The Antimicrobial and Antibiofilm Activity of Copper(II) Complexes

Michael L. Beeton[a], Janice R. Aldrich-Wright[b] and Albert Bolhuis[a*]

[a] University of Bath, Department of Pharmacy and Pharmacology, Claverton Down, Bath BA2 7AY, United Kingdom

[b] University of Western Sydney, School of Biomedical and Health Sciences, Penrith South, Australia.

* Corresponding author: a.bolhuis@bath.ac.uk, phone +44 (0)1225 383813

ABSTRACT

Biofilm-related bacterial infections pose a significant problem, as they are generally more tolerant to antibiotics and the immune system. Development of novel compounds with antibiofilm activity is therefore paramount. In this study we have analysed metal complexes of the general structure $[M(I_L)(A_L)]^{2+}$ (where I_L represents functionalised 1,10-phenanthrolines and A_L represents 1*S*,2*S*- or 1*R*,2*R*-diaminocyclohexane) and $[Cu(I_L)_3]^{2+}$. Antimicrobial activity was tested on a number of bacterial strains, showing that copper(II) compounds were active against both Gram-positive and Gram-negative bacteria, albeit that activity was generally higher for the former. The antibiofilm activity was then determined against a clinical isolate of meticillin-resistant *Staphylococcus aureus* (MRSA). Strikingly, the copper complexes tested showed significant activity against biofilms, and were better in the removal of biofilms than vancomycin, an antibiotic that is currently used in the treatment of MRSA infections.

Keywords: Copper(II) complexes, antimicrobials, meticillin-resistant *Staphylococcus aureus*, biofilms

1. INTRODUCTION

Most bacteria live in complex sessile communities called biofilms. In these, bacteria adhere to surfaces and are embedded in a matrix of self-generated extracellular polymeric substances. Biofilms play a major role in the lifestyle of bacteria and indeed are involved in the majority of bacterial infections [1]. Well-known examples include biofilms forming on living tissue, such as in wound infections, endocarditis or lung infections in cystic fibrosis patients, as well as biofilms on indwelling medical devices such as stents, catheters or prosthetic implants [2].

A particular problem of infections caused by biofilms is that they are difficult to treat. Generally, biofilms are much more tolerant to both antibiotics and the immune system as compared to their planktonic (free-floating) counterparts [3]. Often the resistance of biofilms towards antibiotics may be as much as 100-1000 fold higher than that of planktonic cells [4], and sub-inhibitory concentrations of certain antibiotics may even induce biofilm formation [5]. Several factors play a role in this including an altered physiological state, slow growth rate of bacteria in biofilms [3] and a limited penetration of antibiotics through biofilms [6, 7].

A number of strategies are currently being investigated in order to improve treatment of bacterial infections. In recent years there has been an increased interest in the use of metals such as copper, silver or platinum as antibacterial agents. For instance, silver is increasingly being used in wound dressings and other products [8]. Metallic copper has been shown to be effective as an antimicrobial surface that is useful in e.g. wound dressings or as a material for common hospital surfaces [9, 10]. In addition,

copper incorporated in paints has also been shown to inhibit biofilm formation of bacteria and could thus be employed as an antifouling agent [11].

Coordination complexes of metals with aromatic ligands are also attractive molecules with a demonstrated antibacterial and cytotoxic activity. In these, the metal acts as a scaffold for the ligands, forming a 3D structure that interacts with specific targets. Early work by the group of Dwyer demonstrated that coordination complexes of Ru(II), Ni(II), Cu(II), Fe(II) or Co(II) with ligands such as 1,10-phenanthroline or 2,2'-bipyridine have activity against both Gram-positive and Gram-negative bacteria [12, 13]. As recently reviewed by us, these complexes interact with DNA through intercalation [14]. It is important to note that complex formation is important, as the biological activity is significantly enhanced when compared to ligands or metals alone [15, 16]. In addition, copper(II) complexes may also have chemical nuclease activity [17], and several groups have designed and explored complexes which facilitate DNA transformations [18-20].

In recent years we have investigated the antimicrobial activity of compounds containing iron, ruthenium, copper and palladium [16, 21-24]. All of these compounds have been reported to bind DNA, either through groove binding or intercalation. Several compounds showed good antimicrobial activity on both Gram-negative and Gram-positive bacteria, albeit that the latter are generally more sensitive to metal complexes [16, 21-24]. These complexes have only been tested on planktonic cells, and we therefore sought to investigate the activity of a panel of copper(II) complexes against biofilms, and focussed this specifically on a clinical isolate of meticillin-resistant *Staphylococcus aureus* (MRSA) that is capable of forming stable

biofilms. The copper(II) complexes (compounds **1-9**) are listed in Table 1, and their general structure is shown in Fig. 1. We also assessed the influence of the central metal on biofilms by comparing the copper(II) containing compound **3** with analogous complexes containing either platinum(II) (compound **10**) or palladium(II) (compound **11**).

