH⁻ was observed on the cytotoxic activity of all Cu-L, including the most active Cu-L₂. Hence it seems that the toxicity of L₀ and L₁ is mostly due to the ligands alone and the expected ROS production in presence of AscH⁻ and Cu-L had no additional toxicity, despite Cu-L₁₋₂ were very competent in a buffer solution. L₂ was the only ligand showing a lower activity (IC₅₀ > 100 μM) than the corresponding complex Cu-L₂ (20-40 μM). This would correlate with its highest catalytic activity. However, addition of AscH⁻ did only slightly decrease the IC₅₀. The AscH⁻ addition effect being weak, we can notice that the AscH⁻ concentration was limited to 50 μM as higher concentrations led to toxic effect of AscH⁻ alone. Although 50 μM is a concentration range typical for blood serum, in our experiment it is added once, whereas in a blood serum AscH⁻ is steadily renewed.

The reason for this weak activity in eukaryotic and bacterial cell experiments is not known, but we could exclude instability of the Cu^{II}-L₂ in the medium. Possible reasons could be that: (i) Cu-L is not able to enter the cells and extracellular ROS production is less efficient for killing the cells; (ii) Cu is lost upon entering the cells, i.e. Cu-L dissociate; (iii) AscH⁻ does not localize with Cu-L, so they can't react; (iv) AscH⁻ concentration in AGS cell studies had to be quite low, so maybe higher concentrations would be more efficient. In *E. coli* higher concentrations of AscH⁻ were used, but no effect was observed even at millimolar AscH⁻ concentrations and (v) ROS are produced but this is not enough to kill the cells.

Conclusion

We evaluated a series of Cu-complexes with tripodal tripyridine ligands. The four known ligands L₀ to L₃ bind Cu^{II} with tetra-coordination. They differ only in the number of 5- versus 6-membered metal-chelate rings. These complexes have been characterized in HEPES buffer at pH 7.4, and in line with studies in the literature using different conditions, they showed similar Cu^{II} complex formation and a redox potential dependence correlated to the number and size of chelate rings.

The capacity of the four Cu-L complexes to catalyze the oxidation of AscH⁻ under aerobic conditions, was correlated to their structure. High catalytic activity of Cu-complexes in oxidizing biological reducing agent such as AscH⁻ is often correlated to the production of ROS. High ROS production is a strategy for killing cancer cell or bacteria. The order of activity for this series of complexes was Cu-L₀ < Cu-L₃ < Cu < Cu-L₁ < Cu-L₂. We identified two complexes Cu-L₁ and Cu-L₂ with higher AscH⁻ oxidation activity than Cu in buffer, a feature rarely observed with Cu-complexes of peptides and other ligands^{9,40,41} and on top they have a quite high stability due to the chelate effect of the tetradentate ligand.²¹

Despite the here presented work aiming for increased cytotoxicity, such as the use of tetradentate ligands for high stability, the capacity for fast ROS production and the bioconjugation of an AMP, the complexes were generally not more cytotoxic than the free ligands L. The only exception was L₂, where the chelation of Cu did increase toxicity slightly but only in AGS cells and not in *E. coli* cells. Together with the absence of any significant effect of AscH⁻ addition, this suggest that either ROS production does not occur due to different localization of catalyst and substrates or ROS production rate seems not to be the most critical event. This could point to the importance of other mechanisms for Cu-toxicity, such as thiol-binding and oxidation, transmetalation and protein aggregation.

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Author contributions

Collecting data: M.B.(Catalytic ascorbate tests and cell assay), Y.V.S. (H₂O₂ tests) B.V.(EPR), F.M.(Electrochemistry); Synthesis of compounds: A.S., A.M.S.; Methodology: E.G., F.M., B.V., V.L., L.R., M.I., B.B., P.H., A.S., P.F.; Supervision: E.G., C.O., V.L., L.R. M.I., B.B., P.H., A.S., P.F.; Writing – Review & editing, A.S, P.F., F.M., B.V, C.O.

Conflict of Interest

The authors declare no conflict of interest.

