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# Copper-binding motifs Xxx-His or Xxx-Zzz-His (ATCUN) linked to an antimicrobial peptide: Cu-binding, antimicrobial activity and ROS production

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## ABSTRACT

Depending on the coordination, copper ions can have a very high activity in catalyzing the production of reactive oxygen species. Thus interest arose in increasing the activity of antimicrobial peptides (AMPs) by equipping them with a Cu-binding unit. Several examples, native and engineered, have been investigated with the motif Xxx-Zzz-His, called Amino Terminal Cu(II)- and Ni(II)-binding (ATCUN) motif. Here we investigate a short AMP that was equipped either with Xxx-Zzz-His or Xxx-His. Xxx-His is a shorter motif and yields a more redox active copper complex. The control AMP, Xxx-His-AMP and Xxx-Zzz-His-AMP were investigated toward Cu-binding, Reactive Oxygen Species (ROS) production and antimicrobial activity in *E. coli*. The data indicate that these Cu-binding motifs have very limited impact on antimicrobial activity and low ROS production capability.

## 1. Introduction

Copper (Cu) is an essential element for almost all living beings. It serves mainly as catalytic center in enzymes, typically as a redox center cycling between Cu(I) and Cu(II). A prominent reaction is dioxygen activation, like in cytochrome *c* oxidase [1]. Apart from these Cu-enzymes, organisms have an entire machinery to transport Cu and to control its metabolism. This is necessary as free or loosely bound copper can be highly active in catalyzing the production of Reactive Oxygen Species (ROS) [2,3].

It is well known that, in vitro, peptides can bind Cu(II) quite strongly with conditional  $K_D$  (i.e. pH 7.4) down to the femtoM. The two best known and strongest motifs are the N-terminal binding sites consisting of Xxx-Zzz-His— (XZH, also called ATCUN for Amino Terminal Cu(II)- and Ni(II)-binding motif) and Xxx-His— (XH) with a free N-terminal primary amine (Xxx can be any amino acid, and Zzz any but not Pro) [4–8].

At pH 7.4, the  $K_D$  of XZH and XH motif are between  $10^{-12.4}$ – $10^{-14.7}$  M and  $10^{-11.3}$ – $10^{-13.2}$  M, respectively [7]. To which extent they are bound to Cu(II) in nature is not clear. One well established case is human serum albumin which has a XZH motif with a  $K_D$  of  $10^{-13}$  M [9]. Serum albumin in humans is only loaded to a few % with Cu, indicating that these motifs may be able to bind Cu(II) in vivo [10].

The very small and simple motifs have been utilized for different applications, in particular XZH. This includes application in imaging, ROS quenching, artificial enzymes etc. [7] A very interesting application of Cu-binding to peptides is in the field of antimicrobial peptides (AMPs). Antimicrobial peptides are classically small peptides (up to 50 amino acids long), cationic and target different type of microorganisms [11–13]. Their mechanism of action is mostly based on the interaction with membranes, which are classically more negatively charged in bacteria than in eukaryotes [14]. Different membrane interactions and toxicity mechanisms are reported, such as destabilization or disruption of the membrane, pore formation, and penetration into the cell interior [11].

One of the main interest in Cu-binding to AMPs is based on the high potential of Cu as a catalyst to produce ROS, which could increase the antimicrobial activity due to the damage ROS can perform on biomolecules (DNA, proteins, lipids, etc.) [8,15]. There are several native sequences of AMP known, that contain the XZH motif [16]. There is the possibility that these AMP acquire Cu(II) in the environment and the Cu-bound form has a modulated activity. This is supported by several reports of an increased peptide's antimicrobial (or antifungal) activity when copper is added [17–19]. However, generally modest effects were observed, limited to the same order of magnitude as the AMP without Cu. Another approach was to add a Cu(II)-binding motif to

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AMPs by elongating the AMP with a XZH motif [8,20–24]. In these cases an increased activity by a Cu-ATCUN moiety in AMPs was observed for AMPs proceeding through different mechanisms, suggesting a role for Cu-ATCUN motif not restricted to a single antimicrobial mechanism. It is generally explained by promoting oxidative stress [8,15,21–23,25], whether at the membrane (lipid oxidation) or inside the cell, and or via nucleic acid cleavage [8,20,22,26,27]. Noteworthy, it has been suggested that this nucleic acid cleavage by Cu-ATCUN complexes occurs through an oxidative mechanism [28–31].

