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Ligands as a tool to tune the toxicity of Cu on bacteria: from boosting to silencing

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Abstract

Copper is an essential micronutrient for most living beings including bacteria. This is mainly due to essential roles of catalytic Cu-centers in enzymes. However, an excess of Cu is toxic and thereby used over centuries as a powerful antimicrobial agent. Bacteria developed several systems to detect Cu and protect themselves from high intracellular Cu concentration, namely *via* Cu-extrusion and/or Cu-storage. Several Cu-detoxification systems have been described and new ones are still being discovered. Progress has also been made in understanding the molecular mechanism of Cu-toxicity by identifying target macromolecules. Nevertheless, the importance of each mechanism is bacteria- and environment-dependent paving the way for new findings.

The chemical reactivity of Cu can be modulated by its coordination environment in a complex with a ligand (L) and this opens large opportunities to tune the Cu-ligand complexes (Cu-L) *via* ligand design. For instance, boosting the toxicity of Cu ions toward bacteria was promoted by several small organic ligands. These ligands can make Cu-L complexes with different properties in terms of coordinating atoms (S, N, O), Cu to L stoichiometry, ligand denticity, thermodynamic stability, kinetic inertia and redox potential. However, one common feature shared by Cu-L complexes which efficiently boost Cu-toxicity is the ability of Cu-L to cross the two bacterial membranes and to release the Cu in the cytosol. If this property is required to improve bacteria killing is not clear yet, but considering the ever-growing resistance of bacteria, it would be interesting in the future to try to boost Cu-toxicity via unprecedented mechanisms.

Ligands as a tool to tune the toxicity of Cu on bacteria: from boosting to silencing

1.1 Introduction

Before the Great Oxygenation Event, copper (Cu) was found as copper sulfide minerals such as chalcocite (Cu_2S) and chalcopyrite (CuFeS_2), impairing its bioavailability. In this anoxic environment, earliest anaerobic prokaryotes were not using copper as a metal center for their enzymes to operate [1]. Apparition of oxygen on earth led to the oxidation of copper, increasing its solubility, hence turning Cu into a bioavailable metal [2], [3]. Living organisms had to adapt to this drastic modification of their environment and they subsequently modify their metallome. For instance, in current aerobic organisms, key enzymatic reactions like the reduction of dioxygen into water are performed with copper-containing proteins [4].

However, such benefits came with its part of inconveniency. Indeed, while being a key element, Cu-chemical properties render this metal highly toxic for living organisms. Living cells had to face this paradox of an essential yet toxic element. General strategies have been developed by bacteria to remove any excess of copper. Nonetheless, saturation of these systems induces cell death. Such powerful antimicrobial activity enables beneficial use of Cu in several applications. A promising strategy is the development of synthetic copper complexes (Cu-ligands, referred herein as Cu-L) in which the ligand can drastically modulate the properties of copper ions and their mode of action. In this chapter we will mainly review research exploring the impact of copper and copper complexes on bacteria, and their mode of action. Cu-binding peptides are also attractive options as antimicrobial agents that we will further discuss. Overall, accumulation of recent studies in this field opens

new avenue toward potential use of Cu-based compounds in medicine as antibacterial agents.

1.2 Copper, a biocidal compound

The potential use of Cu-related compounds as antimicrobial agents is not a recent idea. Indeed, four thousand years ago, Egyptians already used Cu to sterilize water. Other historical documents revealed that during antiquity, Cu was also prescribed to treat some diseases. A more detailed overview of historical facts revealing utilization of Cu as biocidal compound can be found in other reports [5]–[7].

Nowadays, Cu is still worldly employed for its biocidal effect to prevent microorganisms spread: materials containing copper are used in wound dressing, filters, hygienic medical devices, and many others ([8], Figure 1).



Fig. 1. Current applications of copper in our society due to its antimicrobial properties.

As often, the use of the antimicrobial property of copper is not an original human invention, as natural copper-based defense systems were evolved to struggle against invading intracellular organisms. It is now clearly established that increased amount of copper (up to 400 μM) were measured in phagolysosomes after bacteria engulfment [9]–[11]. In contrast to other metals like iron or

manganese that are withheld, the immune system induces production and stimulation of copper pumps (Ctr1 and ATP7A) leading to a copper burst to destroy pathogens (Figure 2) [11], [12]. Silencing of the gene encoding for ATP7A clearly attenuated bactericidal activity of macrophages [13]. Accordingly, the deletion of *copA* gene (a bacterial gene encoding a pump to export any intracellular copper excess, see chapter 1.3) renders *Escherichia coli* hypersensitive to macrophage copper-dependent killing [13].

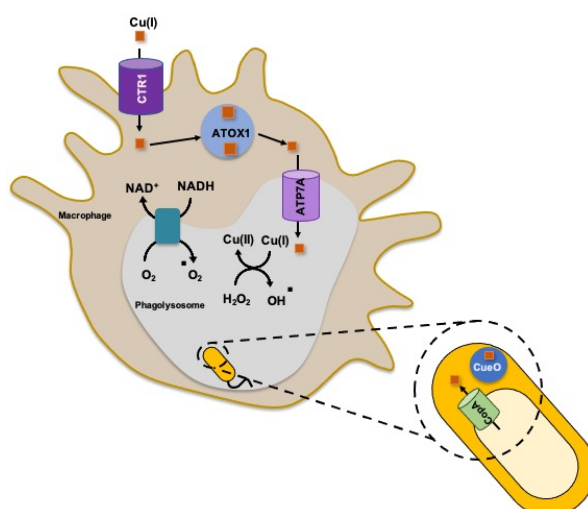


Fig. 2. Cu-dependent strategies of the immune system to kill invading microbes. After pathogenic bacteria engulfment, expression of the gene encoding for Ctr1 pump is stimulated, leading to Cu accumulation in the phagolysosome via the metallochaperone Atox1 and the ATPase ATP7A. Pathogenic bacteria (in yellow) will protect themselves from this increase in Cu content by overexpressing specific systems that pump out, transform or store Cu metals. In this scheme, the CopA pump and the multicopper oxidase CueO are represented as examples.

1.3 Copper homeostasis systems in bacteria

Copper traffic in cells is tightly controlled because on one hand it is essential for the function of key enzymes but on the other hand it will severely impact cell viability in case of intracellular excess. Cu trafficking occurs mainly in the periplasmic/membrane environment of Gram-negative bacteria where most copper enzymes

(superoxide dismutase, cytochrome oxidase, multicopper oxidase) are localized. How the Cu enters the cells is still unknown as no specific copper transporter has been identified so far. Passive diffusion *via* the outer membrane is suggested but needs to be further confirmed [14]. In some bacteria, the metallophores yersiniabactin, methanobactin or staphylopin are secreted to the extracellular environment where it can bind metals, like copper [15], [16]. Such metallophore could play an active role in specific strains to carry metals to the periplasmic compartment [17]. Periplasmic proteins called metallochaperones, will then coordinate any periplasmic Cu ions with the dual goal first to carry Cu to specific enzymes that require copper for their function, and second to prevent unwanted Cu reactions with other biomolecules [18], [19]. CusF is, for example, a well-known periplasmic metallochaperone which limits the amount of free Cu and releases it to pumps for Cu export outside the cells [20].

Any excess of Cu in the environment may lead to an increase of intracellular Cu. To prevent Cu-accumulation and maintain copper homeostasis, specific Cu pumps are overproduced such as CopA or the CusABC system which export Cu out of the different compartments (Figure 3) [18], [20]. The expression of Cu specific defense mechanisms is tightly controlled *via* two regulatory systems, one detecting Cu concentration in the periplasmic environment (CusS/CusR two-component systems in *E. coli*, Figure 3), the other detecting an increase in free copper in the cytoplasm (CueR in *E. coli*, Figure 3) [21], [22]. Other defense mechanisms exist such as the periplasmic enzyme CueO which oxidizes Cu(I) into Cu(II) to prevent the production of toxic reactive oxygen species (ROS) (see chapter 1.5.2 for Cu reactivity) [23]. Cu-storage proteins have also been discovered. The protein Csp binds up to 80 Cu atoms per tetramer and can be found in the periplasmic space and in the cytoplasmic compartment of some bacteria [24], [25]. In addition, non-protein molecules play a central role to maintain copper homeostasis. The cytoplasmic tripeptide glutathione (GSH) not only maintains a reducing environment but also interacts with copper in excess and confers additional copper tolerance [26]. All

these systems found in *E. coli* (Fig 3) are a non-exhaustive list of proteins known to be involved in copper homeostasis in bacteria. Other specialized systems are indeed found in other organisms. For example, in *Rubrivivax gelatinosus*, *copI* is a gene encoding a protein belonging to the cupredoxin family, usually known to be involved in electron transfer [27]. However, in this case, *copI* deletion leads to a strong sensitivity of the strain to copper [28]. The protein CopG found in several organisms including *E. coli* and *Pseudomonas aeruginosa* is involved in the cellular protection toward Cu stress [29]. Even in some strains of *E. coli*, a genomic island encoding for the *pco* systems (with an additional multicopper oxidase (PcoA), other periplasmic chaperones (PcoC and PcoE) and a two component copper sensor (PcoSR)), significantly increases the bacterial resistance to copper [18], [30].