2. MATERIALS AND METHODS

2.1 Strains and growth conditions

The bacterial strains used in this study were *S. aureus* MRSA252 and MSSA209 [25], *Enterococcus faecalis* NCTC775, *Escherichia coli* NCTC86, and *Pseudomonas aeruginosa* ATCC27853. Strains were maintained on Tryptone Soy Agar (Oxoid).

2.2 Antimicrobial compounds

All metal complexes used (listed in Table 1) were synthesised as described [16, 24]. Complexes were either dissolved in water (chloride salts) or DMSO (perchlorate salts) to 10 mg/mL. Vancomycin and chloramphenicol (Sigma-Aldrich) were dissolved in water and 96% ethanol, respectively.

2.3 Minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) values for the complexes were determined with a microdilution broth method using Mueller Hinton Broth (Oxoid) as described [26].

2.4 Biofilm assays in microtitre plates

An overnight culture of *S. aureus* MRSA252 grown in Brain Heart Infusion (BHI; Oxoid) broth supplemented with 0.5% glucose (BHI-G) was diluted 25-fold in fresh BHI-G. This inoculum was dispensed (200 µL) into wells of a 96-well plate (Costar, Corning). Plates were then incubated for 24 h at 37 ^{o}C on an orbital rotating platform at 40 RPM. Following biofilm formation supernatant containing planktonic cells was removed and replaced with either fresh BHI-G (control wells) or BHI-G with appropriate concentration of compound. Plates were then incubated for a further 24 h

in the conditions stated above. Next, supernatants were removed and the biofilms were washed three times in phosphate buffered saline (PBS, Fisher) and allowed to air dry at 60 ºC for 30 minutes. The dried biofilms were then stained with 0.1 % w/v crystal violet for 15 minutes, washed three times by gently plunging into lukewarm tap water and solubilised in 200 µL 30 % v/v acetic acid. The absorbance was then read at 595 nm and the percentage biofilm removal compared with control was determined. All assays were performed in triplicate, with a minimum of 4 wells per experiment.

2.5 Visualisation of biofilms using confocal scanning laser microscopy

An overnight culture of *S. aureus* MRSA252 grown as above (Section 2.4) was diluted 25-fold in fresh BHI-G. This inoculum was dispensed (4 mL) into wells of a 6-well plate (Costar, Corning), and a sterile polyvinyl coverslip (Electron Microscopy Sciences) was placed on the bottom of each well. Plates were then incubated statically at 37 ºC and biofilms were allowed to develop for 24 h. Following biofilm formation supernatant containing planktonic cells was removed and replaced with either fresh BHI-G (control wells) or BHI-G with appropriate concentration of compound. Plates were then incubated for a further 2 h. Next, the coverslips were removed, washed 6 times with sterile H_2O, and stained for 15 min in the dark with 800 µL of BacLight (Invitrogen) following manufacturer's instructions. The coverslips were again washed with sterile H_2O and mounted onto glass slides with nail varnish. Images were collected using a LSM510META Zeiss confocal laser scanning microscope, using a Plan-Apochromat 63x/1.4 oil objective. Biofilm thickness was determined from the number of z stacks with fluorescent pixels. Thickness was measured in three different areas of the biofilms formed.

2.6 Membrane activity assays

To determine the activity of the copper compounds against mammalian membranes, haemolysis of sheep red blood cells (RBCs; Oxoid) was determined. RBCs were collected by centrifugation (800 *g*, 5 min), washed 4-5 times with PBS and diluted to 4%. Next, compound was added at a concentration of 100 µg/mL and the RBCs were incubated for 1 h at 37 ºC. Intact RBCs were then removed by centrifugation and the release of haemoglobin in the supernatant was measured by determining the absorption at 414 nm. As a control for 100% lysis, RBCs were resuspended in water instead of PBS.

3. RESULTS

3.1 Antimicrobial activity

The antibacterial activity of the Cu(II) complexes chosen for this study has been reported before [16, 24], but because the activity was not determined on the same panel of bacterial isolates, we re-evaluated their activity (Table 1). This was compared with the activity of two antibiotics, vancomycin (compound **12**) and chloramphenicol (compound **13**). The copper compounds demonstrate similar activity on two *S. aureus* strains, irrespective of whether they are meticillin-sensitive (MSSA209) or resistant (MRSA252). The copper compounds are also similarly active against another Gram-positive bacterium, *E. faecalis*. Activity against Gram-negative bacteria is lower, with the compounds still showing some activity against *E. coli* but no significant activity against *P. aeruginosa*. Note that neither the ligands [16], nor $CuCl_2$ (compound **14**; Table 1) have a high antimicrobial activity on their own; these have sublethal activity at the concentrations tested, and significant antimicrobial activity is only observed for the complexes of Cu(II) with the ligands.