Regarding ROS production, the shorter motif XH might be more promising than XZH: Cu(II)-XZH is almost impossible to reduce electrochemically to Cu(I) but can be oxidized to Cu(III) at about 1 V (NHE), while Cu(II)-XH can be electrochemically reduced but cannot be oxidized to Cu(III) [32]. Thus Cu(II)-XH is potentially more efficient for catalyzing ROS production in biological environments, where redox couple Cu(I)/(II) is accessible, in contrast to Cu(II)/(III). In addition, it has interesting coordination features. First, Cu-XH has a vacant equatorial coordination site, which can bind to another biomolecule (e.g. protein, amino acid, ions etc) that leads to a higher stability or bind a substrate (e.g. reducing agent) [33–35]. Second, while its affinity for Cu(II) is almost as high as for XZH at pH 7.4, it becomes stronger than XZH when lowering the pH. Lower pH is of interest at several places, like endosomes in cells or the intestine in the body. Despite these advantages, so far no study on the antimicrobial properties of the shorter motif XH has been reported in comparison with XZH.

In the present study we investigate the reported AMP **1** [36], and its derivatives with the two motifs, XH and XZH, respectively **2** and **3** (Table 1). Thus, we were able to compare the two Cu-motifs in terms of AMP activity, with and without Cu. Peptide **1** was chosen from the literature because (i) it is easy to produce as it is short, (ii) it's quite high antimicrobial activity with minimal inhibitory concentrations in the low  $\mu\text{M}$ , (iii) its relatively low toxicity toward human cells and (iv) being a polycationic amphiphilic peptide it is interacting with the membrane and hence could localize Cu-based ROS production to the membranes [36,37].

## 2. Experimental section

### 2.1. Peptide synthesis

Resin (Rink Amide AM,  $0.74 \text{ mmol}\cdot\text{g}^{-1}$ ),  $N_\alpha$ -Fmoc (9-fluorenylmethoxycarbonyl) protected amino acids, coupling agent HCTU (o-(6-chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) and DIPEA (N,N-Diisopropylethylamine) were purchased from Iris Biotech. Trifluoroacetic acid (TFA) and triisopropyl silane (TIS) were purchased from SigmaAldrich. All peptides were synthesized on solid phase following classical Fmoc/tBu strategy (double coupling; 4 eq. Fmoc-aa-OH, 4 eq. HCTU, 12 eq. DIPEA). After deprotection and cleavage (TFA/TIS/ $\text{H}_2\text{O}$  95:2.5:2.5; 2 h), peptides were precipitated and washed two times with cold  $\text{Et}_2\text{O}$ . Mass of the peptide was measured on an ultra-high performance liquid chromatography (UHPLC, Thermo Scientific Accelerate, Hypers II GOLD column  $1.9 \mu\text{m}$   $2.1 \times 50 \text{ mm}$ ) coupled to a mass spectrometer (Thermo Scientific LCQ XL linear ion trap) (Fig. S2). The crude peptides were purified with preparative reverse-phase high performance liquid chromatography (HPLC, VWR LaPrep  $\Sigma$  equipped with a column XBridge C18 BEH 300 Å,  $5 \mu\text{m}$ ,

$19 \times 150 \text{ mm}$ ), using a gradient of solvents A ( $\text{H}_2\text{O}$ , 0.1% TFA) and B ( $\text{MeCN}/\text{H}_2\text{O}$  9:1, 0.1% TFA). The purity of fractions was evaluated with reverse-phase analytical HPLC (Hitachi Primaide equipped with a column XBridge C18 BEH 300 Å,  $5 \mu\text{m}$ ,  $4.6 \times 150 \text{ mm}$ ,  $37^\circ\text{C}$ ) (see Fig. S1), and pure peptides were lyophilized. In order to replace the TFA counter anions by chloride, the peptide powder was dissolved in 2 mM HCl then lyophilized, three times.