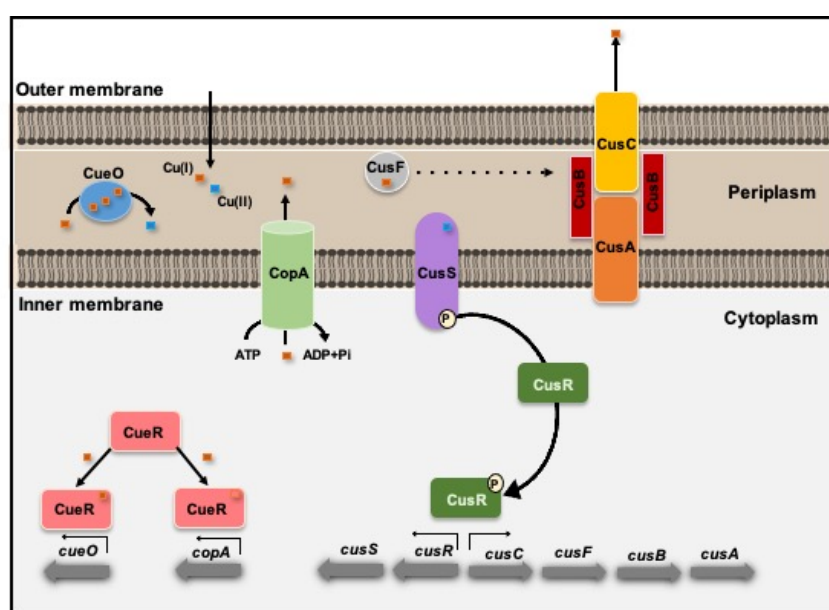


Fig 3. Copper homeostasis systems in *E. coli*. Increase of copper in the periplasmic compartment activates the CusS/CusR two components systems and leads to the induction of the CusR regulon. CusABC system exports periplasmic copper in the extracellular environment. CusF metallochaperone carries any copper excess to the Cus system to further remove any intracellular copper. Any increase in cytoplasmic intracellular Cu content will activate CueR regulator leading to the overexpression of *copA*(Z) encoding for

the inner membrane pump Cop. CueR also induces the expression of *cueO* gene encoding for a periplasmic enzyme, CueO, which catalyzes the oxidation of Cu(I) into Cu(II).

1.4 Copper toxicity: a phenomenon dependent on the bioavailability of copper and on bacteria.

Copper can be encountered in different habitats as a trace element. However, excess of this metal can be found in contaminated environments after mining process or extensive agriculture treatments. In such cases, environmental bacteria are directly impacted [31]. Even though high level of copper can be measured in such environment, its toxicity will be related to its bioavailability which is governed by kinetics and thermodynamics. Cu can bind to substances present in a medium or in the soil. Such complexation of the metal renders difficult to estimate the toxicity of an environment based on metal quantification. In soil for example, the total amount of Cu measured by conventional methods such as inductively coupled plasma - mass spectrometry (ICP-MS) or - optical spectroscopy (ICP-OES) will not provide clear information concerning the amount of bioavailable copper in the sample tested.

Pathogenic bacteria may also encounter high concentrations of copper upon infection of a host. Recent studies on the *Bordetella pertussis* strain have shown that specific copper homeostasis systems have been conserved to withstand copper excess and peroxide stress found in the phagolysosomes[32]. Depending on the complexation properties of the available compounds in its environment, several Cu-derived species may form, with different antimicrobial activities. This may be observed in a simple example: to kill *E. coli* cells, mM concentrations of CuCl₂ or CuSO₄ are required in Luria Broth medium while only μ M are sufficient in a minimal medium (mainly composed of phosphate buffer). Therefore, the most toxic environments correspond to the highest concentration of bioavailable copper which may be quite different from the highest amount of total copper.

In addition, different bacteria species do not survive the same concentration of bioavailable copper. Increasing number of experimental evidences highlight distinct strategies explaining such results. As an example, acidophilic organisms used in bioleaching process can resist copper concentration higher than 100 mM CuSO₄ [33], [34]; in comparison, *E. coli* survives 3 to 5 mM of CuSO₄ in aerobic conditions and in Luria Broth medium [35]. The origin of such resistance has been explained by the duplication of known Cu-resistance genes, new copper-chaperones, surface proteins acting as repellent and an abundant reserve of inorganic polyphosphate (polyP) [34], [36].

1.5 Specificity of copper chemistry

Even though most of the essential d-block metal ions are in the first row (4th period), Cu differs from other metal ions. According to Irving-Williams series, complexes of Cu(II) have the highest stability compared to the other divalent metal ions. Another characteristic is the occurrence of monovalent state Cu(I) (much less common for other biological essential metals like Fe, Mn, etc...), which implies a very high thiophilicity. In biological systems, Cu ions are mainly found as Cu(I) (Cu⁺, or reduced cuprous state) or Cu(II) (Cu²⁺ or oxidized cupric state). Here, we will rapidly introduce Cu coordination in pure water, coordination geometries found in cells at neutral pH, and then Cu reactivity in aerobic conditions.

1.5.1 Cu coordination chemistry

Cu(I) and Cu(II) are cations soluble in polar solvents. In water, Cu(II) is the main form encountered, and is coordinated with six water molecules. At higher pH, copper hydroxide Cu(OH)₂ is formed, with a very low solubility in water. Cu(I) is not stable in pure water: either it is readily oxidized to Cu(II) by dioxygen in aerobic conditions, or it disproportionates into Cu(II) and Cu(0) under anaerobic conditions. Hence, to exist as Cu(I) in aqueous

solvent, it needs to be stabilized by appropriate ligands. Interestingly, Cu(I) and Cu(II) prefer different coordination geometries, as referred in Table 1, which means that each of these two forms can be stabilized by different sets of ligands [37], [38]. Worthy of note, Cu(I) is a soft cation (the softest of the essential metal ions) and hence is very thiophilic. In addition, due to the difference in coordination chemistry of Cu(II) and Cu(I), the redox potential can be tuned over a wide range [39], [40].

	Cu(I)	Cu(II)
Preferred geometry	Digonal, trigonal, tetrahedral or penta-coordinated	Square planar, with one or two weaker axial ligands
Preferred ligands	Thiolates > thioether, amines > oxygen	Amines, sulfur > oxygen
Stability in aerobic condition	Easily oxidized	Stable
Stability in water (pH 7)	Not stable (disproportionation)	Stable
Solubility in water	$K_s^{CuOH} = 1.2 \cdot 10^{-6} (a)$	$(K_s^{Cu(OH)_2} = 2.2 \times 10^{-20})^b$
Interaction with thiols	Can bind strongly to thiols	Is reduced to Cu(I) by thiols

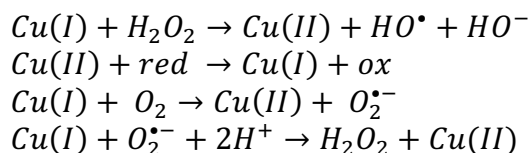
$$^a K_s^{CuOH} = \frac{[Cu^+] \times [OH^-]}{[CuOH]}$$

$$^b K_s^{Cu(OH)_2} = \frac{[Cu^{2+}] \times [OH^-]^2}{[Cu(OH)_2]}$$

Table 1. Key coordination properties of Cu(I) and Cu(II) to understand their behavior in biological environment.

1.5.2 Cu reactivity: the case of ROS production

Cu, like Fe, is a metal ion catalyzing the Fenton reaction. Cu(I) will catalyze the formation of highly toxic hydroxyl radicals (HO•) from hydrogen peroxide, a by-product of the respiratory chain. The Cu(II) generated can be reduced by reducing agent (e.g. ascorbate, glutathione (GSH) or any other thiol found in cells). Cu(I) will then further react with either hydrogen peroxide or directly O₂, as described in the following series of reactions (“red” denotes a reducing agent and “ox” its oxidized form):



Non-controlled Cu amount can thus be a source of reactive oxygen species (ROS) [41]. To prevent such reaction to occur, cells maintain at a low level both Cu(I) and Cu(II) species.

1.5.3 *In vivo* complexation of Cu

In cell, both Cu(I) and Cu(II) are present, in tight interaction with biomolecules (mainly proteins), which can either stabilize Cu(I) or Cu(II) or cycle Cu(I)/Cu(II) in redox active enzymes. Cu(II) and Cu(I) are found in the periplasmic environment of Gram-negative bacteria, whereas Cu(I) predominates in the cytosol. The presence of thiols (as GSH) in mM concentration explains why copper can be maintained in its reduced form [42]. Under growth condition with low level of Cu in the media, intracellular free Cu(I) is estimated to be extremely low. Around 10^{-21} M Cu(I) is found in the bacteria cytoplasm, compared to 10^{-15} M Zn [43], [44]. Actually, keeping intracellular free copper concentration as low as possible constitutes one of the main strategies to prevent intracellular Cu-toxicity.

1.6 Mechanisms of Cu toxicity: macromolecules targeted by copper

The bactericide property of copper is well-established. However, how exactly copper leads to cell death still requires intensive investigation. A pleiotropic impact of copper emerged as the most likely scenario (Figure 4). *In vivo* and *in vitro* studies gathered several evidences that Cu acts on any type of biological macromolecules. As previously mentioned, in aerobic conditions Cu is known to produce *via* the Fenton reaction highly toxic hydroxyl radicals, well known to lead to severe damages on proteins, but also

on DNA and lipids. For long time, Cu toxicity was believed to be mainly mediated by ROS production. However, it became difficult to explain bacteria sensitivity towards Cu under anaerobic conditions. Remarkably, Imlay *et al.* were able to demonstrate in *E. coli* that excess of Cu under anaerobic conditions lead to mismetallation of key enzymes [45]. Fe-S clusters are particularly vulnerable to Cu-transmetallation, in agreement with the high thiophilicity of Cu(I). Nonetheless, it starts to be accepted that accumulation of several intracellular damages (see Figure 4, and explained below) explains the high level of Cu toxicity toward all cell's kind.