3.2 Antibiofilm activity against S. aureus *MRSA252*

A number of copper complexes were chosen for further analysis on biofilms formed by *S. aureus* MRSA252. These included compounds **1-3**, for which the cytotoxic activity is also known [24], and the more hydrophobic and active compounds **7** and **8**. In addition, compound **9** (Fig. 1) was used to analyse the effect of the absence of the diaminocyclohexane. It was observed that all of the copper compounds were capable of removing a significant amount of the biofilm at concentrations of 25 μg/mL or higher (Fig. 2). Compared to the antibiotics vancomycin (**12**) and chloramphenicol (**13**), all of the copper compounds were more effective in removing biofilms. At 25

µg/mL, chloramphenicol only removed 26% of the biofilm, compared to 60-68% for the compounds **1**, **2** and **3**, and 35-42% for compounds **7**, **8** and **9**. Particularly striking was the difference with vancomycin, since the MIC of this antibiotic on MRSA252 (0.25 µg/mL) is considerably better than that of the copper compounds (2-32 µg/mL). Incubation for 24 h in the presence of 25 µg/mL vancomycin (which is 100-fold the MIC value) had even increased the biomass in the biofilm. Only in the presence of 100 µg/mL vancomycin was a reduction (by 44%) of the biofilm observed. Note that $CuCl_2$ has no significant antibiofilm activity at the concentrations tested (compound **14**, Fig. 2).

3.3 Analysis of antibiofilm activity by confocal microscopy

To visualise the effects on MRSA252 biofilms, cells were grown for 24 h on polyvinyl coverslips followed by a 2-h treatment with compound. Next, cells were stained with the BacLight Live/Dead stain in which living cells stain green and dead cells red. As shown in Fig. 3, 2 h after addition of 100 µg/mL of compound **3** the majority of cells stained red, whereas in the presence of the same concentration of vancomycin most cells stained yellow-green. Thus, significantly more bacteria were killed in the presence of compound **3** as compared to vancomycin. In addition, biofilms incubated in the presence of vancomycin had not reduced in thickness (7 µm for non-treated biofilms, 10 µm for vancomycin-treated biofilms), but those incubated with compound **3** were reduced significantly to about 2 µm.

3.4 The copper compounds have no aspecific membrane activity

It is conceivable that the antibiofilm activity of compound **3** is not achieved through DNA binding, but that the compound has non-specific membrane activity instead. To

verify this we analysed the effect of each of the copper compounds on cell membranes by testing for their ability to lyse red blood cells [27]. However, as shown in Table 1 the copper compounds have only a very low haemolytic activity, a level comparable to that of vancomycin and chloramphenicol.

3.5 Effects of the central metal

The antibiofilm activity demonstrated by complex **3** in Fig. 3 is also evident for the other Cu(II) compounds. An important question is whether the central metal influences their activity. To that purpose we tested the activity of three analogous metal complexes [M(5,6-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane]$^{2+}$ where M is copper (compound **3**), platinum (compound **10**) or palladium (compound **11**) [16]. Both compound **10** and **11** were less active than complex **3** in standard MIC tests on planktonic cells, albeit that the Pt(II) compound has a similar activity to complex **3** on *E. faecalis* or *E. coli* (Table 1). Surprisingly, compound **10** was significantly less active on *S. aureus* MRSA252 (MIC>128 μg/mL) than on MSSA, whereas such differences were not observed with other compounds. Strikingly, neither complex **10** nor **11** were very active against biofilms formed by *S. aureus* MRSA252. A 24-h incubation of pre-formed biofilms with complex **10** resulted in only a 17% biomass reduction, while in the presence of complex **11** the biofilms increased in biomass (Fig. 2).