#### 2.1.1. Peptide stock solutions

Peptide solutions (1–2 ml) were obtained dissolving the dry peptide.Cl salts in milliQ water, typically 5–10 mg. The concentration was calculated based on the molecular weight of the peptide and the counter ions (considering 1  $\text{Cl}^-$  per basic residue (His, Lys) and terminal amine), and we confirmed the concentrations of the peptides by absorption at 285 nm using  $\epsilon^{280\text{nm}} = 5690 \text{ mol}\cdot\text{L}^{-1}\cdot\text{cm}^{-1}$  per tryptophan and hence  $17,070 \text{ mol}\cdot\text{L}^{-1}\cdot\text{cm}^{-1}$  for all three peptides, containing each three tryptophans [38]. For peptides **2** and **3**, the concentration of these solutions was corrected by copper titration. These stock solutions were used to prepare aliquots for antimicrobial assays and for all experiments.

#### 2.1.2. Preparation of Cu(II) stock solution

$\text{CuCl}_2$  salt was dissolved in milliQ water 100 mM  $\text{CuCl}_2$  in milliQ water in measuring flask (100 mM final concentration). The concentration was double-checked by UV-Vis absorption spectroscopy, using  $\epsilon^{780\text{nm}} = 12 \text{ M}^{-1}\cdot\text{cm}^{-1}$ .

### 2.2. Copper titrations

Electronic absorption spectroscopy (UV-Vis) titrations of peptides by a Cu(II) solution were performed on an Agilent Cary60 spectrophotometer, in a 100  $\mu\text{L}$  quartz cuvette (Hellma) 1 cm optical path. For each peptide, a solution of c.a. 1 mM was prepared in 100 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer pH 7.4, which was then titrated by a solution of  $\text{CuCl}_2$  of known concentration (10.0 mM).

### 2.3. Ascorbate assays

In a 96 multiwell plate (Half-area,  $\mu\text{CLEAR}^\circ$ , greiner bio-one), a well is prepared for each complex, free copper and blanc: 50  $\mu\text{L}$  HEPES 100 mM pH 7.4, 10  $\mu\text{L}$  Sodium Ascorbate 1 mM (except for blanc), and the volume of mQ water necessary to reach 100  $\mu\text{L}$  final volume (considering the volume of complex that will be added). For each well,  $A^{265\text{nm}}$  is measured with a plate-reader CLARIOstar (BMG LABTECH) ( $31 \pm 1^\circ\text{C}$ ) every minute. After 13 min, 3.25  $\mu\text{L}$  of the copper complex solutions ( $[\text{peptide}] = 307.7 \mu\text{M}$ ;  $[\text{Cu}] = 153.8 \mu\text{M}$ ) or 2.5  $\mu\text{L}$  of  $\text{CuCl}_2$  (200  $\mu\text{M}$ ) is added, wells are homogenized and absorbance measurement is resumed.

### 2.4. Antimicrobial activity assays

*E. coli* bacteria (ATCC25922, Ref 0335-CRM, Thermo Fisher Scientific, Courtaboeuf, France) spread on Mueller-Hinton (MH) agar plates were used to inoculate a 10 ml preculture in MH media pH 7.4 (Fluka Analytical, Sigma Aldrich, Saint-Louis, MO, USA). After overnight growth, the preculture is diluted 10 times, grown up to  $\text{OD}_{550}$  1–2 before inoculation of test cultures (10 ml of MH) at an  $\text{OD}_{550}$  of 0.2. The test cultures were grown for 3–4 h at  $37^\circ\text{C}$  at 250 rpm, the turbidity was again adjusted to an  $\text{OD}_{550}$  of 0.2 and from this, a 1000-fold diluted bacterial suspension was obtained corresponding to  $1\text{--}2 \cdot 10^6$  CFU/ml (colony forming units).

Lyophilized peptide aliquots (100 nanomoles) were solubilized in 50 mM HEPES buffer pH 7.4 or in 90  $\mu\text{M}$  copper/50 mM HEPES buffer

**Table 1**  
Sequences of the antimicrobial peptides used in this study.

Peptide	Sequence	Comment
1	H-WKWLKKWK-NH <sub>2</sub>	Original sequence
2	H-KHLWKWLKKWK-NH <sub>2</sub>	Motif XH added
3	H-LKHLWKWLKKWK-NH <sub>2</sub>	Motif XZH added

pH 7.4 to get stock peptide concentration at 90  $\mu\text{M}$  ( $3 \times [C]_p$ ) and a 1:1 peptide/copper molar ratio.