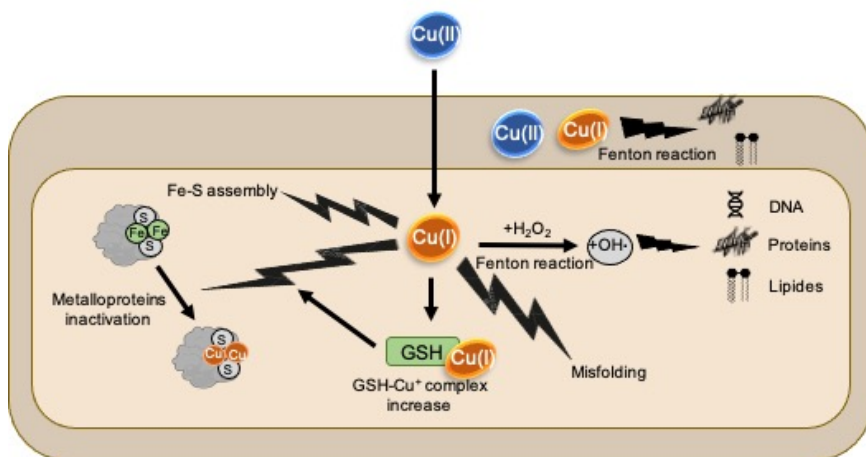


Fig. 4. Intracellular Cu impacts. Copper enters the bacterial cell *via* an unknown pathway. In aerobic conditions, copper can be found in the periplasmic compartment as Cu(I) or Cu(II). However, in the reducing environment of the cytoplasm, Cu(II) will be rapidly reduced into Cu(I). Cu(I) in both compartments can be involved in Fenton-type reactions to produce hydroxyl radicals that are highly reactive and toxic to macromolecules. Copper can also have a direct impact on lipids, proteins and nucleic acid by altering their structure. Free copper or probably the accumulation of glutathione-copper complexes will interfere with metalloproteins by the displacement of the physiological metal and the perturbation of the metallocenter assembly.

1.6.1 Membranes

Cellular membrane is in fact the first component Cu encounters, and lipids have been described as one of the Cu targets. Nevertheless, the action of Cu on membranes appears to be related to indirect effect *via* ROS production or *via* cysteine-binding to proteins, hence, leading to the modification of proteins and/or lipids that will end in the destabilization of the membrane integrity.

Membrane phospholipids such as phosphatidyl ethanolamine (PE) or phosphatidyl serine (PS) have been shown to coordinate Cu(II) ions *via* reactive groups such as the primary amine of PE found at its head group [46], [47]. Hence the production of ROS is locally increased explaining the high level of lipid oxidation in presence of Cu(II) [47]. Other reports confirm that Cu ions bound to the membrane will induce the production of hydroxyl radicals, which will react with lipids and induce lipid peroxidation of the double bonds of unsaturated fatty acids [48]. This phospholipid bilayer modification will further lead to a loss of membrane integrity.

A recent study described the impact of Cu on lipoprotein maturation by impairing its acylation [49]. Cu binding to the cysteine involved in the acylation process, prevented lipoprotein trafficking to the outer membrane. This mislocalization was proposed to additionally damage the membrane integrity and consequently its permeability. The binding of Cu to other key cysteines was also shown to impair peptidoglycan maturation which, per se, perturbs cell envelop and renders it more sensitive to detergents and other compounds [50].

1.6.2 DNA

Cu-toxicity has long been attributed to DNA damages. Indeed, several experimental evidences demonstrate *in vitro* a clear denaturation of DNA fragments in presence of increasing amount of Cu(II) [51]. By binding to phosphate and nitrogen ligands, Cu prevents the correct folding of DNA [52], [53]. Once bound, Cu further reacts and induces DNA break *via* the production of ROS [54]. However, all these earlier studies were performed in a non-cellular environment. In an environment containing millimolar GSH

concentrations, the affinity of DNA to the relevant redox state Cu(I) is low compared to Cu(I) binding to GSH and to other thiolate or sulfide containing proteins (such as those containing FeS clusters). The stronger complex DNA-Cu(II) would never form *in vivo* as Cu(II) will be reduced immediately by GSH. In addition, in *E. coli*, most of Cu ions are found in the periplasm. Very little amount may enter in the cytoplasm where the formation of hydroxyl radicals is unlikely given the high concentration of GSH in this compartment and its potency to stabilize Cu(I). In accordance, Macomber *et al.* demonstrated the absence of severe DNA damages in *E. coli* after Cu treatment [35]. In fact, the Cu binding capacity of DNA might be used by bacteria to scavenge extracellular Cu ions and prevent Cu intracellular damages. Dalecki *et al.* [55] proposed that such strategy could be used by bacteria pathogens to protect themselves against copper by the release of extracellular DNA once inside eukaryotic cells [56] or once they formed biofilm matrix notably composed of extracellular DNA [57]. Further studies might be of interest to better define the role played by DNA among bacterial strategies to survive copper excess.

1.6.3 Proteins

Proteins are one of the most sensitive targets to Cu overload in bacteria cells. Cu ions bind to O- N- and S- donors, elements that are found in the protein backbone as well as in several amino acid side chains (such as cysteine, methionine, histidine, etc..). Such variability of interactions leads to a multitude of impact on proteins and, *in fine*, on cells.

In aerobic conditions, intracellular Cu excess leads to severe oxidative damage linked to hydroxyl radical production *via* the Fenton reaction, as well as to the oxidation of cysteine residues which may form unwanted disulfide bonds. Deletion of periplasmic repair system known to fix abnormal disulfide bonds, decreases drastically *E. coli* survival upon Cu stress in aerobic conditions [58]. This was not observed under anaerobic conditions, underlying the different mechanism of action of Cu under anaerobic versus aerobic

conditions [59]. Cu might also inhibit the reduction of disulfide bonds in the periplasm, a process required to mature cytochrome *c* and this has been shown to perturb key pathways impairing cell survival [28]. Recently, methionine oxidation of the periplasmic protein CusF has been reported [60]. These results confirm the production of ROS in the periplasmic environment of *E. coli*. Under these conditions, periplasmic methionine sulfoxide reductase repairs the oxidized methionines of CusF, demonstrating the importance for cells to keep their copper homeostasis systems active by repairing the oxidation of their residues.

In addition, Cu(II) induces protein misfolding *in vitro* and this was recently demonstrated *in vivo* [59], [61], [62]. As described above, Cu(II) metal ion possesses a strong affinity for proteins compared to other metals, reflecting its place in the Irving-Williams series. Furthermore, Cu(I) shows a high affinity for thiols, such as cysteines. Hence, any intracellular concentration increase of Cu could lead to a strong perturbation of Zn-, Fe- or other metalloproteins, as intracellular concentration of metals have been shown to dictate some proteins metallation [63], [64]. Under anaerobic conditions, direct inactivation of metalloproteins by Cu has been demonstrated *in vivo* by Imlay *et al.* [45]. The authors clearly showed that the iron-sulfur enzyme (isopropylmalate deshydratase) is one of the first Cu-target in *E. coli*. Cu-induced protein inactivation leads to cell death unless amino acids were added to the media. Inactivation of iron-sulfur enzymes or other metalloproteins, as well as perturbation in iron-sulfur biosynthesis or heme biosynthesis has been further confirmed by other authors in other microorganisms [65]–[68].

Intriguingly, under anaerobic conditions, a lower concentration of Cu in the medium is required to kill *E. coli* cells compared to aerobic treatment [35]. This was at first unexpected as Cu-toxicity was thought to act mainly *via* ROS production. Instead, this result further accentuates the adverse impact of intracellular Cu on proteins in a ROS-independent manner. Recently, a correlation between a higher intracellular copper content observed under anaerobic conditions and an increased level of intracellular

aggregated-proteins has been clearly demonstrated [59]. Molecular chaperones have been shown to maintain the cellular proteostasis under copper stress conditions highlighting a central role of this family of proteins to protect cell against copper toxicity [59].

To summarize, the proposed model to explain bacteria death by Cu is more complexed than initially thought. Under aerobic conditions, intracellular copper excess as well as ROS-generated by Cu will interfere with all macromolecules and destabilize several pathways. Accordingly, a recent study highlighted several genes involved in the resistance of *E. coli* toward a prolonged copper exposure confirming the multiple impact of copper on the cell [69]. Under anaerobic conditions, Cu will destroy cells by inducing protein misfolding and/or by mismetalating catalytic sites and this will also lead to cell death. As mentioned in a recent paper by O'Hern *et al.*: "a fundamental understanding of Cu speciation and availability in cells is essential to uncover the cellular consequences of Cu cytotoxicity" [70].

Overall, Cu toxicity in bacterial cells is likely to be multifactorial: ROS production, mismetalation and binding to key aminoacids might be the main features that lead to global cell defect in bacteria. However, in the above-mentioned experimental studies, high Cu salt concentration (CuCl_2 or CuSO_4) was required to observe cell death. With the objective of using Cu as medical treatment, Cu-cytotoxicity needs to be improved. With such aim, Cu-complexes appear as appealing compounds and we will next develop the advances made in this domain.

1.7 Chemistry of Copper-complexes

The emergence of antibiotic-resistant bacteria has urged scientists to find alternative killing strategies in replacement to classic compounds. Developing new antibiotic agents is challenging, time consuming and might end with the apparition of new resistance mechanisms. Cu is currently reconsidered as therapeutic agent in complex or in combination with antibiotics [55].

A variety of ligands (hereafter designated as L) can form complexes with Cu ions through the formation of coordination bonds. Inducing drastic changes in the properties of Cu, a ligand can be rationally designed to obtain complexes for specific applications. For example, depending on its chemical groups, the global charge of Cu-L complexes can be negative, positive or neutral, hence changing the solubility in hydrophilic or hydrophobic environments as well as its affinity toward intracellular components. In this chapter we will give a rapid overview of the chemical properties of Cu-L that can be tuned by modifying the ligand.