4. DISCUSSION

We have previously shown that certain metal complexes have antibacterial activity. Here we demonstrated that in particular Cu(II) complexes are also active against biofilms formed by *S. aureus* MRSA252. Interestingly, even though the antimicrobial activity on planktonic cells was lower with the Cu(II) complexes than with vancomycin (which is used clinically in the treatment of a number of serious infections with Gram-positive bacteria such as *Clostridium difficile*, MRSA and *Enterococcus faecium*), their antibiofilm activity was significantly better than vancomycin. For vancomycin at least 100 μg/mL (equivalent to 400-fold the MIC) is required to get some reduction (~44%) in the biomass in a *S. aureus* MRSA252 biofilm, whereas a concentration of 25 μg/mL complex **3** (equivalent to 3-fold the MIC) reduced these biofilms by 68%. The action of complex **3** is also fairly rapid as demonstrated by CLSM, as an MRSA252 biofilm incubated for 2 h led to the majority of cells being killed as well as a significant reduction in the amount of biomass to basically a single layer of dead cells.

The activity of the different Cu(II) complexes on biofilms was fairly similar, albeit that at 25μg/mL complexes **1-3** were somewhat more active than **7** and **8**. Interestingly, the latter two were the most active on planktonic cells, possibly through enhance uptake or increased affinity with their target due to the increased hydrophobicity, but that did not translate into increased antibiofilm activity. The octahedral compound **9** (which does not contain 1,2-diaminocyclohexane) was similarly active to **7** and **8**, indicating that the diaminocyclohexane is not essential for activity.

One of our initial hypotheses was that the Cu(II) compounds might bind extracellular DNA. This has been found to be an important component of the extracellular matrix of biofilms, and for instance DNAse treatment significantly reduces the biomass of biofilms of *S. aureus* and other bacteria [28]. However, a number of other DNA-binding metallo-complexes that we tested have no or barely any activity on biofilms, including the aforementioned compounds **10** and **11**, as well as other compounds such as major groove-binding complexes of iron [22] or ruthenium [23] (data not shown). Furthermore, as observed by CLSM the majority of cells were killed within 2 h in the presence of compound **3** and binding to extracellular DNA in the biofilm matrix is unlikely to lead to such rapid killing. A more likely target for the Cu(II) complexes is therefore the bacterial chromosome, although we cannot exclude other intracellular targets such as RNA or proteins.

It is well known that many coordination complexes with copper, but not those with platinum or palladium, are redox active and can mediate DNA cleavage [17]. For instance, a 1,10-phenanthroline-copper complex was the first synthetic chemical nuclease reported [29]. This nuclease activity depends on the presence of exogenous reagents such as ascorbate, thiols or peroxide to produce active species that leads to DNA strand scission [30, 31]. We have previously shown that the Cu(II) complexes used here have indeed such nuclease activity [24]. This, together with the observation that replacing Cu(II) in compound **3** with Pt(II) (compound **10**) or Pd(II) (compounds **11)** significantly reduces antibacterial and antibiofilm activity, strongly suggests that this nuclease activity is an important mechanism by which the Cu(II) compounds are active. As mentioned above, this activity is dependent on the presence of reducing agents, which in bacteria could be supplied by low molecular mass thiols that

maintain their cytoplasm in a strongly reducing state. In most Gram-negative bacteria the thiol used is glutathione, whereas in several Gram-positive bacteria including *S. aureus* this is bacillithiol [32]. It is interesting to note that the previously published value of the half maximal inhibitory concentration (IC_{50}) against a mouse cell line (L1210) is much lower for compound **10** (0.0092 μM) than for compound **3** (0.62 μM) or compound **11** (11.5 μM) [24]. Thus, on mammalian cells the Pt(II) compound is far more active than the Cu(II) or Pd(II) compounds, indicating that the mode of action of the complexes in mammalian cells is different from that in bacterial cells.

A question that remains is why the Cu(II) compounds are less active than vancomycin on planktonic cells, yet are significantly more active on biofilms. Important in this is that most antibiotics are particularly effective on rapidly dividing cells, as they inhibit processes that are more active in those cells, such as cell wall synthesis or protein translation. However, cells in biofilms often grow more slowly or are even in a dormant state, explaining the poor activity of antibiotics on biofilms [3]. Indeed, the cell-wall active vancomycin has been shown not to be very effective against *S. aureus* biofilms [33]. However, copper-induced DNA damage may lead to death irrespective of the physiological state or growth rate of the bacterial cells. This could thus explain that, in contrast to antibiotics such as vancomycin, the Cu(II) compounds effectively kill and remove biofilms at concentrations that are relatively close to the MIC values obtained for planktonic cells. It should be noted that, as mentioned before, other factors such as limited penetration into biofilms may also play a role in the antimicrobial resistance of biofilms [6, 7], and it is conceivable that these further exacerbate the differences in antibiofilm activity observed for the Cu(II) compounds and vancomycin.

