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Research paper

Evaluation of *in vitro* and *in vivo* antibacterial activity of novel Cu(II)-steroid complexes

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Dedicated to Ms. Elena Bertacco, former PhD student of D.M.

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ABSTRACT

A pioneer series of copper(II) complexes bearing planar phenanthroline-modified aromatic ligands and steroid (ethynylestradiol and ethisterone) with generic formula $[Cu(N \cap N)(steroid)](NO_3)_2$ where $N \cap N$ is DPQ, DPPZ and DPPN and steroid is estradiol or testosterone, were synthetized, characterised and screened *in vitro* and *in vivo* as antimicrobial agents against *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA). Toxicity studies revealed notable antibacterial activity of the copper – based compounds, which is significantly increased *in vivo* by the presence of the steroid moiety. Toxicity profiling was estimated *in vitro* versus Gram-positive (*Staphylococcus aureus*) and MRSA and *in vivo* in *Galleria mellonella* larvae infected with *S. aureus*. Results showed the complexes to be active against *S. aureus* and MRSA *in vitro* (MIC_{50} average value of 2.46 and 97 μM in *S. aureus* and MRSA, respectively) and to be active when larvae infected with *S. aureus* were administered the agents.

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1. Introduction

The bacterium *Staphylococcus aureus* is a Gram positive body commensal which has the ability to survive in a wide variety of environments [1], and this contributes to its ability to induce a range of superficial and systemic infections. *S. aureus* possesses a number of virulence factors that contribute to its ability to colonise tissue including the presence of a capsule, the expression of adhesins, the secretion of a range of toxins and immunomodulators which disrupt the host's immune response [2]. This bacterium is present on approximately 30% of healthy individuals in the anterior nares and on the skin, but a skin breach resulting from surgery or trauma can result in a variety of skin infections, such as impetigo, or more serious diseases such as toxic shock syndrome and sepsis [3,4]. *S. aureus* infection can be extremely difficult to treat and up to 4% of cases can be fatal [3]. Treatment of *S. aureus* infections is hindered by the emergence of methicillin-resistant *S. aureus* (MRSA) and due to the increasing incidence of resistance to conventional antimicrobials there is a renewed interest in the development of novel metal based drugs as antimicrobial agent [5].

Despite the classic organic based antibacterial agents, metal complexes are an excellent alternative and in particular Cu(phen) derivatives. In the last twenty years the antimicrobial activity of Cu(phen) complexes have been deeply investigated; double charged copper with planar chelating ligands exhibit growth inhibitory activity against *Staphylococcus aureus* ($MIC \geq 4.0–7.9$ μM) and to a lesser extent against *Escherichia coli* [6–13]. It is still not completely clear what role the planar ligands play in the antimicrobial activity of the corresponding metal based drugs. Indeed, metal complexes with substituted planar aromatic ligands (i.e. DPQ, DPPZ and DPPN, Scheme 1) show an increased interaction via intercalation with the DNA and Cu(II) derivatives and also show artificial nuclease activity but this could not be related with the antimicrobial properties [14–16]. Mitochondrial membrane damage and p53 upregulation are also alternative possible pathways of action. Creaven et al. showed that Cu(phen) complexes functionalised with acetic acids are able to inhibit respiration, reduce level of ergosterol and alter the cytochrome c content [12]. Considering these aspects, here we report the syntheses, characterization and antimicrobial evaluation of a series of Cu(II) complexes with chelating planar ligands further functionalised with steroids (testosterone and estradiol). We recently showed that the modification of Au(I)–NHC (*N*-Heterocyclic Carbenes) with steroids derivatives is a promising strategy to enhance the antibacterial activity of the related complexes [17]. In the work presented here the Cu(II) cationic complexes were evaluated as antibacterial agents *in vitro* against *S. aureus* and MRSA and *in vivo* using *Galleria mellonella* larvae.