1.7.1 Reactivity of Cu-complexes

Since Cu-cytotoxicity can arise from different chemical mechanisms (see section 1.6), ligands (L) are a good tool to tune it and/or control its specificity. Indeed, ligands will promote or prevent some of the mechanisms listed below.

a) redox-based: ligands modulate Cu redox potential (see 1.7.4.) modifying Cu redox reactivity. Moreover, a redox reaction often involves the binding of the substrate, replacing a labile ligand (e.g. H_2O), prior to the electron transfer. In that case, which is considered common for Cu-L complexes, the ligand (L) might influence the interaction with the substrate, from hindering it, to attracting or directing/orientating a potential substrate.

b) catalytic redox-independent: Cu(II) (but not Cu(I)) being a quite strong Lewis acid, biological relevant reactivity could include non-redox reactivity, such as hydrolytic cleavage of biomolecules via the modulation of the pK_a of Cu-bound water. In this case, for instance, a ligand can further change the pK_a . Of note, non-redox catalysis cannot be excluded as a potential mechanism involved in Cu-toxicity, but is often considered less likely.

c) pure coordination: the type and structure of the ligand (L) in Cu-L can influence how Cu binds to a biomolecule (BM), often a protein (Figure 5). This is especially relevant for ternary complexes L-Cu-BM. A ligand can modulate the interaction with BM via steric

hindrance or ligand-protein interactions (Figure 5). As a result, some biomolecules will be preferred targets based on the nature (i.e. chemical function) and exposition (i.e. accessibility) of their coordinating groups.

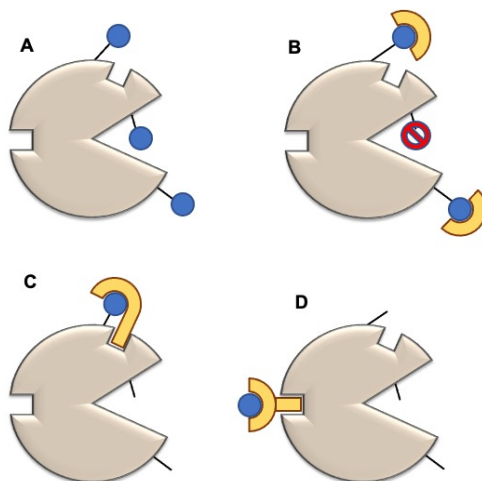


Fig. 5: Influence of ligand L on the interaction of Cu with biomolecule: A) ionic Cu (blue balls) can bind to several coordination sites. L (in yellow) can confer selectivity to Cu binding via ternary complex by different means: B) by steric hindrance of the L which confers selectivity to certain Cu-binding sites, e. g. Cu-L cannot bind to buried sites in a protein; C) by the interaction of the ligand with the target protein together with a Cu-coordination site of the protein or D) by binding of the Cu-L via weak interactions (e.g. H-bonds) but without coordination bond between Cu and target protein.

1.7.2 Stability of the Cu-L complex

The stability of metal-complexes against dissociation is a key parameter to define their intracellular behavior. Complex stability is often expressed as apparent dissociation constants (K_d), which are measured under a certain condition (pH, buffer, concentration, etc...). To compare the affinity among different complexes, these apparent K_d have to be measured at the same pH (often being pH 7.4) without any competitor (such as buffer). Besides, one must care of the stoichiometry of the complexes before comparing their K_d . When appreciating apparent dissociation constant of complexes, important concepts have to be considered, such as: chelate effect,

Pearson's principle (Hard and Soft Acids and Bases, HSAB), coordination geometry, charges (including partial and electronic densities). Of course, another important concern is the physiological context in which the metal-complex will be used. All of this needs to be taken into account in *in vitro* measurements to get a better idea of Cu-L complex intracellular stability and consequently intracellular behavior.

1.7.3 Kinetics of the Cu-L complex

The inertia of the Cu-L complex, *i.e.* how fast L can dissociate from a Cu-L complex and/or how fast an external ligand L_E can bind to Cu-L, can also play a role. Cu(II) and Cu(I) are quite labile metal ions. They can be exchanged quite rapidly in Cu-L, unless the affinity is very high or the ligands are very bulky and shield Cu. The latter is common in some proteins, but more difficult to obtain with smaller ligands. Here again, the design of the ligands will allow to tune the kinetics of dissociation for the purpose expected.

1.7.4 Redox potential

Cu ions in a Cu-L complex can have very different redox potentials. The main biological redox couple is Cu(I) / Cu(II). Depending on the properties of the ligand (geometry, number and type of coordination atoms (O, N, S, etc), and charge or electron density of the ligand, see Table 3), the redox potential can vary from below -0.4 up to $+0.8$ V versus NHE. This means that a range of potentials can be reached in which Cu can switch from quasi-inert behavior in biological environment to a high redox activity.

1.8 Impact of ligands on the biological activity of copper

As previously mentioned, modification of the ligands may change significantly the intrinsic properties of Cu. Drastic intracellular changes and impact on cell survival are expected. Four main

strategies drive the development of new ligands towards the tuning and control of the killing efficiency of copper-based drugs: i) improving membrane permeability, ii) improving the specificity of the reaction, iii) tuning the compartment targeted or iv) tuning the mechanism of action. In this chapter, we will discuss the different intracellular routes Cu-complexes could undertake.

1.8.1 *Localization*

Cu-L cellular localization is largely influenced by its charge: (i) hydrophobic Cu-L might localize into the membranes, (ii) less hydrophobic (yet not too hydrophilic) ones could cross the membranes, and (iii) highly charged ones are expected not to passively penetrate membranes. Noteworthy, a hydrophobic complex (neutral global charge, hydrophobic ligand) might have low solubility in aqueous media. Therefore, experimenters must care about precipitation.

1.8.2 *Presence in biology of potentially competing ligands for Cu(II)*

As mentioned above, biological media possess high amount of potential metal ligands such as glutathione, proteins etc... Concerning the extracellular and the periplasmic environments, where Cu(II) is stable, weak Cu-L can dissociate rapidly. In such case, any biological effects will be due to Cu and/or ligand only. To maintain intracellular Cu-L complex, a strong affinity is required to prevent dissociation. For instance, if a Cu-L is conceived to target bacteria in humans *via* the blood, the Cu-L has to resist to Cu-binding proteins present in that fluid, such as serum albumin (Cu(II) $K_d = 10^{-13}$ M) [71].

As a general rule of thumb, monodentate and bidentate ligands are generally too weak and will be dissociated easily, unless a stable ternary complex with an external ligand is formed (see chapter 1.8.5, Table 2). To keep the integrity of the initial Cu-L complex, stronger ligands, tridentate or tetradentate, should be favored. It is

noteworthy that ligands coordinating metals *via* only oxygen atoms have generally a moderate affinity for Cu(II).

While in the periplasm Cu(II)-L remains oxidized, Cu(II)-L entering the cytosol will be reduced in this compartment which contains high amount of thiols, e.g. 1-10 mM of reduced GSH. Thiols are very efficient reducing agents for Cu(II) and Cu(II)-L and quite strong ligands for Cu(I) (apparent K_d of low μM Cu(I) to 1-10 mM GSH is $\sim 10^{-16}$ M [42]). Actually, most of the Cu-L used as antimicrobial agents are relatively strong Cu(II) ligands, stable in the periplasmic compartment. Once such complex enters the cytosol, Cu-L will be reduced and dissociated if L is a weak ligand for Cu(I). As the coordination chemistry of Cu(II) and Cu(I) are very different, a ligand that strongly binds Cu(II) can be a weak ligand for Cu(I). Ligands that enable such transport of Cu into the cytosol have been referred to as ionophores (Figure 6). Here, the toxicity of the Cu-L will be in fact due to the Cu(I) and ligand released into the cytoplasm rather than directly to the Cu-L complex.

Highly stable Cu(II)-L complexes do exist. They are even maintained in the cytosol in presence of thiol compounds. However, in such a case, they have no redox activity and are less toxic [72] (e. g. Cu-atsm, see chapter 1.9.5).

Furthermore, the formation of ternary complexes can occur already outside the bacteria, in the cell medium or in the blood of the host for example. This can even more modulate the fate and activity of Cu-L. Indeed, extracellular ligands (e.g. amino acids and proteins) may either act as “scavenger/trap” of Cu-L, weakening its effect, or enhance its cellular uptake and hence its activity.

1.8.3 Presence in biology of potentially competing ligands for Cu(I)

Cu(I)-L complexes are unstable in presence of O_2 . They can be rapidly oxidized extracellularly or in the periplasm. In such case, dissociation of the Cu-L complex will occur because strong Cu(I)-

ligands are often weaker Cu(II)-ligands. However, O₂-stable Cu(I)-L exists, and if they are hydrophobic, they can passively enter in the periplasm and/or cytosol. They can be stable in the cytosol. The requirement is a stability constant high enough to resist GSH ($K_d < \text{fM}$) or to prevent the activation of the transcription factor CueR ($K_d < \text{zM}$) and the consequent expression of the Cu extrusion machinery. Such complex might not have strong intracellular toxic effect. Otherwise, Cu(I)-L can dissociate in the cytosol, exerting an ionophore activity.