The antimicrobial assay was performed in 96 well microplates (F-bottom sterile non treated polystyrene, Nunc, Thermo Scientific Nunc A/S, Roskilde, Denmark). The adsorption of the peptides to these microplates was evaluated and estimated to be about 10% for a 4  $\mu\text{M}$  peptide solution, so largely below the error of the measurement. Moreover, all three peptides **1**, **2** and **3** adsorbed to the same degree (see SI for details). In the first column, 100  $\mu\text{L}$  of the peptide solutions (at 90  $\mu\text{M}$  in 50 mM HEPES buffer pH 7.4 with or without copper) are added to 50  $\mu\text{L}$  of MH  $3 \times$  (3 times concentrated). Peptide and media are mixed by up/down pipetting ten times, then 100  $\mu\text{L}$  are transferred to the next column. So a 1.5 fold dilution is successively performed from column 1 to 10 previously loaded with 50  $\mu\text{L}$  of MH  $1 \times$ . For each plate, positive controls for bacterial growth (without peptides) were obtained by adding 100  $\mu\text{L}$  of 50 mM HEPES buffer pH 7.4 with or without copper to the media. Negative controls for contamination (without bacteria) are performed as well.

Finally, the previously prepared bacterial suspension was distributed by adding 50  $\mu\text{L}$  to each well (except for the negative controls) resulting in  $5.10^4$  and  $1.10^5$  CFU/well in final volume of 100  $\mu\text{L}$ . The final peptide concentration ranges from 30  $\mu\text{M}$  to 0.8  $\mu\text{M}$  (columns 1 to 10). Each condition was performed as quadruplicates. Microplates are incubated at 37  $^\circ\text{C}$  for 18 h without stirring in the incubator (Mettler INB-400, Mettler GmbH + Co.KG, Schwabach, Germany).

To monitor the bacterial growth and to determinate the MIC<sub>100</sub> (Minimum Inhibitory Concentration, for complete inhibition of bacterial growth) of each peptide or peptide mixture, a spectroscopic moni-

toring at OD<sub>600nm</sub> as well as a colorimetric assay with resazurin were used [39]. After 18 h of incubation, OD<sub>600nm</sub> is measured. The % of growth for each condition is calculated by comparison to the respective positive controls (after subtraction of the average value of the respective negative controls). The MIC<sub>100</sub> is determined as the lowest peptide concentration inhibiting the bacterial growth.

### 3. Results and discussion

First the Cu(II)-binding of the peptides **2** and **3** was studied by electronic absorption spectroscopy (Fig. 1). The aim was to verify that they coordinate Cu(II) via the designed site, i.e. the XH in **2** (Fig. 1A,B) and the XZH (ATCUN) in **3** (Fig. 1C,D).

In the case of peptide **3**, addition of Cu(II) yielded a band with  $\lambda_{\text{max}} = 520$  nm, which is very typical for the d-d bands of Cu(II)-XZH. The band was increasing linearly up to 1 equivalent, and then a break point was observed (Fig. 1D) [4,7]. This clearly suggests that **3** binds 1 eq. of Cu(II) specifically to the XZH motif (Scheme 1). Noteworthy, above 1 eq. an absorption increase over the entire spectrum was observed (Fig. 1C), as followed at 400 nm and 900 nm, two wavelengths outside the zone of d-d transitions of Cu(II) (Fig. 1D). This increase is attributed to light scattering by precipitation of the excess of copper since (i) precipitation became visible in the cuvette, (ii) absorbance increase at 400 nm was higher than at 900 nm and (iii) previously reported for other Cu-XZH [32].