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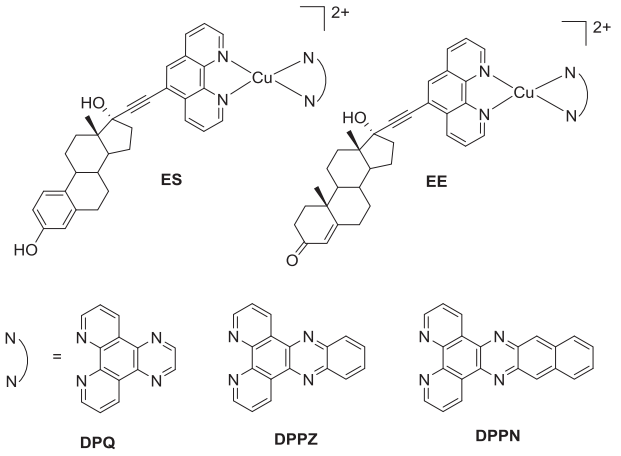


Scheme 1. Structures of $[Cu(ES\text{-}5\text{-}phen)(DPQ)]^{2+}$ **4**, $[Cu(EE\text{-}5\text{-}phen)(DPQ)]^{2+}$ **5**, $[Cu(ES\text{-}5\text{-}phen)(DPPZ)]^{2+}$ **6**, $[Cu(EE\text{-}5\text{-}phen)(DPPZ)]^{2+}$ **7**, $[Cu(ES\text{-}5\text{-}phen)(DPPN)]^{2+}$ **8**, $[Cu(EE\text{-}5\text{-}phen))(DPPN)]^{2+}$ **9**.

The immune system of insects shows many structural and functional similarities to the innate immune response of mammals [18–20] and consequently insects can be used to assess the virulence of microbial pathogens or the *in vivo* efficacy of antimicrobial drugs and give results similar to those obtained using mammals [21,22]. Larvae of *Galleria mellonella* are a popular choice for these types of tests and are inexpensive to purchase, and give rapid results [18]. *G. mellonella* larvae have previously been used to demonstrate the *in vivo* activity of novel metal based drugs against pathogenic bacteria and fungi [23,24] and show a strong correlation with results obtained in rats [25].

The aim of this work was to analyse the *in vitro* effects of the six metal complexes on *Staphylococcus aureus* and MRSA, to evaluate the toxicity of the compounds in *Galleria mellonella* larvae and to examine if the compounds showed any therapeutic properties when larvae had been pre-inoculated with microbial infections.

2. Material and methods

All reagents and reactants used were purchased from commercial sources. The two sources used were Sigma Aldrich and Tokyo Chemical Industry. DPQ (Dipyrido[3,2-*f*:2′,3′-*h*]quinoxaline), DPPZ (Dipyrido[3,2-*a*:2′,3′-*c*]phenazine), DPPN (Benzo[*I*]dipyrido[3,2-a:2′,3′-c]phenazine) and tetrakis triphenylphosphine Palladium(0) were synthetized as previously reported [26–29].

All solvents were used without further purification. The DMF was dried using 4 Ã... molecular sieves, it was then decanted into a round bottom flask and kept under high vacuum using a Schlenk line while immersed in liquid nitrogen. The DMF was then flushed with nitrogen gas. This step was repeated a minimum of three times for the Sonogashira coupling reaction to obtain ES-5-phen and EE-5-phen.

All NMR spectra were recorded on a Bruker Advance spectrometer with the probe at 293 K, operating at 500 MHz for the ^1H nucleus. Proton signals were assigned with the help of 2D NMR experiments (COSY). Spectra were recorded in $CDCl_3$ using Me_4Si as the internal standard. All chemical shifts are reported in ppm.

Infrared (IR) spectra were recorded in the region 4000 – 400 cm^{-1} on a Perkin Elmer precisely spectrum 100 FT/IR spectrometer. The solid samples were run using ATR.

Elemental analyses (carbon, hydrogen and nitrogen) were performed with a PerkinElmer 2400 series II analyzer. ESI mass spectra were recorded in positive mode with a Waters LCT Premier XE Spectrometer.