1.8.4 Reactivity of Cu-L in bacteria

Based on several experimental evidences, some Cu-L show a clear increased bacterial toxicity in comparison to free copper (see chapter 1.9). This can be explained by our previous description of the reactivity of intracellular copper complexes which can be summarized as follows (Figure 6):

- a) Cu-L ionophore activity: the complexation of Cu serves to increase Cu transport efficiency through the membranes. Higher intracellular accumulation of free released Cu can easily explain the higher level of toxicity. In addition, the dissociated ligand could also perturb intracellular pathways. Most of the Cu-L characterized so far, belong to this category (See chapter 1.9).
- b) Gain of function *via* redox reactions: some Cu-L can undergo redox reactions, like reducing O₂ to form ROS or oxidizing essential biomolecules (cysteines, GSH, NADH, etc...). To have a high redox activity, the redox potential should be between ~ 0 to 0.3 V versus NHE, and the Cu-L has to rapidly cycle between Cu(I) and Cu(II). In this case, there is tradeoff between stability and reactivity. Indeed, to obtain a stable and highly reactive Cu-L complex, the ligand needs to bind strongly both redox states (Cu(I) and Cu(II)). This represents a real challenge as the two oxidation states prefer drastically different coordination spheres (in terms of geometry mainly, but also composition) [72].

c) Loss of function *via* binding: a Cu-L complex may be stable enough to react/bind, for instance, to an essential residue of an enzyme, such as cysteine (for Cu(I)-L or Cu(II)-L) or histidine (most likely for Cu(II)-L), which will lead to a loss of function. In that case, one of the ligands of the initial complex must be labile to be replaced by the targeted protein residue. It is also possible that Cu-L binds to a target *via* weak bonds (*e.g.* hydrophobic interaction and hydrogen bonds) in a pocket of its target and inactivates it (see case of Bis-thiosemicarbazones (bTSC) below, chapter 1.9.5).

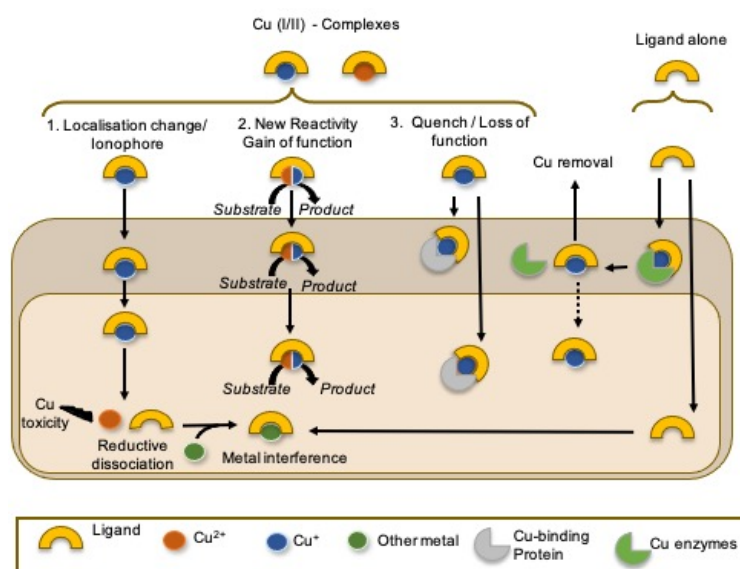


Figure 6: Possible routes and activities of Cu bound to ligands. The left part of the panel describes the activity of a Cu(I/II)-L encountering the bacteria with three conceptual routes. On the right part of the panel, the Cu-free ligand (L) encounters the bacteria, but once inside the cells, it will interact with endogenous Cu enzymes or interfere with intracellular metal homeostasis. For Cu-L, route 1 describes the ionophore activity (also called “mechanism I” in this review). The role of the ligand is to help the Cu to pass the membranes hence increasing the intracellular Cu concentration. Very often the Cu dissociates from the ligand triggered by Cu(II) reduction. The released ligand could bind other metals or interfere with other targets as well the Cu. Route 2 consists of a new function of the Cu-L. Classically, this would be a Cu-L catalysing a reaction, most likely the production of ROS (also called “mechanism II” in this review). In route 3 the Cu-L inhibits an essential function (loss of function) such as binding to an essential Cysteine in a cytoplasmic or periplasmic enzyme.

1.8.5 Reactivity of the free ligand in bacteria

Instead of providing the Cu-L complex directly to the cell, it could be possible to form it intracellularly by adding the ligands in the media that would bind endogenous Cu in the bacteria (Figure 6). This may have biological activity *via* two possible mechanisms:

a) Such interaction could perturb intracellular copper homeostasis and destabilize essential metabolic pathways, in addition to the intrinsic activity of Cu-L. An important prerequisite is the strong affinity of the ligand L for Cu. In the cytosol this is very challenging, as intrinsic protein (CueR) has already a very strong affinity (zM range [43]), and GSH binds Cu(I) in the low μM range [42]. In the periplasmic compartment, strong competitors are available. Indeed, even proteins that do not bind Cu under physiological conditions can have quite strong affinity for Cu(II) to compete against L. As an example, proteins containing a His in 2nd or 3rd position (from the first amino acid in N-terminal) which are quite common in biology (albumin, histatin, antimicrobial peptides ...) have K_d in the range of 10^{-12} M to $10^{-14.5}$ M [73]. Therefore, the design of L may be quite challenging, as its affinity must be suited to compete against endogenous Cu(II)- or Cu(I)-ligands.

b) An interesting and underexplored case is the formation of a ternary complex L-Cu-Protein. Here, a ligand added to the media could, after cellular insertion, bind to a copper center found in an essential enzyme. This will impair the function of the enzyme and kill the cells. Noteworthy, this strategy converges with previously described one, consisting in inhibiting an enzyme by targeting a cysteine or histidine in its active site with Cu-L. In this strategy, it is important to consider the denticity of the ligand and the favored stoichiometry of Cu/L complex(es) (Table 2). For, Cu(II), a tetradentate L has no vacant equatorial coordination site available for a strong bond, and hence ternary L-Cu-Protein is not stable. In contrast a bidentate ligand to Cu(II) can form two strong equatorial bonds in L-Cu-Protein. This could happen also after the release of one L in a Cu(II)-L₂ for example in Cu(II)-1,10-Phen₂ (see chapter 1.9.3).

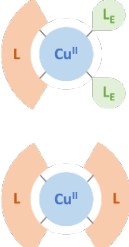

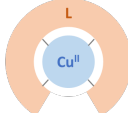




Ligand type	bidentate	tridentate	tetradentate
Complex formed with Cu(II)			
Thermodynamic stability	 High		
Kinetics (ligand exchange)	 Fast		
Formation of ternary complexes (e.g. with proteins)			
Reduction by physiological reducing agent	 Easy/Fast		
Example (for more details see chapter 1.9)	Cu(II)-1,10-Phen2 Cu(II)-HQ2	Cu-terpy ^a	Cu(II)-at-sm Cu(II)-gt-sm

Table 2: Cu(II)-L complexes general properties.

In green L_E correspond to external ligand. External ligands are classically water (or other solvents) or anions during the preparation of the complexes (e.g. halides). Upon addition in cell culture medium or application to living organisms, they are supposed to be exchanged easily with small molecules (water, amino acid, anions) or with proteins and hence form the ternary complex (L-Cu-BM).

a - [74].

As just discussed, several parameters can be modified to specifically modulate the properties of a Cu-complex. Even though several Cu-L have already been designed and show interesting antibacterial properties -the next chapter will provide some relevant examples-, all the possibilities presented above have not yet been

fully exploited and further efforts are required to improve our knowledge on the impact of diverse Cu-L on bacteria.

1.9 Copper complexes as antimicrobial agents, a non-exhaustive list

We will discuss some of the most prominent class of Cu-complexes (Cu-L) used as antibacterials. Some of these complexes are already used to treat cancer or neurodegeneration but they appear well-suited to fight microbes as a very low amount is sufficient to kill them.

1.9.1 Dithiocarbamates compounds

Dithiocarbamates (DTC) are a class of metal chelators that can be formed from thiuram disulfides by a two-electron reduction (Figure 7). They bind Cu(II) bidentately and can form Cu(II)-DTC and Cu(II)-DTC₂ complexes. Cu(II)-DTC₂ is neutral and the redox potential is quite low (Table 3). Thiuram disulfides have a much lower affinity for copper ions than DTCs. Thus in terms of copper-binding, thiuram disulfides are prodrugs, and need to be activated by reduction, which is supposed to occur mainly intracellularly by thiols. The most prominent example is the antabuse drug disulfiram (a neutral thiuram disulfide) that upon reduction yields the active DTC drug, diethyldithiocarbamate (DEDTC).

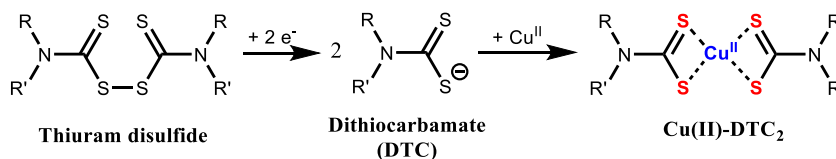


Figure 7: Reduction of thiuram disulfides to dithiocarbamates and subsequent Cu(II)-chelation. Several derivatives are studied depending on the R/R'. (R=R' = -CH₂-CH₃ for DETC, R/R' = cyclic -CH₂-CH₂-CH₂-CH₂- for pyrrolidinedithiocarbamate (PDTC)).

Thiuram disulfides, most likely the intracellular compound DTC, have been shown to have antibacterial activity on *Staphylococcus aureus* [75], on *Mycobacterium tuberculosis* [76] and on *P. aeruginosa* [77]. A link between the antimicrobial activity and DTC ability to bind copper was proposed but not clearly demonstrated. As a thiol agent, DTC might react with cysteine from key enzymes to form a thioester [77]. DTC might also be able to bind Cu(II) in the oxidizing periplasmic compartment of Gram-negative bacteria and per se perturbs Cu trafficking toward Cu-enzymes. Last, DTC might transport Cu(II) into the cytoplasm where it is expected to be released as Cu(I) due to the high thiol content [72]. Coherently, in this reducing environment of bacteria cytoplasm, DTC might not form a complex with Cu(I) as it will not compete with GSH found in mM concentration [72].