Materials and methods

Reagents and synthesis. All solvents and reagents obtained from commercial suppliers were used without further purification. The ligand L₀ (tris(2-pyridylmethyl)amine) was purchased from Sigma Aldrich. Ligands L₁, L₂ and L₃ were synthesized according to the literature.¹⁸ Compound methyl 6-(chloromethyl)nicotinate was obtained in two steps from dimethylpyridine-2,5-dicarboxylate in two steps following the procedure described in the literature.⁴² The synthetic procedures of the other intermediates, peptides and peptide-ligand conjugates are described in the Supporting Information.

UV-Vis absorption spectroscopy. Measurements were performed on a Cary 60 spectrophotometer at room temperature in quartz cuvettes (Hellma; 1 cm optical path). Reactants were mixed inside the quartz cuvette to the final volume of the cuvette. Titrations were performed by adding 0.2 eq (relative to ligand concentration) of copper chloride before each measurement. Absorbance values were corrected by dilution factor caused by copper solution additions.

EPR spectroscopy. Conventional field swept continuous wave EPR spectra were recorded at 100K with a micro X-EMX spectrometer (BrukerBiospin GmbH, Germany) equipped with a high sensitivity resonator (4119HS-W1, BrukerBiospin GmbH, Germany) and a continuous flow liquid nitrogen cryostat. g factor calibration was performed using Bruker standard (strong pitch, $g_{\text{iso}} = 2.0028$). To ensure homogeneous peptide distributions by preventing water crystallization-induced phase separation all samples were supplemented by 10% v/v glycerol (final concentration). Samples (*ca.* 50 μL) were introduced into 4 mm outer diameter EPR quartz tubes (Wilmad-Labglass) and freeze-quenched into liquid nitrogen prior to their introduction into the precooled cavity. Main instrumental parameters were: *ca.* 9.3 GHz frequency; *ca.* 0.1 mW microwave power; 5 G modulation amplitude, 100 kHz modulation frequency, conversion time and time constant were set up at *ca.* 200 and 80 ms, respectively; 1500 G were swept in 5 min. Labmade scripts based on *Easyspin* toolbox⁴³ under Matlab (Mathworks) environment were used to simulate experimental spectra. g strain was used to account for the experimental line-broadening. Superhyperfine couplings arising atoms coordinated N atoms were not considered. Unresolved Cu^{II} background (Sys2, in Figure S2) were taken into account for the simulation (Figure S2). Numerical values of hyperfine coupling constants and g factor were extracted accordingly and summarized into the Table S1.

Cyclic voltammetry. The cyclic voltammetry measurements were carried out in HEPES buffer (100 mM, pH 7.4) with a conventional three electrodes cell connected to a Princeton Applied Research VERSASTAT 4 potentiostat. A 3 mm diameter glassy carbon disk was used as working electrode and a platinum wire as counter electrode. A silver/ silver chloride electrode (3 M KCl) served as reference electrode. The solutions were degassed with argon for 10 min prior to the measurements, and the working electrode was thoroughly polished with 0.3 μm alumina slurry for 10 min before each scan.

Cyclic voltammetry measurements have also been realized in the same conditions, but with Cu(ClO₄)₂ as the Cu source. A negative potential shift of about 60-70 mV has been observed. This indicates the absence of chloride bound to the ionic metal, thus supporting the presence of a Cu-Cl bond with the use of CuCl₂.

Stock solutions preparation. A 100 mM stock solution of copper chloride was prepared in milliQ water from $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$. The concentration was verified by measuring the d-d band absorbance at 780 nm ($\epsilon = 12 \text{ M}^{-1} \cdot \text{cm}^{-1}$). A 500 mM stock solution of HEPES buffer was prepared in milliQ water, the pH was adjusted to 7.4 with addition of a 5M NaOH solution. A 50 mM stock solution of sodium ascorbate in milliQ water was prepared freshly before each experiment. A stock solution of catalase was prepared in milliQ water to a concentration of 1.25 g/L ($\geq 1.25 \times 10^7$ units/L). Stock solutions of ligands were prepared in DMSO at around 40-50 mM and their concentration was verified by spectrophotometric Cu^{II} titrations.