2.1. Assessment of antibacterial activity of novel complexes

The complexes were dissolved in 1 mL DMSO to give a stock concentration of 5 mg/mL. Nutrient broth (100μL) was added to each well of a 96 well plate. Each drug was serially diluted on the plate giving a concentration range 150 – 0.59 μM. Bacteria were grown overnight and the OD_{600} was adjusted to 0.1 (equivalent to cell density of 4×10^7/ml). Bacterial cells (100 μL) were added to each well and the growth was measured at 600 nm after 24 h at 37 °C using a spectrophotometer (BioPhotometer). The MIC_{50} values were calculated as the minimum concentration of drug that inhibited growth by 50%.

2.2. Inoculation of Galleria mellonella larvae

Sixth instar larvae of *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth) (Mealworm Company, Sheffield, England) were stored in the dark at 15 °C. 5 Larvae of the same age and weighing 0.3 g were inoculated with 20 μL of PBS containing cells 4×107 bacterial cell through the last pro-leg using a Myjector U100 insulin syringe (Terumo Europe, Leuven, Belgium).

2.3. In vivo toxicity assay

Larvae were injected with 20 μL of compound solution (150 – 0.59 μM) or 5% (v/v) DMSO through the last left pro-leg as described. Larvae were incubated at 37 °C for 24 h prior to quantifying survival after 24, 48 and 72 h.

2.4. Effect of compounds on survival of G. Mellonella larvae infected with S. aureus

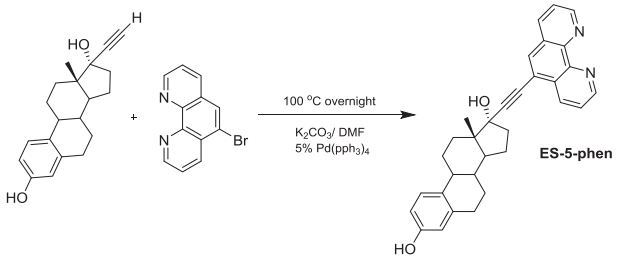
Larvae were injected with *S. aureus* through the last left proleg as described. One hour post infection 20 ul of each compound solution (0.1 or 0.25 mg/ml) was administered. The control consisted of larvae inoculated with the *S. aureus*. Larvae were incubated at 37 °C and survival was assessed at 24 and 48 h.

3. Experimental

The starting $[Cu(N \cap N)(OH_2)_2](NO_3)_2$ (**1**–**3**) complexes (where N ∩ N is DPQ (**1**), DPPZ (**2**) or DPPN(**3**), See Experimental Part in Supporting Information) where obtained by modifying a literature procedure [21–23]. Briefly, $Cu(NO_3)_2{\cdot}3H_2O$ and the corresponding N ∩ N ligand where mixed in a 1.1 to 1 ratio in methanol and refluxed for two hours. The slight excess of the Copper salt prevent the formation of the more stable bis-adduct $[Cu(N \cap N)_2](NO_3)_2$. The solid was obtained by addition of Et_2O, isolated by filtration and dried in vacuum (Supporting Information).

The phen-steroid derivatives of general formula ES or EE-5-phen (where ES-5-phen and EE-5-phen are the Estradiol and Testosterone derivatives, respectively) have been obtained by Sonogashira coupling reaction between 5-Bromophenanthroline and the correspondent commercial available alkyne derivative of the steroids (See Scheme 2 and Supporting Information).

The final complexes $[Cu(N \cap N)(EE \text{ or } ES\text{-}5\text{-}phen)](NO_3)_2$ **(4–9)** are depicted in Scheme 1 and were obtained by mixing the corresponding $[Cu(N \cap N)(OH_2)_2](NO_3)_2$ **(1–3)** and the corresponding steroid-phen in a 1 to 1 ratio in DMF at 50 °C overnight. The solids were isolated by filtration after addition of Et_2O and dried in vacuum (See Supporting Information).