Instead of adding only the ligand DTC, direct addition of Cu(II)-DTC₂ complex in the media has a strong antimicrobial effect. This activity can be explained by intracellular accumulation of Cu due to ionophoric activity (mechanism I). Accordingly, strong Cu-dependent antimicrobial activity of disulfiram/DTC was reported against *M. tuberculosis*, with a minimum inhibitory concentration (MIC) around 0.3 μ M [78], and against *S. pneumoniae* [79]. The authors further showed the ability of Cu(II)-DTC₂ complex to cross bacteria membranes and inactivate cytoplasmic enzymes [78]. In such condition, intracellular Cu stress response is induced. This confirms that Cu(II)-DTC₂ entering the cytoplasm might release high Cu(I) concentration which perturbs metabolic pathways. Such Cu-dependent growth inhibition of disulfiram/DTC has also been observed recently on mollicutes [80]. The addition of a Cu²⁺ chelator in the media completely modifies the MIC values of disulfiram from nanomolar to micromolar range. This result further confirms the critical role of Cu for disulfiram antimicrobial activity.

Besides, Cu(II)-DTC₂ has been shown to interact with zinc finger proteins in cancer cells, and to oxidize Zn(II)-thiolates to form disulfide [81], [82]. Even though this mechanism has not been reported in bacterial cells, its occurrence cannot be ruled out.

1.9.2 8-hydroxyquinoline (8-HQ) compounds

8-HQs are neutral quite hydrophobic compounds able to cross membranes passively [83], [84]. They have been described as antimicrobial agent and have been studied on several organisms demonstrating their efficiency [83], [85]. The 5-chloro-7-iodo-8-HQ, called clioquinol, was developed in 1899 as an antiseptic and later used as an amebicide, but withdrawn from the market in the 70s due to side effects [86]. A high-throughput physiological screen made against *M. tuberculosis* identified 8-HQ compounds as efficient molecules [87]. Although the antimicrobial mechanism is multifaceted, 8-HQ interaction with metal ions including Cu seems to play a role. Addition of Cu ions triggered killing fungal pathogen *Cryptococcus neoformans* by a 8-HQ, and further addition of the strong Cu-chelator BCS forming a membrane-impermeable Cu-BCS₂ complex reversed the toxicity, in line with an important contribution of a Cu-ionophore activity of 8-HQ [83].

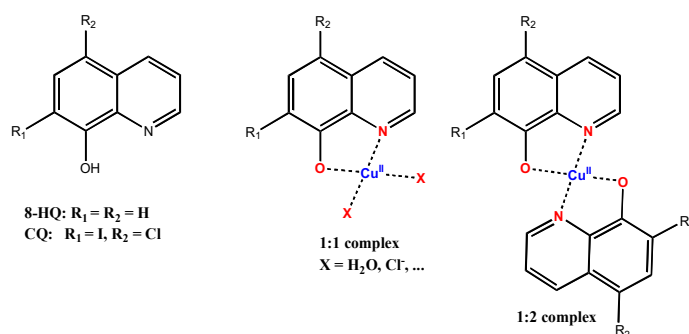


Figure 8 : Structure of 8-HQs and their Cu(II)-complexes.

8-HQ can form Cu(II)-HQ and Cu(II)-HQ₂ complexes (Figure 8). The affinity of Cu(II) for HQ₂ is quite high at neutral pH, whereas the metal ions binding via a phenolate (hard base) suggests a relative low affinity for Cu(I) (soft acid). Despite the quite low redox potential of Cu(II)-HQ₂, it can be readily reduced by the highly concentrated GSH tripeptide found in the cytoplasm, likely via the

formation of a ternary complex. Cu-complexes formation with 8-HQs will consequently depend on the bacteria compartment, i.e. possible with Cu(II) in the periplasm or in the environment, but not with Cu(I) in the cytoplasm. Consequently, 8-HQ is also well suited to serve as a ionophore (mechanism I). Such a mechanism of action was indeed proposed by Festa *et al.* [83] to explain 8-HQ and Cu-8-HQ toxicity on *C. neoformans*. The authors observed an increase in intracellular bioavailable Cu content which can then affect intracellular pathway either by acting directly on proteins or by generating toxic ROS. However, 8-HQ toxicity varies depending on the organism tested [83]. Species-specific targets and different Cu resistance strategies may explain this diversity, requiring further investigations.

It is also important to mention that 8-HQ with their hard phenolate ligand are good Fe(III) binders compared to DTC. Dissociation of Cu(II) might induce some cross talk with Fe(III) pathways which could also be a process involved in 8-HQ toxicity.

1.9.3 Phenanthrolines

Phenanthrolines (Phen) are bidentate ligands forming both 1:1 and 1:2 complexes with Cu(I) and Cu(II) (see Figure 9). 2,9-unsubstituted phenanthrolines (e.g. 1,10-phenanthroline, bathophenanthroline) have generally a high affinity for both Cu redox states.

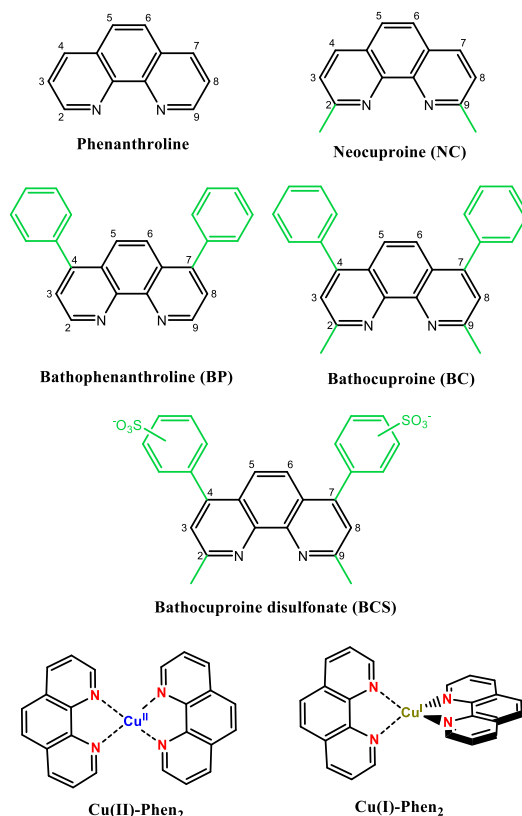


Figure 9: Structure of phenanthroline, its derivatives and their Cu(II)-complexes. Structural difference between the ligands are in green.

In contrast to 8-HQs and DTCs, these phenanthrolines can undergo rapid redox-cycling between Cu(I) and Cu(II) and are potentially very competent in ROS production (mechanism II). However, *in vivo*, they likely act only as ionophores (mechanism I) rather than pro-oxidant. For instance, the Cu(I) and Cu(II)-complexes with neutral phenanthrolines can passively cross the membranes, despite the positive charge of the complex formed. Then, Cu-(Phen)₂ is rapidly reduced by GSH, at least one Phen dissociates and a ternary complex Phen-Cu(I)-GSH might form [88]. However, other proteins with stronger Cu(I) affinity than GSH might be able to totally subtract Cu(I) from Cu-phenanthroline complexes. This has been shown for mammalian metallothioneins

[72], but might be also the case for bacterial metallothioneins or for copper storage proteins such as Csp3 [25].

It is important to distinguish phenanthrolines according to the substitution at position 2 and 9 (Figure 9). Methyl (or larger) groups at these two positions, such as in bathocuproin (BC), neocuproin (NC), cuproin or bathocuproine disulfonate (BCS), stabilize Cu(I) over Cu(II) in the Cu-Phen₂. In other words, they have a stronger Cu(I)-affinity, but lower Cu(II)-affinity compared to 2,9-unsubstituted phenanthrolines. The bulky methyl-groups force the two phenanthrolines into a tetrahedral coordination of the metal ion, a geometry suited for Cu(I) but not for Cu(II). Related to that, Cu(I)-complexes with two 2,9-substituted phenanthrolines (e.g. Cu(I)-BC₂) are difficult to oxidize and are generally air-stable.

It is a general feature that the apparent affinity for a metal ion in a 1:2 (metal:ligand) complex has a more drastic dependence on the ligand concentration compared to a 1:1 complex. Hence 2,9-substituted phenanthrolines that form a 1:2 complex can become stronger than Cu(I)-proteins (that often are 1:1 complexes) at higher concentrations > 0.1 mM [89]. However, at lower ligand concentrations, Cu(I) might dissociate from 1:2 complexes as shown for 10 µM Cu(I)-BCS₂ which dissociated at about 50% in 3 mM GSH and totally with mammalian metallothioneins [72]. Therefore, Cu(I) complexes with two neutral 2,9-substituted phenanthrolines (like BC or NC) can also have ionophore activity at lower concentrations.

The antimicrobial activity of some of these compounds has been demonstrated, which is highly dependent on their ability to cross the membrane. NC exhibited efficient *M. tuberculosis* growth inhibition in presence of increasing amount of copper [90]. Such an effect was also observed on *Mycoplasma* and *Ureaplasma* species [80]. In contrast, bathocuproine disulfonate is membrane impermeable and does not show any effect on *M. tuberculosis* growth.

Even if some of these compounds cannot be used as antimicrobial agent, their ability to tightly bind Cu(I), as NC and BC, have made them widely used as chelating agents *in vitro* or *in vivo* for several experimental purposes.

1.9.4 Pyrithione

Pyrithione (PT) is a neutral ligand which can bind Cu(II) as a Cu(II)-PT₂ complex which is neutral as well, due to the loss of the two hydroxylamine protons (Figure 10). Thus, in terms of charge, PT has properties suited for ionophore activity (mechanism I), as Cu(II)-PT₂ can enter the cytoplasm and PT exit it. The affinity of Cu(I) to PT is unknown, but according to Pearson theory, Cu(I) has a low affinity for N-OH. This means that once reduced in the cytoplasm, Cu(I) will dissociate from PT. This was further confirmed by a recent study [62], where the authors demonstrated the intracellular increase of copper in *E. coli* after treatment with a mix of Cu and PT and in comparison, with Cu alone. Cu-induced cell death is amplified by Cu-PT₂ which most likely might overwhelm copper homeostasis systems. Cu-PT₂ treatment was also tested on *Neisseria gonorrhoeae* which is highly sensitive to this complex with a MIC similar to MIC observed for Cu-gtsm compounds (see 1.9.5) [91]. On *Klebsiella pneumoniae*, the addition of PT with either Zn or Cu increases the antibiotic activity of amikacin. Here, the authors propose an ionophore activity of this compound [92].