This initial work has demonstrated the effectiveness of copper compounds on biofilms of *S. aureus* MRSA252, and effects on biofilms of additional *S. aureus* isolates and other bacteria will be investigated in the near future. Whether the copper compounds can be used for systemic treatment of biofilm-related infections depends on further improvement of activity and specificity. The copper compounds do have some cytotoxicity as previously shown by us using a simple animal (nematode) model and the aforementioned L1210 cell line [24], but it was encouraging to note the lack of non-specific membrane activity on mammalian red blood cells. Even if toxicity is too high, topical treatment of chronic wound infections, which are known to involve biofilms, may still be an option. For instance, the compounds could be used in wound dressings, and we plan to test the copper compounds in combination with novel slow-release matrices [34] that could be used to this purpose. Another application of interest could be the incorporation of the copper complexes in materials of medical devices such as catheters, which are prone to biofilm-related infections [35], in order to prevent biofilm formation.

ABBREVIATIONS

A_L	accessory ligand
I_L	intercalating ligand
BHI	brain heart infusion
BHI-G	BHI supplemented with 0.5% glucose
DMSO	dimethyl sulfoxide
IC50	half maximal inhibitory concentration
MIC	minimal inhibitory concentration

MSSA	meticillin sensitive *S. aureus*
MRSA	meticillin resistant *S. aureus*
ND	not determined
PBS	phosphate buffered saline
RBC	red blood cells
Phen	1,10-phenanthroline
5Mephen	5-methyl-1,10-phenanthroline
56Me_2phen	5,6-dimethyl-1,10-phenanthroline
3478Me_4phen	3,4,7,8-tetramethyl-1,10-phenanthroline
DIP	4,7-diphenyl-1,10-phenanthroline
SS-dach	(1*S*, 2*S*)-1,2-diaminocyclohexane
RR-dach	(1*R*, 2*R*)-1,2-diaminocyclohexane

ACKNOWLEDGEMENTS

We thank Dr Adrian Rogers from the Microscopy and Analysis Suite at the University of Bath for assistance with confocal microscopy. JRAW wishes to acknowledge the University of Western Sydney for funding through internal research grants for the synthesis of the compounds. JRAW would like to acknowledge the awarding of an RSC Travel Grant that enabled the collaboration with AB on this project.

REFERENCES

[1] J.W. Costerton, P.S. Stewart, E.P. Greenberg, Science 248 (1999) 1318-1322.

[2] S.L. Percival, K.E Hill, D.W. Williams, S.J. Hooper, D.W. Thomas, J.W. Costerton, Wound Repair Regen. 20 (2012) 647-657.

[3] T.F.C. Mah, G.A. O'Toole, Trends Microbiol. 9 (2001) 34-39.

[4] N. Høiby, T. Bjarnsholt, M. Givskov, S. Molin, O. Ciofu, Int. J. Antimicrob. Agents 35 (2010) 322-332.

[5] L.R. Hoffman, D.A. D'Argenio, M.J. MacCoss, Z. Zhang, R.A. Jones, S.I. Miller, Nature 436 (2005) 1171-1175.

[6] J.N. Anderl, M.J. Franklin, P.S. Stewart, Antimicrob. Agents. Chemother. 44 (2000) 1818-1824.

[7] R. Singh, P. Ray, A. Das, M. Sharma, J. Antimicrob. Chemother. 65 (2010) 1955-1958.

[8] L.J. Wilkinson, R.J. White, J.K. Chipman, J. Wound Care 20 (2011) 543-549.

[9] L. Weaver, J.O. Noyce, H.T. Michels, C.W. Keevil, J. Appl. Microbiol. 109 (2010) 2200-2205.

[10] M.G. Schmidt, H.H. Attaway, P.A. Sharpe, J. John Jr., K.A. Sepkowitz, A. Morgan, S.E. Fairey, S. Singh, L.L. Steed, J.R. Cantey, K.D. Freeman, H.T. Michels, C.D. Saigado, J. Clin. Microbiol. 50 (2012) 2217-2223.

[11] J.J. Cooney, R.J. Tang, Methods Enzymol. 310 (1999) 637-644.

[12] F.P. Dwyer, E.C. Gyarfas, W.P. Rogers, J.H. Koch, Nature 170 (1952) 190-191.

[13] F.P. Dwyer, I.K. Reid, A. Shulman, G.M. Laycock, S. Dixson, Aust. J. Exp. Biol. Med. Sci. 47 (1969) 203-218.

[14] A. Bolhuis, J.R. Aldrich-Wright, Bioorg. Chem. (2014) doi: 10.1016/j.bioorg.2014.03.009 [epub ahead of print].

[15] M.A. Zoroddu, S. Zanetti, R. Pogni and R. Basosi, J. Inorg. Biochem. 63 (1996) 291–300.