The titration of **2** with Cu(II) yielded a d-d band with a  $\lambda_{\text{max}} = 600$  nm, a typical value for Cu(II) bound in the XH motif [4,7]. Following A<sub>600nm</sub>, no clear breakpoint was observable at 1 equivalent of Cu(II). However, similarly to the titration of **3**, above 1 eq. of Cu(II),

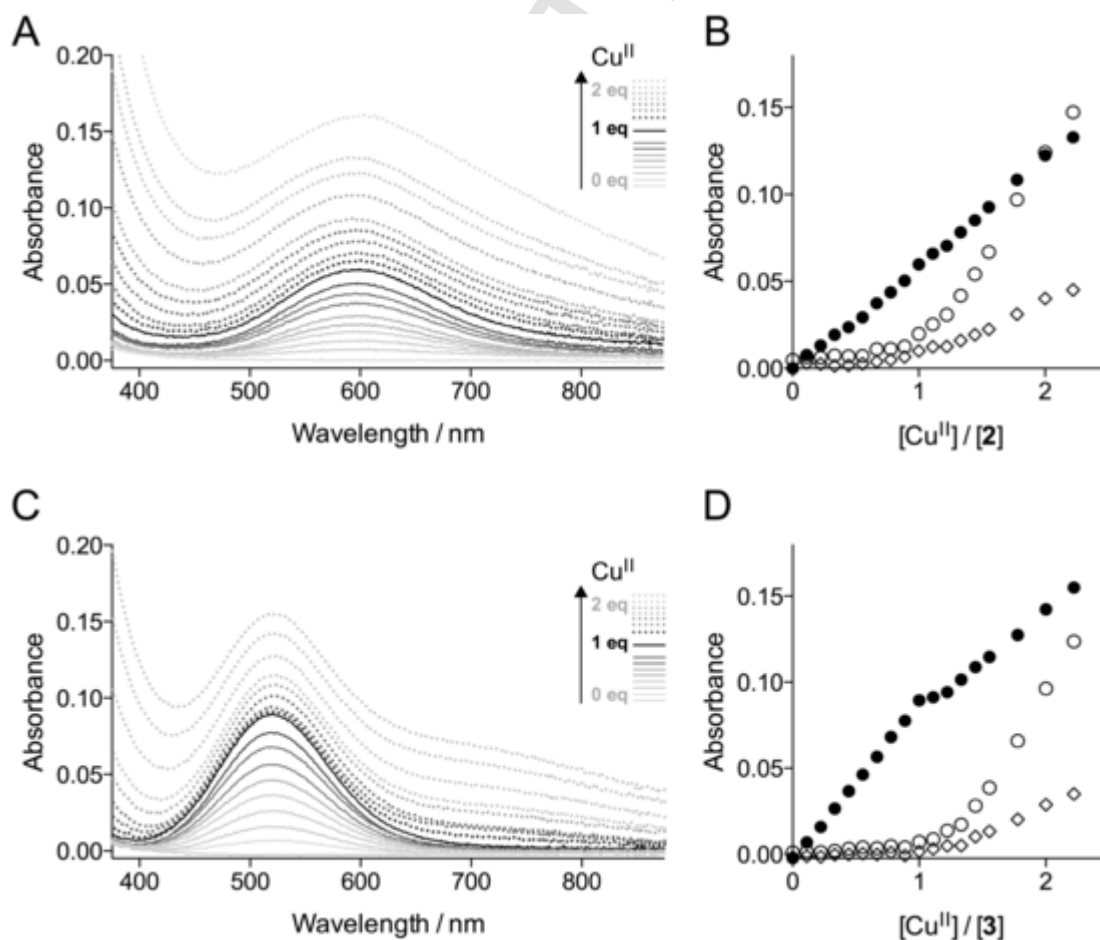
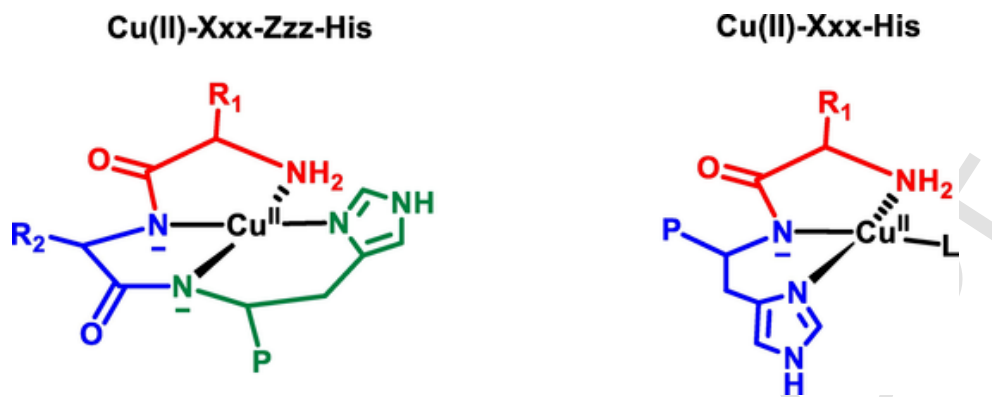