Scheme 2. Synthetic pathway to produce ES-5-Phen using Sonogashira Coupling Reaction.

4. Results and discussion

Recent results from our group showed that the presence of steroids (Estradiol and Testosterone) enhanced the *in vivo* antibacterial activity of Au(I)–NHC carbene complexes [17]. Since Copper and in particular Cu(II)-phenanthroline complexes are important antibacterial agents, we sought to combine these with steroid derivatives to confirm the efficacy of this approach. Furthermore, the complexes were functionalised with aromatic planar phenanthroline derivatives ligands such as DPQ, DPPZ and DPPN.

The coordination sphere of the series of Cu(II) complexes analysed in this work is composed by two modified phenanthroline scaffolds as depicted in Scheme 1. The alkyne steroids derivatives (ES Ethinyl Estradiol and EE Ethiosterone) have been covalently bound to phenathroline using the Sonogashira coupling reaction with 5-Bromophenanthroline as shown in Scheme 2 for the Estradiol steroid (ES-5-phen).

The success of Sonogashira coupling reactions are highly dependent upon the nature of the base and by the catalyst used as well as by the inert and degassed use of solvent. After several attempts we found that the best conditions were the use of the inorganic base K_2CO_3, the use of a freshly prepared *tetrakis*-[$Pd(PPh_3)_4$] catalyst, dry and degassed DMF at 100 °C overnight under nitrogen atmosphere (See Supporting Information). The success of the reaction can be easily visualised in the ^1H NMR spectrum by the disappearance of the alkyne proton of the starting EE and ES at 2.55 ppm and by the shift of the ^1H in position six of the phenathroline that moves upfield of 0.5 ppm in the ES/EE-5-Phen with respect the starting 5-Bromophenanthroline (Fig. 1S and 2S).

The final complexes have been characterised by IR, Elemental Analyses and Mass Spectrometry (Supporting Information). All the mass spectra show a clearly visible peak with the isotopical pattern of Cu in the positive region corresponding to the species $[M-NO_3]^+$ or $[M-2(NO_3)+Cl]^+$ (Figs. 3-8S). The activity of the compounds against *S. aureus* and MRSA was assessed as described and

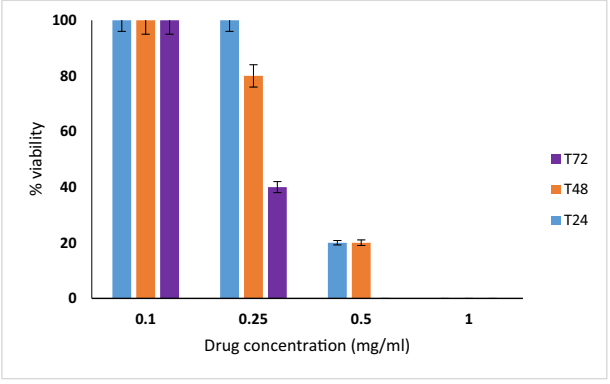


Fig. 1. Viability of *Galleria mellonella larvae* upon treatment with different concentration of complex **4.**

Table 1
Antimicrobial activity of selected compounds. MIC_{50} [μM].

Compound	*S. aureus*	MRSA
4	4.7 ± 0.9	219 ± 10.4
5	1.5 ± 0.2	125 ± 9.5
6	2.1 ± 0.1	102 ± 8.9
7	2.0 ± 0.2	32 ± 3.2
8	2.5 ± 0.4	17.5 ± 1.5
9	2.0 ± 0.3	88 ± 7.8