[16] N.S. Ng, P. Leverett, D.E. Hibbs, Q. Yang, J.C. Bulanadi, M.J. Wu, J.R. Aldrich-Wright, Dalton Trans. 42 (2013) 3196-3209.

[17] D.S. Sigman, A. Mazumder, D.M.Perrin, Chem. Rev. 93 (1993) 2295-2316.

[18] K.L Haas, K.J. Franz, Chem. Rev. 109 (2009) 4921-4960.

[19] M.J. Li, T.Y. Lan, X.H. Cao, H.H. Yang, Y. Shi, C. Yi, G.N. Chen, Dalton Trans. 43 (2014) 2789-2798.

[20] A. Prisecaru, M. Devereux, N. Barron, M. McCann, J. Colleran, A. Casey, V. McKee, A. Kellett, ChemComm 48 (2012) 6906-6908.

[21] A.D. Richards, A. Rodger, M.J. Hannon, A. Bolhuis, Int. J. Antimicrob. Agents 33 (2009) 469-472.

[22] S.E. Howson, A. Bolhuis, V. Brabec, G.J. Clarkson, J. Malina, A. Rodger, P. Scott, Nature Chem. 4 (2012) 31-36.

[23] A. Bolhuis, L. Hand, J.E. Marshall, A.D. Richards, A. Rodger, J. Aldrich-Wright, Eur. J. Pharm. Sci. 42 (2011) 313-317.

[24] A.M. Krause-Heuer, P. Leverett, A. Bolhuis, J.R. Aldrich-Wright, Aust. J. Chem. 65 (2012) 860-873.

[25] J. Collins, J. Rudkin, M. Recker, C. Pozzi, J.P. O'Gara, R.C. Massey, ISME J. 4 (2010) 577-584.

[26] J.M. Andrews, J. Antimicrob. Chemother. 48 Suppl 1 (2001) 5-16.

[27] M.P. Singh, J. Microbiol. Methods 67 (2006) 125-130.

[28] K.C. Rice, E.E. Mann, J.L. Endres, E.C. Weiss, J.E. Cassat, M.S. Smeltzer, K.W. Bayles, Proc. Natl. Acad. Sci. USA 104 (2007) 8113-81138.

[29] D.S. Sigman, D.R. Graham, V. D'Aurora and A.M. Stern, J. Biol. Chem. 254 (1979) 12269–12272 .

[30] K.A. Reich, L.E. Marshall, D.R. Graham, D.S. Sigman. J. Am. Chem. Soc. 103 (1981) 3582-3584.

[31] J.M. Veal, K. Merchant, R.L. Rill. Nucleic Acids Res., 19 (1991) 3383-3388.

[32] G.L. Newton, M. Rawat, J.J. La Clair, V.K. Jothivasan, T. Budiarto, C.J. Hamilton, A. Claiborne, J.D. Helman, R.C. Fahey Nat Chem. Biol. 5 (2009) 625-627.

[33] S.M.Jones, M. Morgan, T.J. Humphrey, H. Lappin-Scott. Lancet 357 (2001) 40-41.

[34] N. Alhusein, P.A. De Bank, I.S. Blagbrough, A. Bolhuis, Drug Deliv. Transl. Res. 3 (2013) 531-541.

[35] B. Liedl, Curr. Opin. Urol. 11 (2001) 75-79.

Table 1. Minimal inhibitory concentrations and haemolytic activity of compounds

Number	Compound[a]	*S. aureus* MRSA252	*S. aureus* MSSA209	*E. faecalis* NCTC775	*E. coli* NCTC86	*P. aeruginosa* ATCC27853	% lysis RBCs (+/- SD)
1	[Cu(phen)(*SS*-dach)]Cl_2	32	32	32	64	>128	2.0 (0.4)
2	[Cu(5Mephen)(*SS*-dach]Cl_2	32	16	8	64	>128	2.1 (0.1)
3	[Cu(56Me_2phen)(*SS*-dach]Cl_2	8	8	4	32	>128	2.6 (0.3)
4	[Cu(56Me_2phen)(*RR*-dach]Cl_2	8	4	2	32	>128	2.2 (0.7)
5	[Cu(3478Me_4phen)(*SS*-dach)]$(ClO_4)_2$	4	4	4	16	>128	2.5 (0.3)
6	[Cu(3478Me_4phen)(*RR*-dach)]$(ClO_4)_2$	4	4	4	16	>128	2.0 (.3)
7	[Cu(DIP)(*SS*-dach)]$(ClO_4)_2$	2	2	2	16	>128	3.1 (0.2)
8	[Cu(DIP)(*RR*-dach)]$(ClO_4)_2$	2	2	2	16	>128	3.0 (0.3)
9	[Cu(56Me_2phen)$_3$]$(ClO_4)_2.2H_2O$	4	8	4	16	>128	3.0 (0.8)
10	[Pt(56Me_2phen)(*SS*-dach]$(ClO_4)_2$	>128	32	4	16	>128	ND[b]
11	[Pd(56Me_2phen)(*SS*-dach]$(ClO_4)_2$	64	64	16	32	>128	ND
12	Vancomycin	0.25	0.5	0.5	ND	ND	2.6 (0.2)
13	Chloramphenicol	16	16	4	2	128	ND
14	$CuCl_2.2H_2O$	>128	>128	>128	>128	>128	2.0 (0.3)