Fig. 1. Copper(II) titrations of **2** (top) and **3** (bottom) followed by electronic absorption spectrophotometry. A,C) Absorption spectra. B,D) Evolution of A<sub>400nm</sub> (open circle), A<sub>900nm</sub> (open diamond) and A <sub>$\lambda_{\text{max}}$</sub>  (black circle), with  $\lambda_{\text{max}} = 600$  nm for Cu(II)•**2** (B) and  $\lambda_{\text{max}} = 520$  nm for Cu(II)•**3** (D).



**Scheme 1.** Representation of the Cu(II)-complexes of the two Cu(II)-binding motifs Xxx-Zzz-His-P and Xxx-His-P. L represents an external ligand (such as H<sub>2</sub>O). R<sub>1</sub> and R<sub>2</sub> represent amino-acid side chains and P stands for the continuation of the peptide.

absorption increase over the entire spectrum (Fig. 1A), as can be followed at 400 nm and 900 nm (Fig. 1B), indicating the precipitation of the excess of copper. Therefore, in line with the interpretation above, after 1 eq. of Cu(II), we attribute the increase of absorption at 600 nm above 1 equiv. to light scattering, and not to Cu(II)-binding.

The interaction of Cu(II) with the control peptide **1** showed the UV-Vis signatures of several d-d bands and some precipitate (not shown). This is in line with the presence of several species of Cu(II)-coordination as expected for peptides with non-coordinating side chains such as poly-Gly peptides [5,40].

Hence these studies strongly support the Cu(II)-binding to **2** and **3** according to the initial design (Scheme 1) and confirmed the 1:1 stoichiometry.

In order to evaluate the antimicrobial activity of **1**, **2** and **3** and their Cu(II)-complexes, the minimal inhibitory concentrations (MIC<sub>100</sub>), at which no bacteria grow, were determined on *E. coli* (Fig. S3). The results are summarized in Table 2. **1** was a relatively potent antimicrobial peptide with a MIC<sub>100</sub> of 4 μM (error ± 2) in line with the value reported in the literature [36]. The two variants **2** and **3** that were elongated by 3 or 4 amino acids to embed a Cu-binding site (Table 1) showed the same MIC, indicating that the addition of these 3 or 4 amino acids did neither increase nor decrease the antimicrobial activity on *E. coli*. The Cu pool available for chelation was measured by the bathocuproine disulfonate (BCS) assay and no labile and accessible pool could be measured with a detection limit of 0.2 μM (see Figs. S4 and S5). This indicates that the MIC<sub>100</sub> determined for **2** and **3** indeed reflects the activity of the free peptides, rather than that of peptides metalated with Cu(II) from the medium. This is in line with the design, in which the phases of positive and hydrophobic amino acids have been conserved (His counts as positive AA). However, the MIC did also not change when the Cu-complexes were preformed, i.e. Cu-peptides Cu(II)•**2** and Cu(II)•**3**. Also, for the control peptide **1** with no specific

Cu-binding site, addition of 1 equivalent of Cu had no measurable impact.