the results indicate that all drugs were active against *S. aureus* with MIC_{50} values < 5 μM (Table 1) that are in line and even smaller with most of the Cu(II) complexes reported in literature. [6–12] The free estradiol and testosterone were non-active as previously reported by us. [17] Among the series, the testosterone derivatives (**5**, **7** and **9**) are more potent (average MIC_{50} of 1.83 μM) with respect the estradiol derivatives **4**, **6** and **8** (average MIC_{50} of 3.1 μM). The planar ligand seems to play an important role because the DPPZ derivatives **6** and **7** are the most active while DPQ (**4** and **5**) are the less potent with DPPN complexes (**8** and **9**) laying between (average MIC_{50} of 2.05, 2.25 and 3.1 μM for DPPZ, DPPN and DPQ, respectively). Two important factors are important: lipophilicity and solubility. It is expected that the most lipophilic complexes would be more potent due to higher intracellular uptake; although DPPN derivatives **8** and **9** are more potent than the DPQ analogues (**4** and **5**) they are less active than the DPPZ (**6** and **7**) because solubility issues arise. Large and steric encumbered DPPN complexes are less soluble than the smaller DPQ and DPPZ and this is the reason why intermediate DPPZ complexes are the most potent. In the case of MRSA the complexes are much less active (average MIC_{50} values of 97.4 μM) with some exception such as compound **8** that has a MIC_{50} in the low micromolar region (Table 1). The complexes were found to be inactive against the Gram negative bacterium *Pseudomonas aeruginosa* and the yeast *Candida albicans* (results not presented).

These promising results prompted us to investigate the *in vivo* antimicrobial properties of these Cu-steroids complexes. MRSA was not studied *in vivo* due to the poor results *in vitro*. *G. mellonella* larvae are a useful tool in this kind of study because, as discussed in the introduction, their immune system is similar to that of mammals, they provide rapid and reliable results and there are no ethical restrictions to their use. [19] The larvae are inoculated by injecting the drug into the last left pro-leg and larval viability can be easily determined by visualisation. Melanisation and lack of movement indicates the inoculation proved fatal. (See Figs. 9 and 10S in Supporting information).

The first study analysed the toxicity of the Cu(II) series in *G. mellonella* larvae. Previous work has established a strong correlation between the toxicity of compounds in insect larvae and rats [30] and compounds such as potassium nitrate [31] and caffeine [32] are metabolised in a comparable manner in *G. mellonella* larvae and mammals. The larvae were injected with increasing concentrations of each compound and the viability was analysed after 24, 48 and 72 h. Administration of doses of 0.1 mg/ml of each drug to larvae had little or no effect on viability over 72 h. In the case of administration of the 0.25 mg/ml dose compounds **4** and **5** with DPQ ligand reduced viability by 60 and 20%, respectively, by 72 h. In the case of the DPPN derivatives **8** and **9** the reduced viability was of 20% (72 h) while no significant decrease in viability at this dose over 72 h was observed for the complexes **6** and **7** with DPPZ moiety. All drugs at a concentration of 0.5 or 1 mg/ml proved toxic to larvae and resulted in 80 – 100% death at 72 h (Fig. 1 for compound **4** and 11-16S in SI for compound **5** – **9**).

In order to determine if the drugs had *in vivo* antibacterial activity, *G. mellonella* larvae were infected with a dose of *S. aureus* (1:2

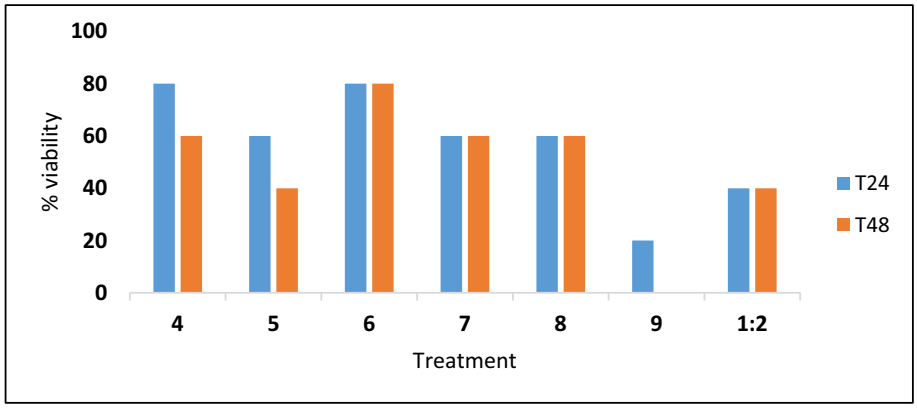


Fig. 2. % Viability of larvae treated with 1:2 dilution of *S. aureus* and 0.1 mg/mL of the tested complexes. Standard error was less than ± 10% of the mean in all cases.