[a] phen: 1,10-phenanthroline; 5Mephen: 5-methyl-1,10-phenanthroline; 56Me_2phen, 5,6-dimethyl-1,10-phenanthroline; 3478Me_4phen, 3,4,7,8-tetramethyl-1,10-phenanthroline; DIP, 4,7-diphenyl-1,10-phenanthroline; *SS*-dach, (1*S*, 2*S*)-1,2-diaminocyclohexane; *RR*-dach, (1*R*, 2*R*)-1,2-diaminocyclohexane. [b]ND, not determined

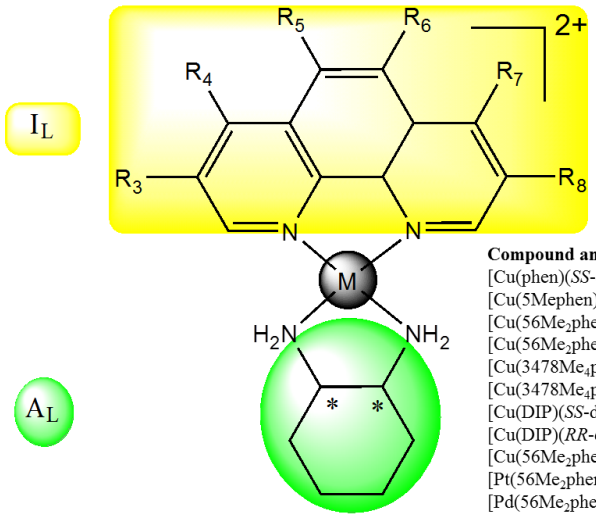
Figure legends

Fig. 1. The general structure of the metal complexes, **1-8**, **10** and **11** where 1,10-phenanthroline is in the rectangle (top; intercalating ligand I_L) and R is either H or CH_3, and the 1,2-diaminocyclohexane is in the oval (bottom; accessory ligand A_L). (*) indicates a stereocentre, either *S* or *R*. Counter-ions have been omitted for clarity. The octahedral complex **9** is also shown.

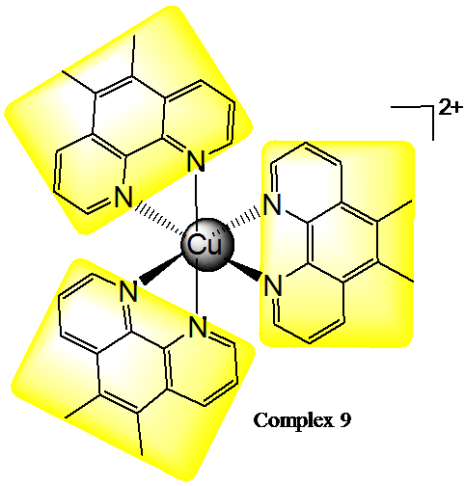
Fig. 2. Activity of complexes 1, 2, 3, 7, 8, 9, 10, 11, vancomycin (**12**), chloramphenicol (**13**), and $CuCl_2.2H_2O$ (**14**) against biofilms of *S. aureus* MRSA252.

Fig. 3. CLSM images of *S. aureus* MRSA252 biofilms (grown for 24 h) that were untreated (left panel), or treated for 2 h with vancomycin (**12**) at 100 μg/mL (centre panel) or compound **3** at 100 μg/mL (right panel). Side views of the biofilms are shown on top and on the right of each panel.