Of note, the structure of Cu-PT is reminiscent of the natural copper-containing antimicrobial agent Fluopsin C, in which Cu is coordinated by two units of N-methylthiohydroxamate. Interestingly, both Cu-PT and Fluopsin C show broad antimicrobial activity against Gram-negative and Gram-positive bacteria, as well as multidrug-resistant strains. By contrast, they are not effective against *P. aeruginosa* PAO1, which produces Fluopsin C to cope with excess Cu stress [93].

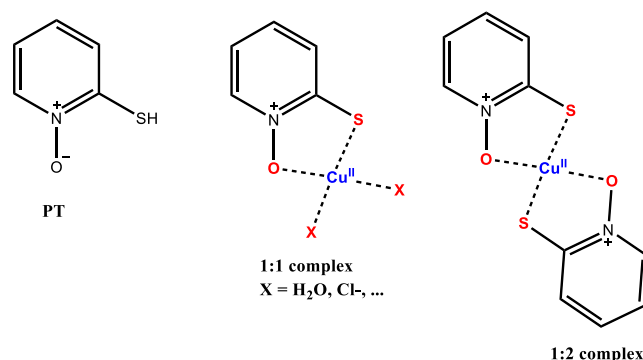


Fig. 10. Structure of PT and its Cu(II)-complexes.

1.9.5 Bis-Thiosemicarbazones (*atsm*, *gtsm*)

Bis-thiosemicarbazones (bTSC) form strong Cu(II)-complexes at neutral pH (log K of the bTSC 3-ethoxy-2-oxobutylaldehyde-bis-thiosemicarbazone (compound called kts) was determined to be 19 at pH 7 [94]). They form much stronger 1:1 complexes with Cu(II) than DTC or 8-HQ, even compared to the 1:2 complex of DTC and 8-HQ in the relevant concentration of low micromolar or below. This is mainly due to the chelate effect as they are tetradentate ligands (Figure 11). Using a drug screen assay, Speer *et al.* [90] demonstrated that bTSC induces a copper hypersensitivity phenotype in *M. tuberculosis* and proposed that copper complexation was the potential mechanism.

Cu-bTSC complexes are quite stable in biological environments. They form neutral, lipophilic compounds that enter the cell by passive membrane crossing. They are weaker Cu(I) than Cu(II) chelators but still have a log K of around 13 at pH 7 for Cu(I)[95]. Once inside the cells, Cu(II) bound to bTCS could be reduced to Cu(I) and transferred from bTCS to GSH. However, depending on the complex formed and on its redox potential, such reduction can be very slow (Table 3, Figure 11). For instance, the Cu(II)-*atsm* complex has been shown to not be reduced *in vitro* even in the presence of millimolar of GSH, in line with Cu(II)-*atsm* low redox potential (see Table 3, Figure 11) [72], [95]. In *E. coli*, Cu seems to

be released from Cu-atism, but with a much slower rate than from Cu-gtism [91]. The difference observed between Cu(II)-atism and Cu(II)-gtism in terms of redox potential and reactivity is reflected in term of level of toxicity. Cu(II)-gtism exerts a higher level of toxicity on bacteria than Cu(II)-atism. This difference can be explained by the fact that Cu-gtism most likely acts as an ionophore (mechanism I): it enters the cells and Cu is rapidly released by the action of intracellular thiols (GSH and others), the fast accumulation of intracellular Cu(I) exerting toxicity. In contrast, Cu(II)-atism is less toxic which is consistent with the fact that Cu(II)-atism is more stable, releases Cu(I) only very slowly and this gives more time to the bacteria to react and to switch on the defense system [91]. Toxicity of cytoplasmic Cu(I) released from Cu(II)-gtism was further confirmed by the increased sensitivity of *E. coli* strain deleted of *copA* gene, encoding the exporter which removes Cu cytoplasmic excess. In contrast, the deletion of *cueO* gene, encoding a periplasmic copper oxidizing enzyme (Figure 3), has no effect on cell survival toward Cu(II)-gtism [91]. This finding suggests that dissociation of Cu(II)-gtism occurs specifically in the cytosol.

A recent study from Totten *et al.* [80] defined the impact of different compounds toward *Mycoplasma* and *Ureaplasma* species. In their assays, they observed a bactericidal activity of gtism compound, however not in a copper dependent manner. Still, the use of Cu²⁺ chelator in the media decreased gtism antibacterial activity, in line with the fact that small amount of Cu in the media is sufficient to boost the growth inhibitory effect of gtism.

The higher toxicity of Cu-gtism compare to Cu-atism applies to other bacteria (Gram-positive and -negative) [91], [96]. However, the susceptibility of the bacteria is very different, and is directly related to the bacterial physiology. Bacteria with a high developed Cu-detoxification system like *E. coli* are quite resistant, other with a poorer Cu extrusion system, like *N. gonorrhoeae* are much more susceptible (IC 50 < μM). This can be explained by the fact that *N. gonorrhoeae* have only one exporter, CopA, whose expression is not inducible by Cu. Moreover, in *N. gonorrhoeae* an additional toxicity mechanism was described related to the inhibition of

dehydrogenases in the respiratory chain [96]. The results suggested that Cu-bTSC complexes bind to membrane proteins, likely in hydrophobic pockets where they interfere with ubiquinol. This mechanism is independent of Cu release or redox activity, in line with experiments showing that Zn-bTSC showed similar activity [96]. However, other mechanisms may explain the toxicity of Cu-gtsm, as bacteria growing under fermentation (*S. pneumoniae*, *E. coli*) are also highly sensitive to Cu-gtsm [91].

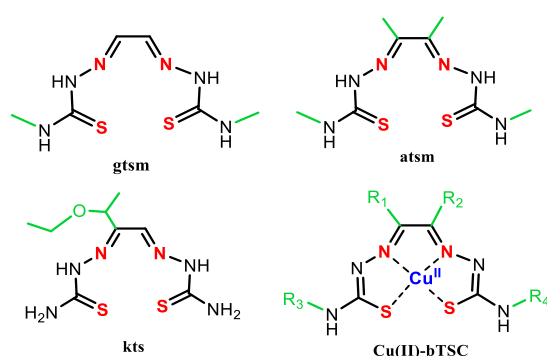


Figure 11: Structure of bis-thiosemicarbazones (bTSC) and their Cu(II)-complexes. Structural difference between the ligands are in green.

Compound class	Representative	Affinity (pH 7.4) in log Kd for		E° Cu(II)/Cu(I) (V/NHE)	Intracellular reactivity (cytoplasm)	Denticity
		Cu(II)	Cu(I)			
Cu alone				0.16	-Fast reduction of Cu(II) - Cu(I) binds to GSH	-
Dithiocarbamate (DTC)	DETC Cu + DETC Cu-DETC + DETC	11.7 ^c 12.2 ^c		-0.14 ^k	- Fast reduction - Complete dissociation - Cu(I) binds to GSH	2
8-hydroxyquinoline (HQ)	Clioquinol (CQ) Cu + CQ Cu + HQ Cu-CQ + CQ Cu-HQ + HQ	10.8 ^f 9.75 ^g 9.2 ^f 8.65 ^g		-0.36 ^h	- Fast reduction - Dissociation - Cu(I) binds to GSH	2
Phenanthrolines	Cu + Phen Cu-Phen + Phen Cu + 2 Phen BCS Cu + 2BCS	9.1 ^l 6.8 ^l 15.9 ^l 12.5 ^d	10.3 ^l 5.5 ^l 15.8 ^l 20.8 ^d	0.17 ⁱ 0.62 ^j	- Fast reduction - Partial dissociation - Cu(I) binds to GSH	2
Pyrrithione	Cu + PT Cu(II)-PT + PT	> 8.5 ^c 5.9 ^c			- Fast reduction - Dissociation - Cu(I) binds to GSH	2
Bis-thiosemicarbazone	Cu + Atsm Cu + Gtsm Cu + Kts	~19 ^b	~13 ^a ~13 ^a	-0.40 ^a -0.24 ^a -0.12 ^b (pH 6.6)	- Stable - Slow - Dissociation	4

Table 3. Thermodynamic and kinetics properties of Cu-L complexes

^a [95]; ^b [94]; ^c in methanol [97]; ^d [98]; ^e [99]; ^f in 80% Methanol and 20% H₂O, [100]; ^g [101]; ^h [102]; ⁱ [103]; ^j [104]; ^k [105]; ^l [106]

1.10 Peptides-based Cu-chelators

Antimicrobial peptides (AMPs) are described as peptides of about 10 to 100 amino acids which can be either naturally produced by the immune system to combat invading pathogens or chemically synthesized with non-natural sequences. For certain natural AMPs, the binding of copper or other metal ions was proposed to be required for their activity. The main idea was that Cu-binding to AMPs may improve the antimicrobial activity either through Cu-catalyzed ROS production or Cu-induced conformational changes enhancing the interaction of the AMP with its target. Two main approaches are reported: (i) identification of native AMPs containing a high affinity-Cu-binding site, (ii) engineering such a site in a native or artificial peptide (recent review on AMPs [107]).