Ascorbate oxidation assay. Ascorbate oxidation was monitored by absorbance at $\lambda = 265 \text{ nm}$ ($\epsilon = 14500 \text{ M}^{-1} \cdot \text{cm}^{-1}$) on a Clario Star plate reader in 100 mM HEPES buffer (pH = 7.4) at 25°C . Reactants were mixed inside a 96-wells plate to a final volume of 100 μL . Starting concentration of AscH^- was 100 μM . When needed, ROS scavengers were added to the medium (catalase 15 mg/L to scavenge hydrogen peroxide or DMSO 5% v/v to scavenge hydroxyl radical). After monitoring during 5 to 10 minutes, copper source (free or complex) was added to reach the targeted concentration (0.3 μM ; 1 μM ; 5 μM depending on the experiment). Complexes with different Cu/ligand ratios (1:1.2 ; 1:2.4 ; 1:5 ; 1:10) were pre-formed in milliQ water before addition to the medium.

Catalase from bovine liver (≥ 10.000 units/mg) was purchased from Sigma Aldrich. Each experiment was performed three times, in different days, with solutions freshly prepared from stock solutions. Initial oxidation rates were obtained by linear regression of the 4 first minutes following copper source addition to the medium.

Antimicrobial activity assay. *E. coli* derived from ATCC® 25922™ Ref 0335P Lot 335-235-2 from Microbiologics, 200 Cooper Avenue North, St. Cloud, Minnesota USA 56303 was spread from glycerol stock on Mueller-Hinton (MH, from Sigma) agar plates then colonies are used to prepare a 10 mL preculture in MH broth at pH 7.4. This preculture is grown overnight at 37°C . The preculture is diluted 10 times (in 10 mL of MH broth) and grown at 37°C for about 3-4 hours to reach an OD_{550} between 1 and 2. OD_{550} is adjusted to 0.2 and diluted 1000 times to obtain a suspension corresponding to $1\text{-}2 \cdot 10^5 \text{ CFU} \cdot \text{mL}^{-1}$.

L_{0-3} stock solutions were diluted in HEPES 50 mM at pH 7.4 with or without copper chloride to reach a ligand concentration of 600 μM (*i.e.* 6 times the highest final concentration) with a 1:1 copper/ligand ratio for concerned samples. Ascorbate was added at concentrations of 3 mM. The antimicrobial assay was performed in 96-well microplates (F-bottom sterile non treated polystyrene, Nunc, Thermo Scientific Nunc A/S, Roskilde, Denmark. In the first column, 50 μL of L_{0-3} or $\text{L}_{0-3}\text{-Cu}$ was added to 50 μL of MH broth 2X (2 times concentrated). The medium is mixed by up/down pipetting ten times, 50 μL of this column is added to the next column. Thus, a 2-fold dilution is performed successively from column 1 to 10 (with column 2-10 previously loaded with MH 1X). In each plate, positive controls (without ligand nor copper complex) were obtained by adding HEPES 50 mM pH 7.4 to MH broth 2X. Negative controls were obtained the same way.

Finally, 50 μL of the bacterial suspension obtained previously were added to each well (except the negative controls where 50 μL of MH 1X was added). Then, 50 μL of culture medium containing or not ascorbate (depending on tested condition) is added.

Thus, each well contains 0.5-1.10⁴ CFU in final volume of 150 μ L. The final ligands/copper complexes concentrations range from 100 μ M to 0.2 μ M. Each condition was performed as triplicates. Microplates were incubated for 18 hours at 37°C without agitation.

After incubation, OD₆₀₀ is measured. Percentage of growth for each condition is calculated relatively to respective positive controls (after subtraction of the average value of respective negative controls). The MIC₁₀₀ is determined as the lowest concentration inhibiting the bacterial growth.

Toxicity against Adenocarcinoma Gastric Cells (AGS). In 96-well plates, 10,000 AGS cells per well were incubated in RPMI medium at 37°C and 5% CO₂ for 24 hours.

Ligands and complexes stock solutions were diluted in RPMI medium to reach final concentrations from 0.5 to 100 μ M. 100 μ L. They were incubated for 48-hour at 37°C and 5% CO₂ atmosphere.

After treatment, cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay. After incubation, medium is removed from each well and 100 μ L of MTT (0.5 mg/mL) in RPMI is added. Cells are incubated 90 minutes at 37°C with 5% CO₂ atmosphere. The medium is then removed and 100 μ L/well of DMSO is added. The absorbance at 590 nm (corresponding to the absorption of formazan formed by MTT reduction) is monitored to assess cell viability.

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