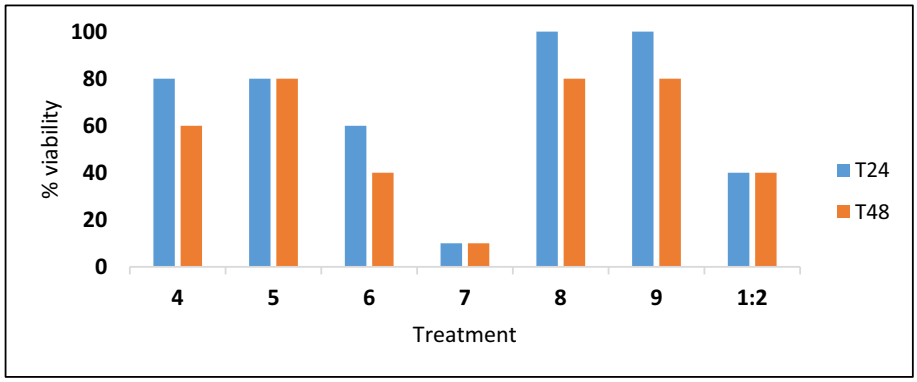


Fig. 3. % Viability of larvae treated with 1:2 dilution of *S. aureus* and 0.25 mg/mL of the tested complexes. Standard error was less than ± 10% of the mean in all cases.

dilution of OD_{600} 1.0) and one hour later administered with a non-toxic dose of each drug at a concentration of 0.1 or 0.25 mg/mL. In the first series of experiments, which used a drug dose of 0.1 mg/mL, administration of the bacteria to larvae resulted in 60% kill after 24 h (Fig. 2). In contrast those larvae administered with complexes **4** to **8** showed viability of 80 – 60% at 24 h and this did not change significantly by 48 h (Fig. 2). In a second series of experiments the ability of the complexes to prevent larvae from infection was assessed with higher concentration, 0.25 mg/mL (Fig. 3). In this experiment complexes **8** and **9** induced 100% survival of larvae at 24 h and 80% at 48 h.

DPPN derivatives **8** and **9** show very high *in vivo* activity only at higher concentration (0.25 mg/ml) while the DPPZ analogues **6** and **7** show the opposite behaviour (more active at lower concentration). The *in vivo* antimicrobial activity of the complexes does not strongly correlate with what was seen *in vitro* but this is quite common since the conditions *in vivo* are much different from those *in vitro*; the same lack of correlation was recently observed by our group with Au(I) complexes [17]. The role of the steroids and of the planar ligands in the activity of this series of Cu(II) complexes is not completely clear; the enhanced lipophlicity due to presence of the steroids that increase the bacterial uptake could explain in part the high antimicrobial activity but other mechanisms can be involved. A deeper investigation that will need the use of proteomics analyses is required and planned to clarify this aspect and will be the object of a future paper.

5. Conclusions

The six novel Cu(II)-steroid complexes showed high potency *in vitro* against *S. aureus* and MRSA and *in vivo* against *S. aureus*. When inoculated with the complexes larval viability noticeably increased in those larvae that were previously infected with *S. aureus*. The effect of the steroids on the antimicrobial activity of the metal complexes is still not clarified and we are currently using proteomics analyses to uncover the mechanism of action. These complexes show great potential to become lead compounds which could eventually be developed into new treatments for *S. aureus* and possibly MRSA infections.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ica.2018.04.054.

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