The rationale of adding a Cu(II) bound to an AMP is that Cu(II) is well known for their efficient catalysis of reactive oxygen species (ROS) due to their redox cycling between Cu(I) and Cu(II) and its potential to activate dioxygen. However, we have recently shown that Cu bound to several peptides with the motif XZH exhibit a very low catalytic activity on ROS production in the presence of dioxygen and the physiological relevant reducing agent ascorbate [41]. To test the ability of our AMPs and to compare the two different Cu-sites we measured the capacity of Cu(II)•**2** and Cu(II)•**3** to oxidize ascorbate leading to the production of ROS. This ascorbate assay was performed as described previously [41]. Here, ascorbate acts as the electron donor, reducing Cu(II) to Cu(I), which is then reoxidized to Cu(II) by O<sub>2</sub>, leading to the production of ROS (O<sub>2</sub>•<sup>-</sup> → H<sub>2</sub>O<sub>2</sub>, ...). Hence, the disappearance of ascorbate correlates with the generation of ROS [42]. As shown (Fig. 2A, black line), the background oxidation of ascorbate by O<sub>2</sub> is slow in an aerated solution at pH 7.4. However, when free copper is added (Fig. 2A, blue line), the initial rate of ascorbate oxidation is 200 times faster (Fig. 2B). Cu(II) in the buffer catalyzes the oxidation of ascorbate because it can cycle fast between Cu(II) and Cu(I). Therefore, the ascorbate oxidation rates of the different complexes were measured with an excess of peptides to avoid any free copper ion to interfere. The premixed Cu(II) + **1** (1:2) yields a similar result as Cu in the buffer. This is in agreement with the absence of metal-binding residues in the sequence of **1**. In contrast, addition of preformed Cu(II)•**3** (1:2), resulted into a very small acceleration (1.7 times, Fig. 2B) of ascorbate oxidation compared to the negative control (absence of copper), illustrating the poor ROS-generation ability of Cu•ATCUN, as reported previously [41]. Addition of preformed Cu(II)•**2** (1:2) to ascorbate resulted in about a two-times faster oxidation of ascorbate compared to Cu(II)•**3**, demonstrating the higher ability of Cu-XH than Cu-XZH for the generation of ROS. However, compared to free Cu the activity is still very low.

#### 4. Conclusions

We confirm here the antimicrobial activity of the nona-peptide **1**, which is active in the low μM range and hence quite interesting for such a short peptide, classical AMP being often longer. Moreover, the addition of KHL- or LKHL- residues at the N-term containing a His-residue did not decrease its activity. Hence two well-known Cu-binding motifs could be attached to **1**. Thus it seems that the antimicrobial activity of **1** is robust and might allow also the addition of other moieties to the N-terminus. However, the Cu(II)-bound forms did not increase or decrease the activity of the peptide. One of the motifs, XZH (or ATCUN) has been widely studied, either as it occurs natively in AMPs or as, like in our case, was added to an AMP. Prominent native AMPs with XZH motifs are histatins, hepcidins or piscidines (for recent review see

**Table 2**

MIC<sub>100</sub> of the peptides and their corresponding copper(II) complexes measured with *E. coli* (ATCC25922), expressed in μM.

	MIC <sub>100</sub> /μM	
	No metal	With Cu(II)
<b>1</b>	4 ± 2	4 ± 2
<b>2</b>	4 ± 2	4 ± 2
<b>3</b>	4 ± 2	4 ± 2
CuCl <sub>2</sub>	–	> 160