Short peptides are often dynamic molecules lacking a well-defined 3D structure. Hence, Cu-binding to peptides is generally weaker than to Cu-proteins with defined 3D-structures, due to entropic penalty for arranging the peptidic ligands in a constrained coordination sphere around the metal ion. To date, no simple Cu(I)-binding peptide motifs with sub-femtomolar affinity (unlike Cu(I)-proteins) are known. Instead, two naturally-occurring His-containing Cu(II)-binding peptide motifs can attain a sub-picomolar affinity at pH 7.4. They require a His residue at either position 2 or 3 in a peptide with a free N-terminus, *i.e.* $\text{NH}_2\text{-Xxx-His-Peptide}$ (XH) or $\text{NH}_2\text{-Xxx-Zzz-His-Peptide}$ (XZH, also known as ATCUN motif) [73] (Figure 12). Most studies focused on these motifs as they show comparable affinity to endogenous Cu(II)-carriers (such as serum albumin in human blood), which makes them apt to bind Cu(II) in biological environments. Antimicrobial activities of several native AMPs containing a XZH motif have been compared with and without Cu (reviewed in [108]). Generally, the impact of Cu was quite low, with a similar antimicrobial activity (measured as MIC values) whether Cu was added to AMPs or not. The modest Cu effect could be explained by the redox properties of Cu bound to XZH which induces a very low ROS production [109]. However, ATCUN peptides bearing an additional Cu(I)-stabilizing site show higher antimicrobial activity. For instance, the salivary peptide Histatin-5 (Hist-5) shows antimicrobial activity against the yeast *C. albicans* and *P. gingivalis* [108] (Table 4). Hist-5 binds Cu(II) in a XZH motif, but has in addition a vicinal Cu(I)-binding *bis*-His (-HH-) motif which might favour the redox cycling and hence the ROS production.

Remarkably, the mutation of Cu-binding His residues from the HH motif significantly affects the antimicrobial activity of Hist-5 [110], [111]. Likewise, ATCUN-bearing AMPs such as Piscidins and Ixosin could show higher ROS production than simple ATCUN peptides thanks to their Cu(I)-binding motifs, i.e. -HH- and -HXZM-, respectively [108] (Table 4). Nevertheless, the impact of such Cu(I)-binding sites on the ROS production by ATCUN-AMPs has never been assessed quantitatively [112], [113]. On the other hand, in the cytosol, Cu(II)-XZH complexes are slowly reduced and dissociated by GSH [114], [115]. In this case, a Cu(I)-binding site could lead to a faster dissociation of Cu from Cu(II)-XZH, via the faster reduction to Cu(I) and subsequent dissociation. Overall, this implies that (i) the potential pro-oxidant activity might take place only in the extracellular or periplasmic compartments (mechanism II) and (ii) these complexes might act as ionophores (mechanism I), even though no report has yet shown any AMP with an efficient ionophore activity.

Recently, the shorter XH motif in an AMP peptide (XH-AMP) was investigated and compared to XZH-AMP and AMP only. Cu(II)-XH is slightly more active in ROS production than Cu(II)-XZH, although still less compared to Cu in buffer or in a well redox active Cu-complex such as Cu-Phen₂ [72]. Nevertheless, the same antimicrobial activity as pure AMP against *E. coli* was observed with Cu-bound or unbound XH- or XZH-AMP [116].

Besides, structural changes upon Cu-binding to XH and XZH motifs are likely not drastic as these motifs are short and located at the N-terminus.

PEPTIDE	Cu binding motifs
Hist-5	DS HAKR HH ...
Piscidin 1	FF HH ...
Piscidin 3	FI HH ...
Ixosin	GL H K V M ...

Table 4. Peptides mentioned in this chapter and their Cu-binding motifs (red for Cu(II), blue for Cu(I), violet for both)

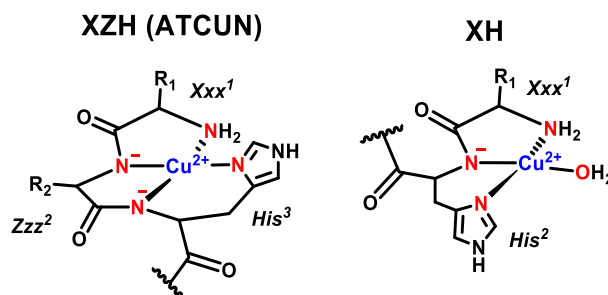


Figure 12: Structure of Cu(II)-XZH and Cu(II)-XH complexes.

1.11 Recent advances toward the future use of copper in medicine

Copper is a potent antimicrobial agent and even though used since thousands of years, it regains interest as a promising compound to increase therapeutic efficiency toward recalcitrant microbial infections. Indeed, as increasing amount of copper in bacteria environment leads to cell death, such property is even used by macrophages to kill invading pathogens. Cu-mechanism of action to explain toxicity as well as strategies developed by cells to survive such thread start to be better understood and these fundamental knowledges will pave the way toward the discovery of copper related compounds with application in medicine. Copper-complexes, as described in this chapter, show interesting properties demonstrating their potential for clinical treatment. They present a higher level of bactericide activity compared to Cu or the ligand alone. For most of them, Cu-L permit to cross bacteria membranes with a higher efficiency than “free” Cu and once in the reducing cytoplasmic compartment they dissociate and the released Cu accumulates and perturbs intracellular pathways. This ionophore activity has been mainly described for most of the Cu-complexes. Other mechanisms of action could occur, however, such as a gain of function to induce ROS production by the Cu-L once inside the periplasmic compartment. This requires further experimental evidences to be confirmed.

Cu-gtsm is a promising Cu-complex, highly effective toward multidrug resistant bacteria, giving some hope to cure disease related to highly resistant strains. Very interestingly, antimicrobial Cu-gtsm doses used to kill the extracellular mucosal pathogen *N. gonorrhoeae* did not show significant toxicity toward cervical epithelial cells [91]. Recently, other molecules, like the 1-hydroxy-5-*R*-pyridine-2(1*H*)-thiones (with *R*: COOMe, COOEt, CF₃), have been shown to efficiently inhibit *M. tuberculosis* at very low amount and in a copper-dependent manner [117].

However, the next challenge in this area of study will be to develop specific antibacterial compounds to target pathogenic bacteria but neither host cells nor non-pathogenic bacteria. Even if some complexes might be interesting to further study for clinical applications, most of the Cu-complexes described in this issue have no selectivity towards bacteria versus mammalian cells. Some of these compounds are even used to treat cancer and are known to negatively impact eukaryotic cells [118], [119]. To increase selectivity, several strategies can be envisioned.

Festa *et al.* [83] used a compound which gets activated once inside macrophages. They took advantage of the high level of ROS as well as of copper in macrophages to transform a non-toxic compound into a highly toxic one. In particular, oxidation of a pro-drug released the 8-HQ ligand that complexed Cu and then acted as ionophore to aid the immune system to kill the pathogens. They were able to show that such conditional activation worked *in vitro* and in mouse model on the pathogen *C. neoformans* [83].

Another strategy followed by Wolschendorf and coworkers was to screen different compounds in combination with copper against *S. aureus* [120]. They were able to highlight the potential of new copper-dependent molecules such as thiourea inhibitors which were able to inhibit efficiently *S. aureus* growth and seemed tolerated in cell culture. They also demonstrated the toxicity of PZP-915 (5-Benzyl-3-(4-chlorophenyl)-2-methyl-4*H*,7*H*-pyrazolo [1,5-*a*] pyrimidin-7-one) toward *S. aureus* resistant strains with a certain selectivity toward bacteria over eukaryotic cells [121].

The use of peptide with Cu-binding motifs is another way to obtain higher selectivity toward bacteria as it is exemplified by the natural AMPs. Peptides could also be used for targeting a Cu-site to compartments or proteins. Accordingly, cytoplasm-targeted peptides could act as ionophores by adding a Cu(II)-site to the peptide. This strategy would need optimization of the peptide sequence toward cell-penetration, so far an area of research poorly explored. However, it is not clear whether peptides can be as efficient as small organic ligands. For the antimicrobial strategy consisting in employing a Cu-site on an AMP to catalyze ROS production, reports are mainly limited to Xxx-Zzz-His motifs. This motif is quite redox inert as it stabilizes Cu(II) and hence ROS production is low. Other high affinity Cu-binding sites are needed to increase ROS production. This seems to be very challenging for short peptides, and protein-like folding and/or organic ligands are needed to reach the balance between stability and redox reactivity. Moreover, peptides form a scaffold which can be conjugate to other entities of interest, such as fluorophore for tracking other antimicrobial molecules.

Synergic effect of copper/copper derivatives with antibiotics is also an alternative way of finding highly specific molecules toward pathogenic bacteria [55]. ATP-6K in presence of copper and in combination with ampicillin was shown to restore ampicillin antimicrobial activity against resistant strains without affecting eukaryotic cells [122].

These strategies among others should allow to overcome the limitation of copper use in medical applications. Indeed, study on copper and its derivatives is of high interest to develop novel efficient antimicrobial agents. However, the use of metals to kill pathogenic bacteria has to be carefully thought. Indeed, feeding animals by copper in industry lead to the spread of resistance mechanism such as the *pco* genomic island in *E. coli* [123] as well as of a functional *cus* locus in *S. enterica* that increases its resistance toward metal [124]. This raises the concern of the presence of metal

in food which acts as a disseminating agent of resistance mechanisms.

The development of Cu-dependent inhibitors might impose selection pressure to bacteria and lead to the development of metal resistance strategies. Efficient copper pumps, for instance, could help bacteria to resist Cu-complexes ionophore activity. However, as described in this review, copper induces bacteria cell death by targeting a variety of cellular processes. These multiple targets make Cu-dependent inhibitors very attractive compared to some antibiotics which target a single process that bacteria can easily overcome. A good balance is required: the use of small amount of copper specifically dedicated to kill invading microorganisms should be clearly envisioned while decreasing the excessive use of copper as food additive and biocide.

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