Figures



Compound and number	
[Cu(phen)(*SS*-dach)]Cl_2 **(1)**	M= Cu, R_{3-8} = H
[Cu(5Mephen)(*SS*-dach]Cl_2 **(2)**	M= Cu, R_5 = CH_3 and $R_{3,4,6-8}$ = H
[Cu(56Me_2phen)(*SS*-dach]Cl_2 **(3)**	M= Cu, $R_{5,6}$ = CH_3 and $R_{3,4,7-8}$ = H
[Cu(56Me_2phen)(*RR*-dach]Cl_2**(4)**	M= Cu, $R_{5,6}$ = CH_3 and $R_{3,4,7-8}$ = H
[Cu(3478Me_4phen)(*SS*-dach)]$(ClO_4)_2$ **(5)**	M= Cu, $R_{3,4,7-8}$ = CH_3 and $R_{5,6}$ = H
[Cu(3478Me_4phen)(*RR*-dach)]$(ClO_4)_2$ **(6)**	M= Cu, $R_{3,4,7-8}$ = CH_3 and $R_{5,6}$ = H
[Cu(DIP)(*SS*-dach)]$(ClO_4)_2$ **(7)**	M= Cu, $R_{4,7}$ = phenyl and $R_{3,5,6-8}$ = H
[Cu(DIP)(*RR*-dach)]$(ClO_4)_2$**(6)**	M= Cu, $R_{4,7}$ = phenyl and $R_{3,5,6-8}$ = H
[Cu(56Me_2phen)$_3$]$(ClO_4)_2.2H_2O$ **(9)**	M= Cu, $R_{5,6}$ = CH_3 and $R_{3,4,7-8}$ = H
[Pt(56Me_2phen)(*SS*-dach]$(ClO_4)_2$ **(10)**	M= Pt, $R_{5,6}$ = CH_3 and $R_{3,4,7-8}$ = H
[Pd(56Me_2phen)(*SS*-dach]$(ClO_4)_2$ **(11)**	M= Pd, $R_{5,6}$ = CH_3 and $R_{3,4,7-8}$ = H



Complex 9

Figure 1

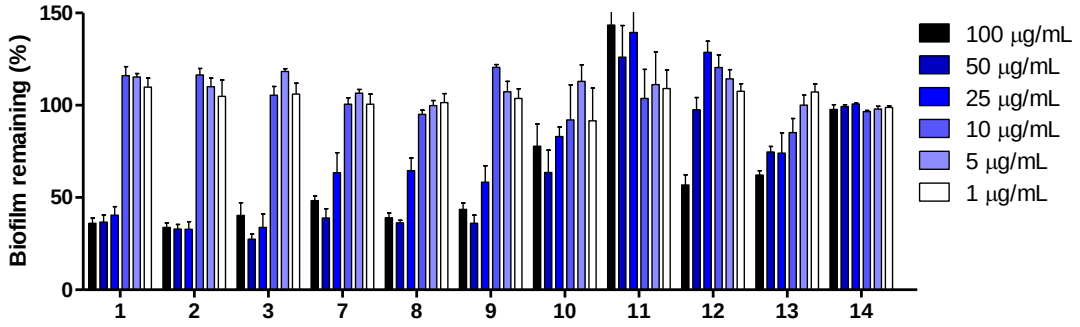
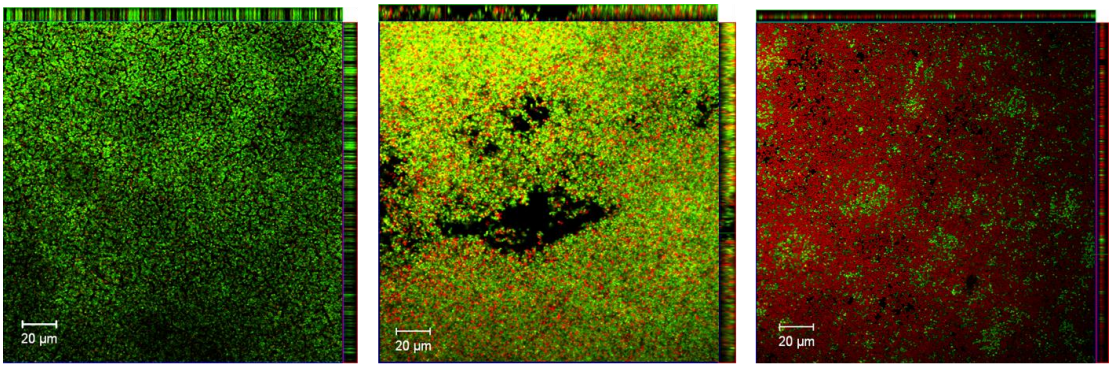

Biofilm remaining (%)
150
100
50
0
1
2
3
7
8
9
10
11
12
13
14
100 μg/mL
50 μg/mL
25 μg/mL
10 μg/mL
5 μg/mL
1 μg/mL

Figure 2



Positive control | Vancomycin (**12**) | compound **3**

Figure 3