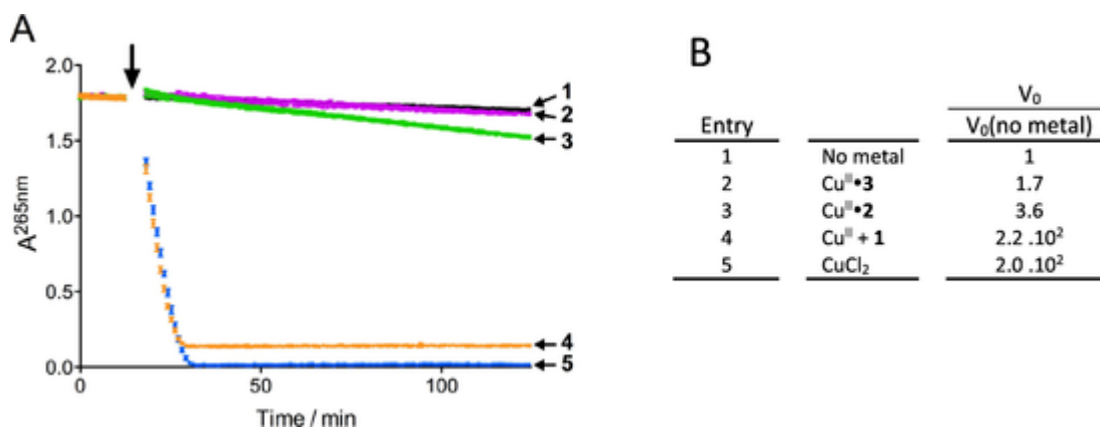


Fig. 2. A) Comparison of ascorbate oxidation by the copper-peptide complexes followed by absorption spectroscopy (at 265 nm). The bold arrow indicates when the Cu-peptide complexes (or controls) were added: mQ water (black),  $\text{CuCl}_2$  (blue),  $\text{Cu} + \mathbf{1}$  (orange),  $\text{Cu}\bullet\mathbf{2}$  (green),  $\text{Cu}\bullet\mathbf{3}$  (purple). Conditions: buffer was 50 mM HEPES pH 7.4, final concentrations are 100  $\mu\text{M}$  ascorbate, 5  $\mu\text{M}$   $\text{CuCl}_2$  and 10  $\mu\text{M}$  Peptide. Experiment was performed in triplicate. Error bars show the mean square deviation. B) Table depicting the initial rates of ascorbate oxidation relative to the uncatalyzed aerobic oxidation of ascorbate. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

Alexander et al.) [16]. However, when going through the literature the antimicrobial activity was rarely improved by the  $\text{Cu}(\text{II})$ -bound form, and in the case it was, the effect was modest (less than an order of magnitude). One of the potential explanation is that the  $\text{Cu}(\text{II})$ -bound to the ATCUN is very redox inert and hence  $\text{Cu}$ -bound to AMP does not produce efficiently ROS as it is also the case with  $\text{Cu}(\text{II})\bullet\mathbf{3}$  as shown here [41]. Moreover, upon reduction,  $\text{Cu}(\text{I})$  does not bind strongly to the ATCUN and hence is easily outcompeted by native  $\text{Cu}(\text{I})$  binders like cysteine or glutathione [41]. This points to the importance of peptides able to also bind  $\text{Cu}(\text{I})$ , like in the case of Histatins which showed one of the best improvement by  $\text{Cu}(\text{II})$  addition ( $\text{EC}_{50}$  diminished by a factor c.a. 4) [18].

In contrast to XZH (ATCUN), the antimicrobial potential of the motif XH has so far not been evaluated, despite the very different properties.  $\text{Cu}(\text{II})$ -XH has an open equatorial site, that potentially can bind a substrate or other molecule to make ternary complexes (Scheme 1). Moreover,  $\text{Cu}(\text{II})$ -XH can be reduced to  $\text{Cu}(\text{I})$  electrochemically. Indeed,  $\text{Cu}(\text{II})\bullet\mathbf{2}$  has a higher activity than  $\text{Cu}(\text{II})\bullet\mathbf{3}$  in ascorbate oxidation and concomitant ROS production (Fig. 2). Despite that, no gain in activity was observed upon  $\text{Cu}(\text{II})$  complexation of  $\mathbf{2}$ . A potential explanation is that first the ascorbate activity is still quite low, and probably more important, the affinity of XH for  $\text{Cu}(\text{I})$  is low and dissociation occurs rapidly.

In conclusion both motifs XZH and XH, which are so far the strongest  $\text{Cu}(\text{II})$  binding motifs for unstructured peptides, strongly bind and stabilize  $\text{Cu}(\text{II})$  but are poor  $\text{Cu}(\text{I})$ -binders. Hence they exhibit low redox activity (low ROS generation) and dissociation of copper upon reduction to  $\text{Cu}(\text{I})$ . Other motifs with better redox cycling are known, but do have a lower binding-constant. Thus, the modulation of AMP activities by  $\text{Cu}(\text{II})$ -ATCUN reported in the literature, are likely to be attributed to two non-exclusive mechanisms i) structural changes of AMP upon  $\text{Cu}(\text{II})$ -binding or ii) ionophore type activity, i.e. AMP helps copper to penetrate into the cell, where  $\text{Cu}(\text{II})$  is released in the cytoplasm after reduction to  $\text{Cu}(\text{I})$  e.g. by glutathione. Then, after this dissociation, AMP and  $\text{Cu}$  could have different targets.

## Uncited references

[43–45]

## Declaration of competing interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jinorgbio.2020.111255>.

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